Erratum

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We apologise for this mistake.
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Summary

RECQL4 belongs to the conserved RecQ family of DNA helicases, members of which play important roles in the maintenance of genome stability in all organisms that have been examined. Although genetic alterations in the RECQL4 gene are reported to be associated with three autosomal recessive disorders (Rothmund-Thomson, RAPADILINO and Baller-Gerold syndromes), the molecular role of RECQL4 still remains poorly understood. Here, we show that RECQL4 specifically interacts with the histone acetyltransferase p300 (also known as p300 HAT), both in vivo and in vitro, and that p300 acetylates one or more of the lysine residues at positions 376, 380, 382, 385 and 386 of RECQL4. Furthermore, we report that these five lysine residues lie within a short motif of 30 amino acids that is essential for the nuclear localization of RECQL4. Remarkably, the acetylation of RECQL4 by p300 in vivo leads to a significant shift of a proportion of RECQL4 protein from the nucleus to the cytoplasm. This accumulation of the acetylated RECQL4 is a result of its inability to be imported into the nucleus. Our results provide the first evidence of a post-translational modification of the RECQL4 protein, and suggest that acetylation of RECQL4 by p300 regulates the trafficking of RECQL4 between the nucleus and the cytoplasm.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/8/1258/DC1

Key words: RECQL4, RecQ helicases, Genome stability, p300, Protein acetylation

Introduction

Genome instability plays a major role in the development and progression of cancer. All organisms have developed pathways to mitigate DNA damage by employing enzymes that are involved in all DNA metabolic processes, including replication, recombination and repair (Tuteja and Tuteja, 2001). The fundamental importance of these enzymes, such as DNA helicases and acetyltransferases, is highlighted by the variety of genetic instability disorders caused by enzymes defective in these functions (Iyer et al., 2004; Khakhar et al., 2003).

One family of proteins required to maintain genome stability is the RecQ helicase family (Hickson, 2003). Humans possess five RecQ homologues: RECQL1, BLM, WRN, RECQL4 and RECQL5. Five autosomal recessive disorders (which have been characterized by genomic instability, cancer progression and developmental abnormalities) have been associated with defects in the gene products BLM (causing Bloom syndrome) (Ellis et al., 1995), WRN (causing Werner syndrome) (Gray et al., 1997) and RECQL4 [causing Rothmund-Thomson (RTS), RAPADILINO and Baller-Gerold (BGS) syndromes] (Dietschy et al., 2007; Kitao et al., 1999b; Siitonen et al., 2003; Van Maldergem et al., 2006). RTS is an unusual autosomal recessive condition and is associated with poikiloderma, growth deficiency, juvenile cataracts, premature aging and a predisposition to malignant tumours, particularly osteosarcomas (Vennos et al., 1992). Interestingly, mutations in the RECQL4 gene cause only 60% of all RTS cases (Kitao et al., 1999a). Accordingly, RTS seems to be a heterogeneous disease and mutations in other, yet unidentified gene(s) seem to be responsible for the phenotype of the remaining 40% of RTS patients. Mutations found in RECQL4 range from nonsense, frameshift and splice-site mutations to intronic insertions and deletions (Siitonen et al., 2003). Most of them result in premature termination of protein translation, yielding truncated RECQL4 proteins that often lack a large part of the helicase domain (Lindor et al., 2000). Cells derived from RTS patients show genomic instability, including trisomy, aneuploidy and chromosomal rearrangements (Der Kaloustian et al., 1990; Vennos et al., 1992). Additionally, RTS cells are hypersensitive to ionizing radiation and oxidative stress (Vennos and James, 1995; Werner et al., 2006).

RAPADILINO syndrome is another autosomal recessive disease associated with mutations in the RECQL4 gene (Siitonen et al., 2003). The acronym stands for the characteristic clinical features: RA for radial hypoplasia or aplasia; PA for patellae hypoplasia or aplasia, and for cleft or highly arched palate; DI for diarrhoea and for dislocated joints; LI for little size and for limb malformation;
NO for slender nose and for normal intelligence. The most common mutations of the RECQL4 gene in RAPADILINO patients represent deletions of exon 7, which do not affect the helicase domain of RECQL4 protein (Siitonen et al., 2003). Although RAPADILINO patients share some clinical features with RTS patients, such as photosensitivity with extra pigmentation of skin and growth deficiency, there are unique diagnostic findings, such as joint dislocations and patellar hypoplasia or aplasia. In contrast to RTS, RAPADILINO syndrome is more common in females than in males. Furthermore, only one out of the 15 RAPADILINO patients diagnosed so far has developed malignant tumours, mainly osteosarcomas (Kellermayer et al., 2005). However, to date, no study has documented the risk of osteosarcoma over time.

BGS is the third recently reported autosomal recessive disorder linked to mutations in the RECQL4 gene (Van Maldergem et al., 2006). The clinical hallmarks of BGS are radial aplasia or hypoplasia and craniosynostosis. To date, most mutations of RECQL4 found in BGS patients represent a R1021W missense mutation and a 2886 base pair deletion (Kuo and Allis, 1998). Traditionally, HATs have been associated exclusively with the acetylation of histone tails and with transcriptional regulation through chromatin remodelling. By alleviating repressive histone-DNA binding, and thus facilitating the association of transcription factors with DNA, histone acetylation can lead to increased transcriptional activity (Nakatani, 2001). One such family of HATs is p300/CBP, which includes the two distinct but related proteins p300 (also known as p300 HAT and EP300) and CBP; these proteins participate in many physiological processes, including proliferation, differentiation and apoptosis (Goodman and Smolik, 2000). Although p300 was originally identified as acetylating lysine residues on core histones (Bannister and Kouzarides, 1996; Ogyrko et al., 1996), in the past decade a growing body of evidence has suggested that it acetylates other non-histone targets, including proteins involved in the regulation of transcription, such as p53, E2F1, EKLF, TFIEβ, TFIEF, TCF, GATA1, HMGI(Y) and ACTR (Kouzarides, 2000), as well as DNA-repair and -replication proteins, such as PCNA (Hasan et al., 2001a), FEN-1 (Hasan et al., 2001b), TDG (Tini et al., 2002), APE1 (Bhakat et al., 2003), NEIL2 (Bhakat et al., 2004), DNA polymerase-β (Hasan et al., 2002) and human 8-oxo-guanine-DNA glycosylase (OGG1) (Bhakat et al., 2006). Acetylation by p300 can modulate the activity of DNA-repair and -replication proteins, either positively or negatively. For example, acetylation of FEN1 by p300 reduces the nuclease activity of FEN1, presumably as a result of reduced DNA binding (Hasan et al., 2001b). Similarly, acetylation of NEIL2, an oxidized pyrimidine-specific DNA glycosylase, was shown to inhibit its enzymatic activity (Bhakat et al., 2004). By contrast, acetylation of human OGG1 by p300 significantly increases the activity of OGG1 activity in vitro in the presence of AP-endonuclease by reducing its affinity for the abasic (AP) site product (Bhakat et al., 2006). Also, p300-mediated acetylation of WRN enhances its helicase and exonuclease activities in vitro and regulates the function of WRN in base excision repair (Mutluoglu et al., 2008). All these examples highlight the importance of p300 in the DNA base excision repair process.

