The Large Subunit of Replication Factor C Interacts with the Histone Deacetylase, HDAC1*

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§ The abbreviations used are: HAT, histone acetyltransferase; RFC, replication factor C; C/EBPα, CCAAT/enhancer-binding protein α; HDAC1, histone deacetylase 1; PCNA, proliferating cell nuclear antigen; RB, retinoblastoma protein; BRCA1, breast cancer susceptibility gene 1; ATM, ataxia telangiectasia mutated; CAF1, chromatin assembly factor 1; WTB, Wellcome Trust Biocentre.

The large subunit of RFC, RFC (p140), is a pentameric complex of five distinct subunits that functions as a clamp loader, facilitating the loading of proliferating cell nuclear antigen (PCNA) onto DNA during replication and repair. More recently the large subunit of RFC, RFC (p140), has been found to interact with the retinoblastoma (RB) tumor suppressor and the CCAAT/enhancer-binding protein α (C/EBPα) transcription factor. We now report that RFC (p140) associates with histone deacetylase activity and interacts with histone deacetylase 1 (HDAC1). This complex is functional and when targeted to promoters as a Ga4 fusion, RFC (p140) is a strong, deacetylase-dependent repressor of transcription. Further analysis revealed that RFC (p140) contains two distinct transcriptional repression domains. Moreover, both of these domains interact separately with HDAC1.

A common problem faced during the assembly of protein complexes on eukaryotic chromosomal DNA is the inhibitory effect of chromatin structure. For example, the assembly of a gene into chromatin represses transcription by limiting access of the transcriptional machinery to the DNA template (1). Transcriptional regulatory proteins therefore recruit chromatin remodeling activities to either facilitate or inhibit access of DNA-binding proteins to their target sequences (2, 3). It can be predicted, therefore, that proteins associated with DNA replication and repair will also have to be able to regulate chromatin structure. Consistent with this, the largest subunit of the human origin recognition complex, ORC1, and the replication factor minichromosome maintenance protein 2 (MCM2) have both been found to interact with factors containing histone acetyltransferase (HAT)1 activity (4, 5).

This observation suggested that ORC1 might fulfill an important function regulating chromatin structure and facilitating the binding of other components of the DNA replication machinery. Acetylation of the amino-terminal tails of core histones alters nucleosome structure and facilitates both the recruitment of DNA-binding proteins and other chromatin remodeling activities (2, 6, 7). In the context of transcriptional regulation, HAT activity is therefore generally associated with the stimulation of gene expression, and it can be assumed that it will be similarly associated with the positive regulation of DNA replication and repair (8). Proteins with HAT activity, however, do not always acetylate histones. Many non-histone substrates have been identified, including DNA-binding proteins such as the tumor suppressor p53, components of the basal transcription apparatus, and the architectural transcription factor high mobility group protein I(Y) (HMG I(Y)) (9–11). Interestingly, in this latter case, acetylation of HMG I(Y) on lysine 71 by GCN5 stimulates the ability of the protein to promote transcription from the β-interferon promoter. In contrast, acetylation on lysine 65 by CREB-binding protein (CBP) inhibits HMG I(Y) function and is associated with the termination of β-interferon transcription (11). Thus the association of HAT activity with a protein should not automatically be interpreted as an indication of an effect on chromatin structure.

In contrast to proteins with HAT activity, histone deacetylases are often associated with transcriptional repression (12, 13). However, proteins that participate in DNA replication and repair have also been described associating with histone deacetylase activity. For example, the ataxia telangiectasia-mutated (ATM) kinase and breast cancer susceptibility gene 1 (BRCA1) have been shown to interact with histone deacetylases (14, 15). Furthermore, the RbAp48 subunit of chromatin assembly factor 1 (CAF1), a protein complex that facilitates the assembly of nucleosomes onto newly replicated DNA (16, 17), has also been identified as a protein known to interact with histone deacetylases and associate with the tumor suppressor protein Rb (16, 18, 19).

In this study we have investigated the interaction of RFC (p140) with histone deacetylase activity. RFC (p140) is a component of the pentameric replication factor C (RFC) complex (20). RFC functions as a clamp loader and facilitates both the loading and unloading of PCNA at sites of DNA synthesis (20). PCNA functions both to recruit DNA polymerase and CAF1, giving it a role both in stimulating DNA synthesis and in subsequent chromatin assembly (20, 21). It is apparent, however, that RFC (p140) has the potential to regulate other cellular processes and participate as a component of many protein complexes. RFC (p140) has been described in a large protein complex containing both BRCA1 and ATM, termed the BRCA1-associated genome surveillance complex (BASC), which has been postulated to act as a sensor for DNA damage (22). Moreover, RFC (p140) contains an LXXCXE motif and can bind directly to Rb (23). Interestingly, and consistent with it having a dynamic regulatory function, RFC (p140) binding to Rb was shown to have a prosurvival function following ultraviolet light stimulation (23). Both Rb and BRCA1 have important func-
ions as transcriptional regulators (24, 25). This raises the possibility that RFC (p140) might also have a dual role as a regulator of gene expression. Consistent with this, RFC (p140) was recently described as a transcriptional coactivator for the bZIP transcription factor C/EBPβ (26).

