Human CRSP interacts with RNA polymerase II CTD and adopts a specific CTD-bound conformation

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Activation of gene transcription in mammalian cells requires several classes of coactivators that participate in different steps of the activation cascade. Using conventional and affinity chromatography, we have isolated a human coactivator complex that interacts directly with the C-terminal domain (CTD) of RNA polymerase II (Pol II). The CTD-binding complex is structurally and functionally indistinguishable from our previously isolated CRSP coactivator complex. The closely related, but transcriptionally inactive, ARC-L complex failed to interact with the CTD, indicating a significant biochemical difference between CRSP and ARC-L that may, in part, explain their functional divergence. Electron microscopy and three-dimensional single-particle reconstruction reveals a conformation for CTD–CRSP that is structurally distinct from unliganded CRSP or CRSP bound to SREBP-1a, but highly similar to CRSP bound to the VP16 activator. Together, our findings suggest that the human CRSP coactivator functions, at least in part, by mediating activator-dependent recruitment of RNA Pol II via the CTD.

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Results and Discussion

To identify putative human cofactors that interact selectively with the RNA Pol II CTD, we screened HeLa nuclear extract (NE) using an affinity resin composed of the human RNA Pol II CTD [52 heptad repeats] fused to glutathione-S-transferase (GST–CTD). More than 30 polypeptides from HeLa NE were specifically retained on the GST–CTD column as compared with control resins [Fig. 1A, lane 1; data not shown]. Fractionation of the HeLa NE over a phosphocellulose (PC) column prior to CTD affinity purification revealed that the polypeptides bound to the CTD column could be separated into two populations eluting at 0.5 M KCl (PC 0.5M) and 1 M KCl (PC 1M), respectively [Fig. 1A, lanes 4 and 5]. Intriguingly, the polypeptide pattern from the PC 1M fraction closely resembled that of the CRSP coactivator identified previously in our laboratory [Ryu et al. 1999]. To investigate a possible relationship between the PC 1M-derived CTD-binding polypeptides and CRSP, we examined whether the CTD-binding fractions [from PC 0.5M and PC 1M] could substitute for CRSP in a chromatin-based in vitro transcription assay. This assay utilized a LDLR-derived chromatin template driven by the SREBP-1a and Sp1 activators that require the CRSP coactivator component of ARC for transcriptional activation [Näär et al. 1999; Taatjes et al. 2002]. The CTD-binding polypeptides purified from the PC 0.5M phosphocellulose fraction were largely inactive [Fig. 1B, cf. lanes 2 and 4]; however, the PC 1M-derived polypeptides strongly potentiated [>100-fold] SREBP-1a/Sp1-dependent activation [Fig. 1B, cf. lanes 2 and 6], suggesting that this class of polypeptides harbors a functional CTD-binding coactivator. Further purification of the PC 1M-derived CTD-associated polypeptides using glycerol gradient sedimentation [Fig. 1C] confirmed that they were components of a large ~1-MD multiprotein complex [Fig. 1D, lanes 3–5]. The transcriptionally inactive PC 0.5M-derived CTD-binding polypeptides also purify as a large, multisubunit complex. Peptide microsequence analysis of individual subunits indicates that this complex is composed of novel gene products that are unrelated to subunits of known transcriptional coactivator complexes and may not be directly involved in regulation of transcription initiation [A.M. Näär, unpubl.]. We have not pursued the characterization of this CTD-binding complex further.

Direct comparison of SDS–polyacrylamide silver-stained gels of the PC 1M-derived CTD-binding complex and CRSP confirms that these two coactivator complexes are highly related or identical [Fig. 2A, cf. lanes 1 and 2]. Immunoblotting confirmed the identity of several of the polypeptides found in the CTD-binding complex as bona fide CRSP subunits [Fig. 2B, cf. lanes 1 and 2]. Interestingly, no ARCl–specific polypeptides [ARC240, ARC250, cdc8] cyclin C] appeared to bind the CTD affinity column, despite their presence in the PC 1M fraction. This suggests that the ARC-L complex, in contrast to CRSP, is unable to interact with the CTD of RNA Pol II. This result is consistent with previous reports indicating that SMCC and NAT, which are highly related to ARCl, are also unable to associate directly with the RNA Pol II CTD [Sun et al. 1998; Gu et al. 1999]. Because ARCl lacks the CRSP70 subunit, it is possible that the CRSP–CTD interaction is mediated by CRSP70. However, it is notable that a CRSP70 homolog is absent in yeast Mediator. Alternately, we propose that the additional subunits in ARCl may occlude a CTD-specific binding surface on the CRSP complex. Some additional protein density is present near the CTD-binding region in ARCl [Taatjes et al. 2002].