In spite of several recent studies showing that acetylation of DNA repair and replication proteins by p300 results in altered DNA repair and substrate specificity, acetylation of RECQL4 as well as its physiological relevance have not been addressed so far. In this paper, we report the physical and functional interaction between RECQL4 and p300. p300 specifically acetylates RECQL4 on a stretch of lysine residues, previously shown to function as the NOS-NLS (nucleolar and/or nuclear localization signal) sequence of RECQL4 (Burks et al., 2007; Woo et al., 2006). Furthermore, we demonstrate that mutation of these lysine residues to alanine (Lys 376, 380, 382, 385, 386 Ala), but not to arginine (Lys 376, 380, 382, 385, 386 Arg), completely abolishes the nuclear import of RECQL4 in human cells. Additionally, fusion of the RECQL4 aa 376-386 to the N-terminus of β-galactosidase leads to the nuclear import of this 100-kDa protein in mammalian cells, indicating the importance of the positively charged amino acid residues for the nuclear import of the RECQL4 protein. Overexpression of p300, but not the catalytically dead mutant (p300 ΔHAT), leads to a significant accumulation of RECQL4 protein in the cytoplasm. The same effect could be observed by treating the cells with histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and nicotinamide (NA), suggesting that acetylation of RECQL4 by p300 is involved in the RECQL4 cytoplasmic localization. Taken together, our data help to understand the mechanism by which RECQL4 is localized to the cytoplasm in mammalian cells.

Another class of enzymes, known as acetyltransferases, are also responsible for maintaining genome stability (Freeman and Tjian, 2003; Hasan and Hottinger, 2002). Histone acetyltransferases (HATs) catalyze the reversible transfer of an acetyl group from acetyl coenzyme A to the ε-NH₃⁺ of lysine residues on core histone tails (Kuo and Allis, 1998). Traditionally, HATs have been associated exclusively with the acetylation of histone tails and with transcriptional regulation through chromatin remodelling. By alleviating repressive histone-DNA binding, and thus facilitating the association of transcription factors with DNA, histone acetylation can lead to increased transcriptional activity (Nakatani, 2001). One such family of HATs is p300/CBP, which includes the two distinct but related proteins p300 (also known as p300 HAT and EP300) and CBP; these proteins participate in many physiological processes, including proliferation, differentiation and apoptosis (Goodman and Smolik, 2000). Although p300 was originally identified as acetylating lysine residues on core histones (Bannister and Kouzarides, 1996; Ogyrko et al., 1996), in the past decade a growing body of evidence has suggested that it acetylates other non-histone targets, including proteins involved in the regulation of transcription, such as p53, E2F1, EKLF, TFIEβ, TFIEF, TCF, GATA1, HMGI(Y) and ACTR (Kouzarides, 2000), as well as DNA-repair and -replication proteins, such as PCNA (Hasan et al., 2001a), FEN-1 (Hasan et al., 2001b), TDG (Tini et al., 2002), APE1 (Bhakat et al., 2003), NEIL2 (Bhakat et al., 2004), DNA polymerase-β (Hasan et al., 2002) and human 8-oxo-guanine-DNA glycosylase (OGG1) (Bhakat et al., 2006). Acetylation by p300 can modulate the activity of DNA-repair and -replication proteins, either positively or negatively. For example, acetylation of FEN1 by p300 reduces the nuclease activity of FEN1, presumably as a result of reduced DNA binding (Hasan et al., 2001b). Similarly, acetylation of NEIL2, an oxidized pyrimidine-specific DNA glycosylase, was shown to inhibit its enzymatic activity (Bhakat et al., 2004). By contrast, acetylation of human OGG1 by p300 significantly increases the activity of OGG1 activity in vitro in the presence of AP-endonuclease by reducing its affinity for the abasic (AP) site product (Bhakat et al., 2006). Also, p300-mediated acetylation of WRN enhances its helicase and exonuclease activities in vitro and regulates the function of WRN in base excision repair (Mutluoglu et al., 2008). All these examples highlight the importance of p300 in the DNA base excision repair process.