In this study, we report that RFC (p140) is associated with histone deacetylase activity and can directly bind HDAC1. Moreover, we demonstrate that when directly targeted to a promoter, RFC (p140) functions as a potent, histone deacetylase-dependent repressor of transcription. These results suggest that the cellular function of RFC (p140) is complex, and that in addition to its role as a clamp loader it also has the potential to regulate chromatin structure and repress transcription.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The full-length RFC (p140) cDNA was isolated from human foreskin fibroblast cell RNA by reverse transcription polymerase chain reaction (PCR) and inserted into the KpnI site of pCDNA3 or pCGN, which inserted an HA tag at its amino terminus and the NcoI/NotI sites of pVR1012Gal4 (27). Fragments of RFC (p140) were isolated by PCR from the original clone and inserted into the NotI/XhoI sites of pGEX CD1 (to make glutathione S-transferase (GST) fusion proteins), the NotI/HindIII site of pCMV5 (to make HA epitope-tagged proteins), or the SalI/XhoI sites of pVR1012Gal4.

RFC (p140) F1A–36 was generated by PCR using the following oligonucleotide primer: CTGCGGGCGCGCAAAGAAAGGAAATTAAAAGGAATTC. RFC (p140) F3 LXXGXXK was generated by overlap extension PCR using the following primers to create the mutation: ctggtgggctag-aagttgggatacagctacgtggaactg and cttctgacccaccagggaagctgtggtggttttgc-GGAAATC. RFC (p140) F1 and F3 described above, the wild type versions were remade using the same sites. These latter plasmids were used only in Fig. 3, D and E and were functionally identical to the original Gal4 fusion proteins. All PCR products and mutations were confirmed by sequencing.

The pCDNA3 HA-tagged HDAC1 and pING 14A-HDAC1 plasmids were supplied by Dr. Andy Bannister and Professor Tony Kouzarides (University of Cambridge). The reporter plasmids G5 TK-CAT and G0 TK-CAT were supplied by Dr. Stefan Roberts (University of Cambridge). The full-length RFC (p140) cDNA was isolated from human foreskin fibroblast cell RNA by reverse transcription polymerase chain reaction (PCR) and inserted into the KpnI site of pCDNA3 or pCGN, which inserted an HA tag at its amino terminus and the NcoI/NotI sites of pVR1012Gal4 (27). Fragments of RFC (p140) were isolated by PCR from the original clone and inserted into the NotI/XhoI sites of pGEX CD1 (to make glutathione S-transferase (GST) fusion proteins), the NotI/HindIII site of pCMV5 (to make HA epitope-tagged proteins), or the SalI/XhoI sites of pVR1012Gal4.

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constructed (Fig. 3A). RFC (p140) F1 (amino acids 1–369) encodes the amino terminus of the protein. RFC (p140) F2 (amino acids 367–493) encodes a domain with homology to DNA ligases and has a BRCT domain also found in BRCA1 and other proteins involved in DNA repair (20). RFC (p140) F3 (amino acids 480–882) is responsible for binding to PCNA and also contains the domain homologous to other RFC subunits, an LXCXE motif required for binding to Rb and a caspase-3 cleavage site (20, 23, 35). RFC (p140) F4 (amino acids 728–882) encodes the amino terminus of the protein. RFC (p140) F2 and F4 had only a weak inhibitory effect. RFC (p140) F1 was also expressed to the promoter to repress transcription. HEK293 cells were transfected with 3 μg of G5 TK-CAT together with pVR1012Gal4 alone (1 μg) or 10 ng, 100 ng, 500 ng, and 1 μg of pVR1012Gal4-RFC (p140) as indicated. Results shown are representative of three separate experiments. B, RFC (p140) needs to be targeted to the promoter to repress transcription. HEK293 cells were transfected with 3 μg of G5 TK-CAT or G0 TK-CAT, together with 100 ng of pVR1012Gal4 alone or pVR1012Gal4-RFC (p140). Results shown are representative of three separate experiments. C, repression of transcription by RFC (p140) is reversed by TSA. HEK293 cells were transfected with 3 μg of G5 TK-CAT together with 5 μg of Gal4-RFC (p140). Cells were either treated with 300 nM TSA for 24 h before harvesting or left untreated. Results are expressed as fold repression (CAT activity seen with Gal4 alone divided by that seen with Gal4-RFC (p140)) in the absence or presence of TSA. Results represent the means ± S.E. of three separate experiments.
(36). Deletion of this domain, which had no effect, demonstrated that transcriptional repression was not mediated by PCNA binding to this region (Fig. 3D). Recently, RFC (p140) has been shown to interact with the Rb tumor suppressor through an LXXCE motif present within the F3 region (23). Since Rb is a strong repressor of transcription that has also been shown to interact indirectly with histone deacetylases (18, 37) it was important that its involvement in repression by the RFC (p140) F3 region be determined. To facilitate this, a mutant form of RFC (p140) in which the LXXCE motif was mutated to LXGXXK was generated. This mutation has previously been found to disrupt the interaction between Rb and RFC (p140) (23). As a Gal4 fusion however, RFC (p140) F3 LXGXXK still repressed transcription to the same extent as wild type RFC (p140) F3, suggesting that association with Rb does not account for the effects seen here (Fig. 3E).