Although the CTD-binding complex was found to be a potent coactivator in our in vitro transcription assays and exhibited a subunit composition similar to CRSP, we nevertheless wished to directly compare their coac...
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tivator activities in our chromatin-based transcrip-

tion system. As shown in Figure 2C, the CTD-binding complex and CRSP exhibit simi-

lar specific activities in this assay [cf. lanes 4 and 6 for single-point analysis, titrations not

shown]. These results further suggest that the CTD-binding complex and CRSP are function-

ally related. Accordingly, we will refer to the CTD-binding complex [derived from the PC 1M

fraction] as CTD–CRSP throughout the rest of the text.

Other biochemical similarities between CTD–CRSP and CRSP were established by specific

activator-binding experiments. We documented previously the ability of SREBP-1a and VP16 ac-

tivation domains to bind CRSP [Taatjes et al. 2002]. Here, we find that affinity resins bearing

the SREBP-1a or VP16 activation domains efficiently deplete CTD–CRSP from the PC 1M

fraction (Fig. 2D, cf. lanes 2 and 3 with 5 and 6). In contrast, GST control resins failed to bind

CRSP from this same fraction (Fig. 2D, cf. lanes 1 and 4). Conversely, prior depletion of PC 1M

with GST–CTD significantly reduced the amount of CRSP bound to GST–SREBP-1a and

VP16 activation domain resins [data not shown].

Together, these findings establish that the activator-targeted human CRSP coactivator can

interact with the CTD of RNA Pol II. By virtue of this interaction with the CTD, CRSP [but not

ARC-L] may help recruit RNA Pol II to the promoter. We observed previously that CRSP and

ARC-L possess contrasting transcriptional properties in vitro; CRSP displayed potent, co-

activator activity, whereas ARC-L was inactive [Taatjes et al. 2002]. Given that the RNA Pol II

CTD has been implicated in the activation of transcription [Gerber et al. 1995], we speculated

that the CRSP-specific interaction with the CTD may mediate its coactivator function. To

substantiate this, we examined whether disruption of this interaction would inhibit CRSP-de-

pendent transcriptional activation. An excess of free GST–CTD was added to the transcription

reactions, which potently inhibited CRSP-dependent transcriptional activation in a dose-de-

pendent manner [Fig. 2E, lanes 3–8]. In contrast, addition of GST alone had no significant effect

[Fig. 2C, cf. lanes 5 and 6 with E, lanes 1 and 2]. These results further show the functional im-

portance of the CTD in potentiating transcript initiation. However, given the essential role of

the CTD in multiple aspects of the transcription process, including transcript elongation,

we cannot exclude the possibility that the ex-

ogenously added CTD may also titrate other ac-

tivities required for transcription.

In addition to the biochemical characteriza-

tion of CTD–CRSP, we determined its struc-

tural characteristics using EM and single par-

ticle reconstruction techniques [see Materials

and Methods]. A micrograph of a typical nega-

tively stained CTD–CRSP sample is shown in

Figure 3A. The three-dimensional structure of

CTD–CRSP, reconstructed from 3662 single



Figure 2. Comparative analysis of VP16-purified CRSP and the PC 1M-de-

rived CTD-binding complex. (A) Silver stain of SDS-PAGE-separated VP16-

CRSP [lane 1] and the CTD-binding complex [lane 2]. The bands correspond-

ing to GST–CTD and GST–VP16 are indicated at left, along with nonspecific

proteins (*). The molecular weights of the subunits are indicated at right [in kilodaltons]. (B) Immunoblot analysis of VP16–CRSP [lane 1] and the PC