In spite of several recent studies showing that acetylation of DNA repair and replication proteins by p300 results in altered DNA repair and substrate specificity, acetylation of RECQL4 as well as its physiological relevance have not been addressed so far. In this paper, we report the physical and functional interaction between RECQL4 and p300. p300 specifically acetylates RECQL4 on a stretch of lysine residues, previously shown to function as the NOS-NLS (nucleolar and/or nuclear localization signal) sequence of RECQL4 (Burks et al., 2007; Woo et al., 2006). Furthermore, we demonstrate that mutation of these lysine residues to alanine (Lys 376, 380, 382, 385, 386 Ala), but not to arginine (Lys 376, 380, 382, 385, 386 Arg), completely abolishes the nuclear import of RECQL4 in human cells. Additionally, fusion of the RECQL4 aa 376-386 to the N-terminus of β-galactosidase leads to the nuclear import of this 100-kDa protein in mammalian cells, indicating the importance of the positively charged amino acid residues for the nuclear import of the RECQL4 protein. Overexpression of p300, but not the catalytically dead mutant (p300 ΔHAT), leads to a significant accumulation of RECQL4 protein in the cytoplasm. The same effect could be observed by treating the cells with histone deacetylase (HDAC) inhibitors trichostatin A (TSA) and nicotinamide (NA), suggesting that acetylation of RECQL4 by p300 is involved in the RECQL4 cytoplasmic localization. Taken together, our data help to understand the mechanism by which RECQL4 is localized to the cytoplasm in mammalian cells.
Results
In vivo acetylation of RECQL4 by p300 and interaction of the full-length proteins
We wanted to investigate whether RECQL4 is acetylated in mammalian cells. To identify a potential acetyltransferase that acetylates RECQL4 in vivo, RECQL4 containing (His)_6-Xpress epitope tag was overexpressed in HEK 293T cells with each of the four major human acetyltransferases (p300, PCAF, GCN5 and HAT1). Notably, we were forced to use overexpressed RECQL4 protein in this assay owing to a low expression level of the endogenous RECQL4 gene. An Omni-probe antibody raised against a peptide mapping between the (His)_6 and the polylinker sequence of Xpress vectors was used to immunoprecipitate the recombinant RECQL4 protein from whole-cell extracts (Fig. 1A, upper left panel). A control IgG antibody failed to precipitate RECQL4 and confirmed the specificity of the Omni-probe antibody for (His)_6-Xpress-RECQL4 (Fig. 1A, lower left panel). Although western blot analysis revealed that all four acetyltransferases (p300, PCAF, GCN5 or HAT1) were expressed to similar levels (Fig. 1A, right panel), RECQL4 was found to be strongly acetylated only in cells overproducing p300, suggesting that p300 might be an acetyltransferase that acetylates RECQL4 in vivo. Additionally, neither cell-cycle status nor treatment with different DNA damaging agents (cisplatin, camptothecin, etoposide) significantly affected RECQL4 treatment with different DNA damaging agents (cisplatin, camptothecin, etoposide) significantly affected RECQL4 acetylation in vivo (supplementary material Fig. S1A,B).

We next analyzed whether RECQL4 and p300 interact in human cells. Because we could not detect complex formation between endogenous RECQL4 and p300 owing to a low expression level of both proteins, we switched to the overexpression approach. For this, FLAG-p300 and (His)_6-Xpress-RECQL4 expression constructs were transiently transfected into HEK 293T cells, and the total cell extract was immunoprecipitated with Omni-probe antibody and analyzed by immunoblotting using an anti-FLAG antibody. We found that FLAG-p300 was efficiently co-precipitated with (His)_6-Xpress-RECQL4 (Fig. 1B, lane 1), but was not detected when the control IgG antibody was used (Fig. 1B, lane 2). Notably, 500 μg of whole cell extract needed to be used in order to detect the interaction, which might be because of the transient nature of the interaction between p300 and RECQL4. To further confirm the interaction between RECQL4 and p300 in human cells, the reciprocal co-immunoprecipitation experiment was carried out using a FLAG-antibody. (His)_6-Xpress-RECQL4 could be specifically co-immunoprecipitated with FLAG-p300 (Fig. 1B, lane 6), but not with the control IgG antibody (Fig. 1B, lane 5). It should be noted that cell extracts used in the above-mentioned experiments were supplemented with ethidium bromide, excluding the possibility that association of RECQL4 and p300 is mediated by DNA.

We next performed an ELISA-based protein-binding assay using purified recombinant proteins to determine whether RECQL4 and p300 interact directly. Increasing concentrations of purified recombinant RECQL4 (0–40 nM) were incubated in wells that had been precoated with purified p300 at a concentration of 20 nM and subsequently blocked with BSA to prevent nonspecific interactions. After extensive washing, the bound RECQL4 was incubated with a specific anti-RECQL4 polyclonal antibody, followed by a colorimetric assay to quantify the binding. In control experiments, RECQL4 was incubated in wells precoated only with BSA. We found that RECQL4 was bound to p300-coated wells in a dose-dependent manner, but was not bound in wells precoated with BSA, indicating a direct interaction (Fig. 1C).

Taken together, these data indicate that p300 acetylates RECQL4 in vivo, that RECQL4 and p300 form a complex in human cells, and that the proteins interact directly in vitro.

Mapping of the RECQL4 and p300 interaction regions
To map the p300-interaction domain in RECQL4, RECQL4 was incubated in wells precoated only with BSA, and the total cell extract derived from these cells was immunoprecipitated with anti-RECQL4 antibody (α-RECQL4) or control IgG (IgGctrl) and analyzed by SDS PAGE. One-tenth (100 μg) of the same total cell extract was used as input control (lane 1). Immunoprecipitated FLAG-p300 and RECQL4 were detected by western blotting using anti-FLAG (α-FLAG) and anti-RECQL4 (α-RECQL4) antibody, respectively (lane 3). Reciprocal co-immunoprecipitation is shown in the right panel: lane 4, input; lane 5, immunoprecipitation with the control IgG; lane 6, immunoprecipitation with anti-p300 antibody (α-p300) using total cell extracts derived from HEK 293T cells overexpressing (His)_6-Xpress-RECQL4. (C) Binding of RECQL4 to p300 as a function of RECQL4 concentration. Increasing concentrations of RECQL4 (0–40 nM) were incubated at 37°C for one hour in wells of an ELISA plate that were pre-coated with the p300 protein (20 nM) and subsequently blocked with 3% BSA. After extensive washing, bound RECQL4 protein was detected as described in the Materials and Methods. Absorbance values were corrected by subtracting background values obtained with BSA-coated wells. Triangles represent the average of measurements from duplicate samples.
cloned and expressed as fusions with glutathione S-transferase (GST) (Fig. 2A,B). GST pull-down experiments with these fragments were performed using total extract from HEK 293T cells overexpressing FLAG-p300. Western blot analysis of bound proteins using anti-FLAG antibody indicated that only RECQL4-2 was able to interact with full-length p300 (Fig. 2C, lane 4). This fragment spans aa residues 1-408 of RECQL4 and contains the RECQL4 NOS sequence previously mapped to aa 376-386 (Woo et al., 2006) and a part of the helicase domain (see also Fig. 2A).