We next investigated whether these domains could interact with HDAC1. Consistent with their ability to repress transcription, both Gal4-RFC (p140) F1 and F3 co-immunoprecipitated with HDAC1, whereas no association was seen with Gal4-RFC (p140) F2 and F4 (Fig. 4A). Finally, we determined whether this interaction could be seen in vitro in a GST pull-down assay. Bacterially expressed GST-RFC (p140) F1 and F3 bound reticulocyte lysate-translated HDAC1 (Fig. 4B). No significant interaction was seen with GST alone or GST-RFC (p140) F1–36, which lacks the PCNA binding site. A sample of input material (10% of the total protein used in each pull-down assay) is shown. E, mutation of the Rb binding motif of RFC (p140) F3 does not affect binding to HDAC1 in vitro. The experiment was performed as in B using GST-RFC (p140) F1 and GST-RFC (p140) F1A1–36, which lacks the PCNA binding site. A sample of input material (10% of the total protein used in each pull-down assay) is shown. D, mutation of the Rb binding motif of RFC (p140) F3 does not affect binding to HDAC1 in vitro. The experiment was performed as in B using GST-RFC (p140) F1 and GST-RFC (p140) F1A1–36, which lacks the PCNA binding site. A sample of input material (10% of the total protein used in each pull-down assay) is shown.
sociation for these proteins, suggesting that DNA might have an inhibitory effect on their association.

**DISCUSSION**

In this report we have demonstrated that the large subunit of replication factor C, RFC (p140), can interact with the histone deacetylase HDAC1 through two distinct domains. It cannot be ruled out that RFC (p140) might interact with other cellular deacetylase activities, because these were not investigated. Although the in vitro association between these proteins suggests a direct interaction (Fig. 4), it cannot be ruled out at this time that an intermediary protein exists that facilitates the association of HDAC1 with RFC (p140). These interactions are functional in vitro because RFC (p140) can repress transcription in a TSA-sensitive manner when targeted to a promoter. The actual function of this RFC (p140)-associated deacetylase complex in vivo is unclear, however. RFC (p140) has been previously implicated as a transcriptional regulator. For example, it has recently been shown to interact with the bZIP transcription factor C/EBPs and was reported to stimulate transcription (26). This observation is not obviously consistent with an interaction between RFC (p140) and histone deacetylases, suggesting that such effects might be context specific or influenced by the cell type in which the study is performed. Significantly, as a Gal4 fusion we did not find that RFC (p140) stimulated gene expression (data not shown), suggesting transcriptional activation is not an intrinsic property of RFC (p140). More consistent with our observations is the report that RFC (p140) can interact with the tumor suppressor Rb (23). Rb is an important repressor of E2F transcriptional activity. Moreover, it accomplishes this in part through interacting with proteins that recruit histone deacetylase activity (18, 37). It is possible, therefore, that RFC (p140) might participate in this process.

Although we show that the RFC (p140) histone deacetylase complex has the potential to repress transcription as a Gal4 fusion, it is perfectly possible that this activity could be involved in a process other than the regulation of gene expression. Replication factor C also participates in the removal of PCNA following replication (20). As such, it might have a function in regulating newly assembled nucleosomes and re-establishing chromatin structure following DNA synthesis. Alternatively, this deacetylase activity might regulate the function of an RFC (p140)-associated protein. The Gal4 fusion experiments were performed principally to allow the activity of RFC (p140)-associated histone deacetylase activity to be analyzed in vivo, and it would be an overinterpretation to assume that it must therefore have a role in transcriptional regulation.

In conclusion, there are a number of possible roles that the RFC (p140)-associated deacetylase activity might perform, and these will require further experimentation to fully understand. Nonetheless, this study adds to a growing body of evidence that the function of RFC (p140) is more complex and dynamic that originally thought. It also highlights the similarities and possible dual functions of proteins associated with replication and transcription. Given the complexity of chromatin-remodeling activities associated with transcriptional regulators, it can be anticipated that many similar interactions remain to be discovered.

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