1M-derived CTD-binding complex [lane 2]. The ARC-L/CRSP subunits ana-

lyzed are indicated at right. (C) Direct comparison of coactivator activity

associated with equal quantities of VP16-purified CRSP and CTD-purified

complex. SREBP-1a/Sp1-dependent activation of the LDLR-derived chroma-

tin template [lanes 1,5, no activator; lanes 2,4,6, SREBP-1a and Sp1] was

analyzed in the absence of added protein [lanes 1,2], in the presence of 0.5 nM

CTD-binding complex [lanes 5,6], or in the presence of 0.5 nM VP16-purified

CRSP [lanes 3,4]. The primer extension product is indicated by the arrowhead

at right. (D) Analysis of presence of CTD-binding complex in activator- or

control-depleted PC 1M. Silver-stain analysis shows depletion of CTD-com-

plex from PC 1M by CRSP-targeting activation domains. The PC 1M fraction

was depleted (see Materials and Methods) using resins containing GST [lane

1], GST–SREBP1a [lane 2], or GST–VP16 [lane 3]. Lanes 1–3 show bound

material after first depletion. After depletion, the PC 1M fractions were in-

cubated with GST–CTD resin and bound material was then analyzed by

SDS-PAGE and silver staining [lanes 4–6]. (E) Addition of exogenous CTD

inhibits gene activation dependent on the CTD-purified complex. SREBP-1a/

Sp1-dependent activation was analyzed as in C after the addition of 5 pmole

[lanes 3,4], 15 pmole [lanes 5,6], or 50 pmole [lanes 7,8] of GST–CTD, or 50

pmole of GST alone [lanes 1,2]. The primer extension product is indicated at

right by an arrowhead.
particle images, following multiple rounds of angular refinement, is shown in Figure 3B. The complex is somewhat elongated and possesses three distinct regions, the head region, which contacts the protein-dense body in two areas to form a lobular density with a central cavity, and a hook-like leg domain that contacts the body from the opposite side. The relatively large size of the complex (340 Å × 160 Å × 135 Å) suggests it may be capable of mediating many protein–protein interactions at the promoter. TFIID, for example, is considerably smaller by comparison (200 Å × 135 Å × 110 Å) (Andel et al. 1999).

In a previous study, we used EM single-particle reconstruction techniques to identify structural characteristics of the CRSP coactivator bound to different activators (Taatjes et al. 2002). This study revealed that CRSP was conformationally flexible and capable of adopting multiple activator-dependent conformations. Specifically, CRSP assumed three distinct conformations when unliganded, or bound to VP16 or SREBP-1a. Interestingly, CRSP adopts a conformation very similar to VP16–CRSP (for comparison, see Fig. 3C ) when bound to the CTD, as evident by visual comparison and cross-correlation analysis (see Materials and Methods). The fact that CTD–CRSP adopts a conformation similar to VP16–CRSP suggests that this conformational state may represent a particular activated form of the CRSP complex that efficiently potentiates transcript initiation. Such structurally dynamic transitions may facilitate activation by allowing CRSP to associate with other ligands, such as specific activators or other components of the transcriptional apparatus.

The CTD-binding site was localized on the CRSP complex using CTD–CRSP samples labeled with anti-GST antibodies (CTD is present as a GST fusion protein). Samples of CTD–CRSP were prepared as described (Fig. 1C), followed by addition of antibody in a fivefold excess. Subsequent three-dimensional reconstruction and difference mapping of antibody-labeled versus unlabeled samples localized the CTD-binding site to a relatively small region between the head and body of the complex (Fig. 4A). Both polyclonal and monoclonal antibodies against GST were used for this analysis and yielded similar results in independent experiments. Incidentally, VP16 binds a similar, but not identical, region on the CRSP complex (Fig. 4B; Taatjes et al. 2002). Thus, the CTD and VP16 bind proximal regions and induce similar conformations in the CRSP coactivator. This suggests that VP16 may be able to substitute for, but not compete with, a potential function of the CTD in activating transcription. By inducing a CTD-bound conformation in the CRSP coactivator, VP16 may circumvent CTD-dependent regulatory mechanisms that would otherwise moderate transcript initiation. Although it is likely that VP16 and the CTD target different peptide sequences in CRSP, these sequences may reside in the same subunit. VP16 is proposed to bind CRSP77 (TRAP80) (Ito et al. 1999); interestingly, the homolog of this subunit is essential for viability in Drosophila (Boube et al. 2000). Although the subunit that mediates CRSP interaction