To map the RECQL4-interaction region in p300, p300 was also divided into several fragments (p300-1 to p300-5), which were subsequently expressed and purified as GST fusions (Fig. 2D,E). GST pull-down experiments were performed using whole cell extracts of HEK 293T cells overproducing (His)6-Xpress-RECQL4. Western blot analysis of bound proteins using Omni-probe antibody indicated that only p300-4 was able to interact with RECQL4 (Fig. 2F, lane 6). This fragment spans aa 1459-1892 and contains a part of the p300 HAT domain that was previously shown to interact with human FEN-1 (Hasan et al., 2001b).

Identification of acetylation sites of RECQL4

Several studies revealed that p300 preferentially acetylates stretches of lysine residues of substrate proteins (Bai et al., 2005; Faiola et al., 2007; Hasan et al., 2001b; Hassa et al., 2005; Topper et al., 2007). The RECQL4 amino-acid sequence contains 32 lysine residues, and the only lysine stretch is located in the previously

Fig. 2. Mapping of interaction domains between p300 and RECQL4. (A) Schematic representation of RECQL4 and its deletion variants used in this study. (B) SDS-PAGE analysis of bacteriologically expressed and purified GST-RECQL4 fragments 1-4. Gel was stained with Coomassie blue. (C) GST pull-down assay showing binding of FLAG-p300 to bacteriologically expressed GST-RECQL4 fragments 1-4. GST-RECQL4 fragments bound to glutathione-Sepharose beads were incubated with whole cell extract (500 μg of total protein) derived from HEK 293T cells overexpressing FLAG-p300. Binding of p300 was analyzed by western blotting using anti-FLAG antibody (α-FLAG). Enhanced chemiluminescence (ECL) reagent was used for detection, and the film was exposed for 2 minutes. (D) Schematic representation of p300 and its deletion variants (p300-1 to p300-5). (E) SDS-PAGE analysis of bacterially expressed and purified GST-p300 fragments 1-5. (F) GST pull-down assay showing binding of RECQL4 to bacterially expressed GST-p300 fragments 1-5. GST-p300 fragments bound to glutathione-Sepharose beads were incubated with whole cell extract (1 mg of total protein) derived from HEK 293T cells overexpressing (His)6-Xpress-RECQL4. RECQL4 binding was analyzed by western blotting using Omni-probe antibody (α-Omni).
identified NOS sequence of RECQL4, with five lysine residues at positions 376, 380, 382, 385 and 386 (Woo et al., 2006). Two RECQL4 NOS mutants, with all five lysine residues mutated to either alanine (K→A) or to arginine (K→R), were generated using site-directed mutagenesis in order to identify the extent of acetylation of the NOS lysine residues within RECQL4 (Fig. 3A). Wild-type, K→A or K→R (His)6-Xpress-RECQL4 were subsequently expressed in HEK 293T cells in the presence or absence of FLAG-p300, and immunoprecipitated from whole cell extracts using Omni-probe antibody (Fig. 3B, upper left panel). A control IgG serum confirmed the specificity of the Omni-probe antibody for (His)6-Xpress-RECQL4 in the immunoprecipitation reaction (Fig. 3B, lane 7). The extent of RECQL4 acetylation was visualized by western-blot analysis using an anti-acetylated lysine antibody (Fig. 3B, lower left panel). Although co-expression of wild-type RECQL4 and p300 resulted in acetylation of RECQL4 (Fig. 3B, lane 6), the acetylation of the K→A (Fig. 3B, lane 2) and K→R (Fig. 3B, lane 4) mutants of RECQL4 was not detected, suggesting that at least one of the lysine residues at positions 376, 380, 385 and 386 of RECQL4 is required for acetylation by p300. To further investigate which of the five lysine residues is acetylated in vivo, we generated single (K382R) (Fig. 3B, lanes 9 and 10), double (K385,386R) (Fig. 3B, lanes 11 and 12) and triple (K376,380,382R) (Fig. 3B, lanes 13 and 14) mutants of RECQL4 by site-directed mutagenesis. The three mutants were co-transfected in the presence or absence of p300 in HEK 293T cells and analyzed as described above (Fig. 3B, left panel). Co-transfection of all three mutants and p300 resulted in acetylation of RECQL4 (Fig. 3B, lanes 10, 12 and 14), suggesting that more than one of the lysine residues at positions 376, 380, 385 and 386 is acetylated by p300.

To confirm that the stretch of five lysine residues in RECQL4 at positions 376, 380, 382, 385 and 386 is acetylated by p300, RECQL4 was divided into several fragments (RECQL4-a to RECQL4-e) that were subsequently expressed and purified as GST fusion proteins (Fig. 3C). Subsequently, p300 purified from SF9 insect cells was incubated with the above-mentioned GST-RECQL4 fragments and [14C] acetyl coenzyme A. The reactions were analyzed by SDS PAGE. After staining with Coomassie blue (Fig. 3D, left panel), the gel was subjected to autoradiography and revealed that p300 was self-acetylated and was able to acetylate fragment RECQL4-b (Fig. 3B, right panel). This fragment spans amino acid residues 359-478, including the putative NOS sequence (aa 376-386) of RECQL4. Furthermore, this fragment also contains a part of the p300 interaction region (aa 1-408) that was mapped in the experiment shown in Fig. 2B. Importantly, there was no detectable acetylation of RECQL4-c, which covers the same region as RECQL4-b but has all five lysine residues mutated to alanines. The difference in electrophoretic mobilities of fragments b and c can be explained by the lack of positive charge in the alanine mutant. It should be noted that Coomassie-blue staining of the gel confirmed that comparable amounts of RECQL4 fragments were used in each acetylation reaction (Fig. 3D).