Figure 3. CTD–CRSP and VP16–CRSP are structurally similar. [A] Negatively stained electron micrograph of CTD–CRSP sample. Bar, 800 Å. (B, C) Three-dimensional reconstruction of CTD–CRSP and VP16–CRSP at 32 Å resolution. Complexes are rendered to 1.25 MD, their approximate predicted molecular weight. Dimensions shown. Rotation of the volumes 90° gives the second side view of the coactivator.

Figure 4. (A) Localization of the CTD binding site (yellow) on the CRSP coactivator. This site was identified via EM analysis and difference mapping of structures generated from CTD–CRSP samples incubated with anti-GST antibodies, which target the GST–CRSP fusion protein bound to the CRSP complex. As in A, the CTD-binding site is shown in yellow. The VP16-binding site is indicated by the white arrowhead.
with the CTD is unknown, it also may be essential for viability given the likely importance of the CRSP–RNA Pol II interaction in activating gene expression.

Our findings identify the CRSP complex as a probable link between RNA Pol II and human transcriptional activators, analogous to the suggested function of Mediator in yeast. Furthermore, yeast Mediator selectively binds the hypophosphorylated form of RNA Pol II CTD, phosphorylation appears to prevent CTD–Mediator association [Myers et al. 1998]. This is consistent with our current results, insofar as we observe strong interaction between recombinant, unphosphorylated CTD and the human CRSP coactivator complex. Thus, despite substantial divergence in subunit composition and structure, some core biochemical functions of yeast Mediator and human CRSP, such as interaction with activators, RNA Pol II CTD binding, and transcriptional coactivation, appear to have been maintained over evolutionary time.

Materials and methods

GST-pull-down assays

A total of 1 mL of HeLa NE or PC fractions was applied to 25 µL of GST–CTD beads (Peterson et al. 1992) and mixed at 4°C for 3 h. Beads were washed 7 x 1 mL with 0.5 M KCl HEGN (20 mM HEPES at pH 7.6, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 1 mM benzamidine, 0.25 mM PMFS, 2 µg/mL aprotinin) and 1 x 1 mL of 0.1 M KCl HEGN + 0.02% NP-40. The beads were then eluted with a Tris-buffered 20 mM glutathione solution (0.1 M KCl). For depletion experiments, 500 µL of PC 1M was mixed with 100 µL of GST, GST SREBP-1a (amino acids 1–50), or GST–VP16 (amino acids 413–490) beads for 2 h. The supernatant was then transferred to 100 µL of fresh beads and mixed for another 2 h at 4°C. The double-depleted fraction was then incubated with GST–CTD and analyzed as above. For elution of GST–CTD-associated proteins, 2 x 1 bead volumes of 0.1 M KCl HEGN with 0.25% sarkosyl or 20 mM glutathione were added and mixed at 4°C for 1 h each.

Purification of CTD–CRSP

HeLa NE was prepared as described [Dignam et al. 1983] and loaded onto a P11 PC column equilibrated in 0.1 M KCl HEGN (20 mM HEPES at pH 7.6, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and protease inhibitors). Eluted fractions were dialyzed against 0.1 M KCl HEGN. Typically, 10 mL of the PC 1M fraction was mixed with 100 µL of GST, GST SREBP-1a (amino acids 1–50), or GST–VP16 (amino acids 413–490) beads for 2 h. The supernatant was then transferred to 100 µL of fresh beads and mixed for another 2 h at 4°C. The double-depleted fraction was then incubated with GST–CTD and analyzed as above. For elution of GST–CTD-associated proteins, 2 x 1 bead volumes of 0.1 M KCl HEGN with 0.25% sarkosyl or 20 mM glutathione were added and mixed at 4°C for 1 h each.