Thus, we conclude from the in vivo and in vitro acetylation assays that p300 acetylates RECQL4 on one or more of the lysine residues at positions 376, 380, 382, 385 and 386, previously characterized as the NOS-NLS sequence.

Role of lysine residues 376, 380, 382, 385 and 386 in RECQL4 localization in mammalian cells

We next investigated the functional significance of acetylation of lysine residues 376, 380, 382, 385 and 386 by analyzing the subcellular distribution of RECQL4 protein in human cells. To that end, we transiently overexpressed (His)6-Xpress-RECQL4 in HeLa cells and examined its subcellular localization by indirect immunofluorescence using an Omni-probe antibody (Fig. 4A, top row). We found that, in 70% of the cells, RECQL4 was localized...
predominantly in the nucleus. In 22% of the cells, RECQL4 was equally distributed between nucleus and cytoplasm. In 8% of the cells, predominant cytoplasmic localization of RECQL4 protein was observed (Fig. 4B, bars on the left). Our observation is consistent with previously published biochemical and immunofluorescence data on overexpressed and endogenous RECQL4, showing that RECQL4 is found both in the nucleus and in the cytoplasm of the different cell lines examined (Burks et al., 2007; Petkovic et al., 2005; Yin et al., 2004). Therefore, we considered the (His)6-Xpress-RECQL4 fusion protein to be an adequate tool for further characterization of the cellular localization of RECQL4. We next studied the localization of the K→A and K→R mutants of RECQL4 in HeLa cells, because the RECQL4 N-terminal basic motif spanning aa 376-386 (KQAWKQKWRKK) was previously reported to have nucleolar-localization activity (Woo et al., 2006). Each of the mutants were transiently transfected in HeLa cells and the expressed proteins were detected by indirect immunofluorescence using Omni-probe antibody (Fig. 4A, middle and bottom rows). No significant difference in subcellular localization between the wild-type RECQL4 and its K→R mutant was observed (Fig. 4A, middle row; Fig. 4B, centre histograms). Interestingly, the RECQL4 K→A mutant was mostly localized to the cytoplasm of HeLa cells. Similar results were obtained using U2OS cells, and cell-type-specific observations could therefore be excluded (supplementary material Fig. S2). Importantly, our findings seem contradictory to the observations of Woo et al., who found nucleolar exclusion (but nucleoplasmic localization) of GFP-RECQL4 lacking the basic motif (Woo et al., 2006). To further verify the validity of our results, we fused the nucleotides encoding the basic motif (aa 376-386) of RECQL4 (NLS) or the K→A mutant sequence (NLS K→A) N-terminally to the Escherichia coli lacZ cDNA (to give the proteins NLS β-Gal and NLS K→A β-Gal, respectively; Fig. 4C). Transiently overexpressed E. coli β-galactosidase localized almost entirely to the cytoplasm in HeLa cells (Fig. 4D, top row; Fig. 4E). Intriguingly, the RECQL4 wild-type basic motif fused to the β-galactosidase protein was able to import this 100-kDa protein to the nucleus (Fig. 4D, middle row; Fig. 4E), whereas the NLS K→A mutant (D) were co-expressed with FLAG-p300 or FLAG-p300 ΔHAT proteins in HeLa cells. RECQL4 proteins were visualized with Omni-probe antibody (α-Omni; red), and the p300 and p300 ΔHAT proteins were visualized with anti-FLAG antibody (α-FLAG; green). DAPI-staining (blue) shows nuclear DNA. Right-hand column show merged pictures (Merge). (C,E) Quantification of B and D. The histograms show the prevalence of RECQL4 in the cytoplasm (N>C) or a prevalence of RECQL4 in the nucleus (N<C). The plotted data represent the mean ± standard deviation of two independent transfection experiments in which more than 200 transfected cells were analyzed each time. Scale bars: 10 μm.

**Fig. 5.** p300 activity-dependent accumulation of RECQL4 protein in the cytoplasm. (A) p300 and p300 ΔHAT, a catalytic dead mutant, localize to the nucleus in mammalian cells. FLAG-tagged p300 or FLAG-p300 ΔHAT proteins were transiently expressed in HeLa cells and visualized by indirect immunofluorescence using anti-FLAG antibody (green, centre column). DAPI-stained nuclei are shown in the left column (blue), and the merged pictures (Merge) are shown in the right column. (B,D) Hist-Xpress-RECQL4 (B) or its K→R mutant (D) were co-expressed with FLAG-p300 or FLAG-p300 ΔHAT proteins in HeLa cells. RECQL4 proteins were visualized with Omni-probe antibody (α-Omni; red), and the p300 and p300 ΔHAT proteins were visualized with anti-FLAG antibody (α-FLAG; green). DAPI-staining (blue) shows nuclear DNA. Right-hand column show merged pictures (Merge). (C,E) Quantification of B and D. The histograms show the prevalence of RECQL4 in the cytoplasm (N>C) or a prevalence of RECQL4 in the nucleus (N<C). The plotted data represent the mean ± standard deviation of two independent transfection experiments in which more than 200 transfected cells were analyzed each time. Scale bars: 10 μm.

**RECQL4 accumulates in the cytoplasm upon acetylation by p300.** We next wanted to examine the cellular localization of RECQL4 protein in human cells that express either the wild-type or mutated p300. We first transiently expressed, in HeLa cells, FLAG-p300 and FLAG-p300 ΔHAT, a catalytically dead mutant lacking part of the HAT domain, and visualized both proteins by indirect immunofluorescence. As expected, both proteins were localized entirely to the nucleus of HeLa cells (Fig. 5A). We then investigated the functional consequence of the RECQL4 acetylation in vivo. Immunofluorescence experiments on co-transfected HeLa cells [Hist-Xpress-RECQL4–FLAG-p300 and (Hist)6-Xpress-RECQL4–FLAG-p300 ΔHAT] showed that the catalytic activity of p300 enhances the number of cells in which RECQL4 is mainly cytoplasmic by about threefold (Fig. 5B,C). In accordance with this observation, p300 catalytic activity did not drive the accumulation of RECQL4 K→R mutant in the cytoplasm, confirming that these lysine residues are responsible for p300-induced RECQL4 re-localization to cytoplasm (Fig. 5D,E). We conclude that overexpression of the catalytically dead mutant of p300 (FLAG-
Histone deacetylase inhibitors cause RECQL4 to accumulate in the cytoplasm

To further confirm that the observed cytoplasmic accumulation of RECQL4 is indeed caused by its acetylation, HeLa cells were transfected with (His)₆-Xpress-RECQL4 and (His)₆-Xpress-RECQL4 K→R mutant were transiently overexpressed in HeLa cells. Trichostatin A (TSA) and nicotinamide (NA) were added 24 hours post-transfection and the cells left for an additional 30 hours. Cells were subsequently fixed and expressed proteins were visualized as in Fig. 4A. (B) Quantification of A. The plotted data indicate the mean ± standard deviation of two independent transfection experiments in which more than 200 transfected cells were analyzed each time. Scale bar: 10 μm.

Histone deacetylase inhibitors promote translocation of RECQL4 to the cytoplasm. (A) (His)₆-Xpress-RECQL4 and (His)₆-Xpress-RECQL4 K→R mutant were transiently overexpressed in HeLa cells. Trichostatin A (TSA) and nicotinamide (NA) were added 24 hours post-transfection and the cells left for an additional 30 hours. Cells were subsequently fixed and expressed proteins were visualized as in Fig. 4A. (B) Quantification of A. The plotted data indicate the mean ± standard deviation of two independent transfection experiments in which more than 200 transfected cells were analyzed each time. Scale bar: 10 μm.

Fig. 6. Histone deacetylase inhibitors trichostatin A and nicotinamide promote translocation of RECQL4 to the cytoplasm. (A) (His)₆-Xpress-RECQL4 and (His)₆-Xpress-RECQL4 K→R mutant were transiently overexpressed in HeLa cells. Trichostatin A (TSA) and nicotinamide (NA) were added 24 hours post-transfection and the cells left for an additional 30 hours. Cells were subsequently fixed and expressed proteins were visualized as in Fig. 4A. (B) Quantification of A. The plotted data indicate the mean ± standard deviation of two independent transfection experiments in which more than 200 transfected cells were analyzed each time. Scale bar: 10 μm.

We also showed that lysine residues 376, 380, 382, 385 and 386 of RECQL4 within the N-terminal basic motif (KQAWKQKRKK) are essential for the nuclear localization of RECQL4. Because p300 catalytic activity did not drive the accumulation of RECQL4 K→R mutant in the cytoplasm, our data indicate that acetylated lysine residues 376, 380, 382, 385 and 386 of RECQL4 are crucial for the cytoplasmic localization of RECQL4 in the two different human cell lines examined. In addition, the exposure of these cell lines to histone deacetylase inhibitors TSA and NA (both of which increase the level of protein acetylation) induced the cytoplasmic localization of the wild-type RECQL4 but not of the RECQL4 K→R mutant. This further confirmed the importance of the lysine residues 376, 380, 382, 385 and 386 of RECQL4.
p300-dependent acetylation of RECQL4. For example, it is possible that deacetylation of RECQL4 by the as-yet-unidentified deacetylase regulates its acetylation status. Indeed, Li et al. recently reported that deacetylation of WRN helicase by the SIRT1 deacetylase regulates WRN-mediated cellular responses to DNA damage (Li et al., 2008). Additional experiments are planned in our laboratory to determine the possible effects of deacetylation of RECQL4 on its cellular localization and other roles in DNA metabolism.

Exactly how p300 regulates the subcellular localization of RECQL4 is an intriguing problem. As shown in Fig. 5A, p300 is localized exclusively to the nucleus of HeLa cells. Therefore, we hypothesize that the acetylation of RECQL4 by p300 takes place in the nucleus, followed by subsequent export of the acetylated RECQL4 to the cytoplasm. Once in the cytoplasm, the acetylation of the RECQL4 NLS prevents the helicase from being imported into the nucleus again, leading to its accumulation in the cytoplasm. As shown in Fig. 6A,B, treatment of cells with HDAC inhibitors TSA and NA, thus shifting RECQL4 towards an acetylated state, led to an accumulation of RECQL4 in the cytoplasm. Our hypothesis is further supported by previous work showing that p300-mediated acetylation of the transcription factors HMG1 (Bonaldi et al., 2003) and E1A (Madison et al., 2002) negatively affects the function of their NLS, thus disrupting their association with the nuclear import machinery and leading to subsequent cytoplasmic accumulation of these transcription factors. Furthermore, we would like to emphasize that the RECQL4 K→A mutant, mimicking the acetylated state of the RECQL4 NLS by charge depletion, could not be imported to the nucleus in the different cell lines examined. It has been recently demonstrated that RECQL4 is found in complex with UBR1 and UBR2, at least partially, in cytosolic extracts of HeLa cells (Yin et al., 2004). UBR1 and UBR2 belong to the family of E3 ubiquitin ligases, which is part of the ubiquitin proteasome system (Hershko et al., 2000). It is therefore tempting to speculate that, after a portion of RECQL4 is acetylated by p300 in the nucleus, RECQL4 is translocated to the cytoplasm where the interaction with UBR1 and UBR2 takes place and leads to a subsequent degradation by the proteasome. Finally, we note that we cannot, at the present time, rule out the possibility that RECQL4 has some other role in the cytosol that still awaits further investigations.