Immunoblotting

Immunoblotting was performed essentially as described [Näär et al. 1999] using the specified antibodies.

Chromatin-based in vitro transcription

The template plasmids were assembled into chromatin as described [Näär et al. 1998]. Drosophila embryo cytosolic extract (S-190), purified Drosophila core histones, Mg/ATP, and an ATP-regenerating system were added to supercoiled DNA template, and assembly was performed for 4.5 h at 27°C. Transcription reactions were performed as described [Näär et al. 1998].

Electron microscopy and single particle reconstruction

Electron micrographs were obtained using a Tecnai 12 TEM at 30,000× magnification. Glycerol gradient-purified CTD–CRSP samples were applied to a glow-discharged carbon grid and negatively stained with a 4% uranyl acetate solution. Each sample was dialyzed versus a 5% trichose solution (20 mM HEPES, 0.1 mM EDTA, 0.1M KCl) prior to analysis. Micrographs [38] were digitized with a scan step of 13.3 µm (4.4 Å/pixel). Image pairs of tilted (35°–45°) and untitled (0°) complexes were obtained and analyzed via random conical tilt [Radermacher et al. 1987] using the SPIDER and WEB software packages [Frank et al. 1996]. Untitled images were subjected to reference-free alignment and merged into 24 distinct classes (indicative of their orientation on the grid) following in-plane shifts and rotations [Penczek et al. 1992]. Three-dimensional structures for each class were calculated by back projection using corresponding tilted images. These three-dimensional structures were then correlated against each other to establish a homogeneous data set. Related classes (comprising 82% of the data set and having a correlation coefficient of 0.80 or higher) were subsequently merged to generate an initial three-dimensional reference structure. This structure was subjected to multiple rounds of angular refinement by use of the previously defined homogeneous data set (3662 particles). Experimental images were matched to reference projections. On the basis of highest cross-correlation, a refined volume was then calculated with the newly identified Euler angles [Penczek et al. 1994]. This process was repeated multiple times until the angles did not change and the resolution of the reconstruction did not improve. Final angular refinement was performed by generating 796 reference projections with an angular step of 5°. The CTD–CRSP structure was reconstructed to a resolution of 32 Å, on the basis of the 0.5 Fourier shell correlation criteria [Harauz and van Heel 1996]. At this resolution, no CTF correction was needed.

Antibody labeling experiments

After eluting the CTD–CRSP complex from the affinity column, a five-fold excess of anti-GST antibodies were added and mixed for 1 h at 4°C. This sample was then run over a glycerol gradient to remove unbound antibody. Antibody-labeled CTD–CRSP samples were then analyzed by electron microscopy as described above, except that no tilted images were obtained, as the unlabeled CTD–CRSP structure was used as reference.

Cross-correlation analysis

All 24 classes within the CTD–CRSP data set had correlation coefficients between 0.78 and 0.88. Classes at the lower end of this range may represent degraded complexes, alternate conformers, or distorted complexes. Such classes were excluded from angular refinement because they reduced the quality (resolution) of the reconstruction. The average correlation coefficient of the classes used for angular refinement of CTD–CRSP was 0.83, which serves as a reference indicative of conformational similarity. Cross-correlation of CTD–CRSP and VP16–CRSP yielded a correlation coefficient of 0.88. For comparison, the correlation coefficient between conformationally distinct VP16–CRSP and SREBP–CRSP structures is 0.77.

Structural analysis of CTD–CRSP and VP16–CRSP was done completely independently (via random conical tilt) without reference bias. The dynamic nature of the CRSP coactivator suggests that, despite adopting specific and distinct conformational states, a degree of flexibility is maintained in each. Consequently, structures resolved by electron microscopy likely represent an average conformation about which the structure oscillates. For these reasons, it is likely that the structures of VP16–CRSP and CTD–CRSP are not 100% identical, although they clearly represent the same conformational state.

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