Materials and Methods

Plasmid construction

Full-length RECQL4 cDNA was subcloned in the mammalian expression vector pcDNA3.1/Hisc (Invitrogen) between EcoRI and XhoI sites to generate N-terminal fusion of RECQL4 with a (His)6-Xpress epitope tag. This construct was subsequently used for all immunofluorescence experiments. The mammalian expression vector for FLAG-p300 was described previously (Hasan et al., 2001a). Various portions of the RECQL4 cDNA (Fig. 2A) were amplified by PCR and cloned in pGEX6P1 (GE Healthcare) between the NheI and XhoI sites of the pET21b-RECQL4 plasmid, resulting in a construct encoding for a GST-RECQL4 (His)6 fusion protein.

Expression and purification of RECQL4 and p300 proteins

GST-p300 and GST-RECQL4 protein fragments were expressed in E. coli BL21(DE3) (Novagen) using the plasmid constructs described above. Overnight cultures grown in Luria broth at 37°C were diluted 1:100 into fresh medium and incubated until OD600 reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM, and the cultures were incubated at 16°C for 20 hours. Cells were harvested by centrifugation and re-suspended in buffer A [50 mM Tris-HCl (pH 8), 50 mM NaCl, 20% (v/v) glycerol supplemented with 1 μg/ml benstatin, leupeptin and pepstatin, (v/v) protease inhibitors (1.5 mg of total protein) were incubated with 10 mM Tris-HCl (pH 8.1), 30 mM K2HPO4, 0.5 mM NaCl, 10% (v/v) glycerol, 0.5 mM PMFS, 20% (v/v) glycerol] and stored at −80°C.

The full-length RECQL4 protein was produced in BL21(DE3)Rosetta-pLyS5 cells using the pET21b-GST-RECQL4 (His6), construct under conditions described above. Cells from a 1-liter culture were harvest by centrifugation, resuspended in 300 ml of buffer B [10 mM Tris-HCl (pH 8.1), 30 mM K2HPO4, 0.5 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole supplemented with 1 mM PMFS and 2 μg/ml of each benstatin, leupeptin and pepstatin] and disrupted by French press. After centrifugation (20,000 rpm for 2 hours at 4°C, SS-34 rotor), the supernatant was loaded on a 1-ml HiTrapFusion column (GE Healthcare). The column was washed with 30 ml of buffer C supplemented with 60 mM imidazole. Protein was eluted with buffer D supplemented with 300 mM imidazole. RECQL4-containing fractions were pooled, dialyzed against buffer D [50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM DTT, 10% (v/v) glycerol, 0.5 mM PMFS and 1 μg/ml of each benstatin, leupeptin and pepstatin] and applied onto a 1-ml GST-Sepharose HiTrap column (GE Healthcare). After washing with 30 ml buffer D containing 0.2% Triton X-100, bound proteins were eluted by buffer D supplemented with 10 mM glutathione. Fractions containing RECQL4 protein were pooled, dialyzed against buffer E [50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 40% (v/v) glycerol and protease inhibitors] and stored at −80°C.

Full-length p300 was produced in Sf9 cells and purified as previously described (Hasan et al., 2002).

GST pull-down assays

HEK 293 whole-cell extracts, containing overexpressed full-length RECQL4 or p300 (500 μg of total protein), were incubated with 10 μg of purified GST-p300 or GST-RECQL4 protein fragments (or GST alone), 50 μl glutathione-Sepharose 4B beads (GE Healthcare), and 100 μg/ml ethidium bromide in buffer B in a total reaction volume of 600 μl. Bound proteins were eluted with 30 μl Laemmli buffer, boiled for 5 minutes at 100°C and subjected to western blot analysis using anti-RECQL4 (rabbit polyclonal, ab34800-100, Abcam) or anti-p300 antibodies (mouse polyclonal, ab3164-500, Abcam).

Cell culture and indirect immunofluorescence microscopy

U2OS, HEK 293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% bovine fetal serum and 1% penicillin-streptomycin in 5% CO2 atmosphere at 37°C. At 50% confluency, cells grown on glass slides were transiently transfected with 2 μg of appropriate plasmid DNA using Metafectene (Biontex).

For indirect immunofluorescence assays, cells growing on glass slides were incubated in PBS for 5 minutes at room temperature, fixed with methanol (30 minutes at −20°C), and washed in acetone (30 seconds at −20°C). After a blocking step (5% BSA in PBS, 20 minutes at room temperature), slides were incubated with a mixture of appropriate antibodies (anti-OMNI-probe, 1:200, goat polyclonal, sc-499-6, Santa Cruz; anti-FLAG, 1:2000, mouse polyclonal, F-1804, Sigma; anti-β-galactosidase, 1:5000, rabbit polyclonal, ab616, Abcam). Rabbit antibodies were detected with Alexa-Fluor-488-conjugated goat anti-rabbit IgG (1:400 in blocking buffer, Molecular Probes), the goat antibodies were detected with Alexa-Fluor-546-conjugated donkey anti-goat IgG (1:400 in blocking buffer, Molecular Probes), and mouse antibodies were detected with AlexaFluor-548-conjugated donkey anti-mouse IgG (1:400 in blocking buffer, Molecular Probes). To visualize nuclear DNA, slides were incubated with DAPI (0.4 μg/ml). After washing, slides were mounted in Vectashield (Vector Laboratories) and viewed under a Zeiss Axiosvert 200M microscope. Images were processed by Axiovision software. For statistical analysis at least 200 cells were counted in two independent experiments.

Synchronization of cells and DNA damaging treatments

HEK 293T cells growing exponentially (25% confluency) were transiently transfected with the plasmids (5 μg each) expressing (His6)-Xpress-RECQL4 or FLAG-p300, using the calcium phosphate transfection method. For G1-S phase synchronization, 24 hours after transfection, cells were grown for 16 hours in the presence of 2 mM 5′-iodo-deoxyuridine. After block release, cells were collected by trypsination up to 12 hours. Cell-cycle status of the individual samples was determined by flow cytometry analysis of propidium iodide-stained cells. To induce DNA damage, 24 hours after transfection, cells were treated with 1 μM camptothecin, 10 μM cisplatin or 10 μM etoposide for 12 hours before harvesting.

Co-immunoprecipitation experiments

Whole cell extracts, supplemented with ethidium bromide (100 μg/ml), containing ectopically expressed (His6)-Xpress-RECQL4 (1 mg of total protein) or FLAG-p300 (1.5 mg of total protein) were incubated with 1.5 μg of anti-p300 antibody (mouse...
polycyclon, ab3164-500, Abcam) or 1.5 μg of anti-RECQL4 antibody (rabbit polyclonal, ab34800-100, Abcam), respectively. Immune complexes were bound to protein A/G-Agarose beads (Santa Cruz) for 4 hours at 4°C. The final volume was raised to 1 ml with IP buffer [20 mM HEPES (pH 7.5), 5 mM MgCl2, 150 mM NaCl, 0.1% (w/v) NP40, supplemented with 1 μg/ml bacteria, leupeptin and pepstatin]. Immunoprecipitated p300 or RECQL4 proteins were detected by western blot analysis using the above antibodies, and co-immunoprecipitated (His)6-Xpress-RECQL4 and one of the selected histone acetyltransferases.

ELISA-based protein binding

Purified recombinant p300 was diluted to a concentration of 20 nm in carbonate buffer [10 mM NaCO3, 34 mM NaHCO3 (pH 9.6)] and added to wells of a 96-well microtiter plate (50 μl/well). Plates were incubated overnight at 4°C. For control reactions, wells were precoated with an equivalent amount of bovine serum albumin (BSA). After aspiration of the samples, the wells were blocked with blocking buffer (phosphate-buffered saline, 0.5% (v/v) Tween 20, and 3% (w/v) BSA) for 2 hours at 37°C (200 μl/well). Following blockage, the wells were incubated with increasing concentrations of purified recombinant RECQL4 protein for 1 hour at 37°C. Wells were washed four times with blocking buffer to eliminate unbound proteins and incubated with RECQL4 antibody (rabbit polyclonal, 1:2000, ab3164-500, Abcam) diluted in blocking buffer. Plates were incubated for 1 hour at 37°C. After four washings with blocking buffer, horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10,000 in blocking buffer) was added and the plates were incubated at 37°C for 30 minutes. After extensive washing with blocking buffer, the protein complexes were detected using o-phenylenediamine dichloride (Sigma) dissolved in 0.1 M citrate phosphate buffer (pH 5.0) containing 0.03% hydrogen peroxide and 0.0001% H2O2. The reactions were terminated after 10 minutes by adding 50 μl of 1 M H2SO4. The plates were scanned in a microplate reader (Molecular Devices) for absorbance at 450 nm. The A450 values, corrected for background signal in the presence of BSA, were plotted as a function of the concentration of RECQL4 protein using the GraphPad Prism software.

In vivo acetylation assay

Total protein extract from HEK 293T cells (1 mg of total protein), ectopically expressing (His)6-Xpress-RECQL4 and one of the selected histone acetyltransferases (p300, GCN5, PCAF, HAT1), was incubated for 4 hours at 4°C with 2 μg of Omni-probe antibody. The reactions were terminated after 10 minutes by adding 50 μl of 2 M H2SO4. The plates were scanned in a microplate reader (Molecular Devices) for absorbance at 450 nm. The A450 values, corrected for background signal in the presence of BSA, were plotted as a function of the concentration of RECQL4 protein using the GraphPad Prism software.

In vitro acetylation assay

Purified GST-RECQL4 fragments (1 μg) were incubated with 0.1 μCi [14C]-acetyl coenzyme A (CFA390, Amersham Biosciences) and purified p300 (1 μg) in 30 μl HAT-Buffer [50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 150 mM NaCl, 1 mM coenzyme A (CFA390, Amersham Biosciences) and purified p300 (1 μg)] expressing (His)6-Xpress-RECQL4 and one of the selected histone acetyltransferases.


Figure S1. DNA damage and cell cycle status have no significant influence on RECQL4 acetylation (A) (His)_6-Xpress-RECQL4 and FLAG-p300 expression vectors were transiently transfected in 293T cells and the cells subsequently treated with different DNA damaging agents including cis-diaminedichloro-platinum (CDDP), camptothecin (CPT) and etoposide (ETO). (His)_6-Xpress-RECQL4 was then immunoprecipitated with Omni-probe antibody and analyzed by Western blot (upper panel). The membrane was then stripped and re-probed with anti-acetyl-lysine (α-Ac-Lys) antibody (lower panel). (B) (His)_6-Xpress-RECQL4 and FLAG-p300 vectors were transiently transfected in 293T cells. Transfected cells were synchronized at G1/S transition by treatment with hydroxyurea (HU) for 16 hours and then released to S phase by adding fresh medium without HU. At indicated time points, RECQL4 acetylation status was analyzed by Western blot as described above. (C) In parallel, cells were subjected to FACS analysis. The resultant cell cycle profiles for each time point are shown. AS, asynchronous cells population; x-axis: DNA content.; y-axis: number of cells (arbitrary units, set to maximum value). Positions of the G1 and G2 peaks are indicated.
Figure S2. Mutation of RECQL4 lysine residues Lys-376, Lys-380, Lys-382, Lys-385 and Lys-386 to alanine but not arginine relocates RECQL4 to the cytoplasm of U2OS cells. (His)6-Xpress-RECQL4 and its K→R and K→A mutants in the NLS sequence were expressed in U2OS cells by transient transfection and visualized with the Omni-probe antibody by indirect immunofluorescence (red). The left panels show DAPI-stained nuclei (blue) and the right panels show the merged images. 10 μm.
Figure S3. RECQL4 sequence spanning amino acids 376-386 fused to β-Galactosidase leads to its nuclear import in 293T cells. β-Galactosidase and the indicated N-terminal fusion constructs (NLS-β-Gal and NLS-β-Gal K→A) were transiently transfected in 293T cells and visualized with the β-Galactosidase antibody by indirect immunofluorescence (middle panels, α-β-Gal, red). DAPI-staining shows nuclear DNA (left panels) and right panels show merged pictures (Merge). Bar, 10 μm.