Identification of a Cyclin T-Binding Domain in Hexim1 and Biochemical Analysis of Its Binding Competition with HIV-1 Tat*

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The active form of the positive transcription elongation factor b (P-TEFb) consists of cyclin T and the kinase Cdk9. P-TEFb stimulates transcription by phosphorylating the C-terminal domain of RNA polymerase II. It becomes inactivated when associated in a tetrameric complex with the abundant 7SK small nuclear RNA and the recently identified protein Hexim1. In this study, we identified a stable and soluble C-terminal domain (residues 255–359) in Hexim1 of 12.5-kDa size that binds the cyclin boxes of Cyclin T1. Functional assays in HeLa cells showed that this cyclin T-binding domain (TBD) is required for the binding of Hexim1 to P-TEFb and inhibition of transcriptional activity in vitro. Analytical gel filtration and GST pull-down experiments revealed that both full-length Hexim1 and the TBD are homodimers. Isothermal titration calorimetry yielded a weak multimer for the TBD with a multimerization constant of 1.3 × 10^3 M. The binding affinity between the TBD and cyclin T1 was analyzed with fluorescence spectroscopy methods, using a dansyl-based fluorescence label at position G257C. Equilibrium fluorescence titration and stopped flow kinetics yield a dissociation constant of 1.2 μM. Finally, we tested the effect of the HIV-1 Tat protein on the cyclin T1-TBD complex formation. GST pull-down experiments and size exclusion chromatography exhibit a mutually exclusive binding of the two effectors to cyclin T1. Our data suggest a model where HIV-1 Tat competes with Hexim1 for cyclin T1 binding, thus releasing P-TEFb from the inactive complex to stimulate the transcription of HIV-1 gene expression.

The positive transcription elongation factor b (P-TEFb)† is a key regulator of the productive transcription elongation of nascent pre-mRNA molecules into mature mRNA by RNA polymerase II (1). The action of P-TEFb has been studied most intensively in the regulation of the human immunodeficiency virus (HIV) gene expression, which is the paradigm for the control of transcription at the level of elongation. Shortly after promoter clearance, RNA polymerase II stalls due to the action of the negative elongation factor, which consists minimally of the sensitivity-inducing factor 5,6-dichloro-1-6-D-ribofuranosyl-benzimidazole riboside (DSIF) and the negative elongation factor (2, 3). During this postinitiation block, only short transcripts arise that may be prematurely terminated (1, 4). In HIV-infected cells, this block is relieved by the recruitment of P-TEFb to the paused RNA polymerase II by the viral Tat (transcriptional transactivator protein). P-TEFb phosphorylates the C-terminal domain of the RPB1 subunit of RNA polymerase II as well as the Spt5 subunit in DSIF and the RD subunit in negative elongation factor. As a consequence, transcription elongation ensues. In addition to HIV gene expression, cellular genes require P-TEFb as well. On one hand, short transcripts have been also found during transcription of many genes, including c-myc, c-myc, c-fos, and HSP70. On the other hand, several eukaryotic transcription factors bind P-TEFb to stimulate transcription (for a review, see Ref. 5). Finally, DRB and flavopiridol, two ATP analogs that inhibit the kinase activity of Cdk9 (cyclin-dependent kinase 9), inhibit nearly all transcription by RNA polymerase II in human cells (6).

The active P-TEFb complex is composed of two subunits, Cdk9 and its regulatory subunit, cyclin T1, T2, or K. However, unlike other Cdks and their cyclin partners, P-TEFb is constitutively expressed, and its protein levels vary little throughout the cell cycle (7). Accordingly, a large inactive P-TEFb complex has been identified only recently, which comprises four subunits: Cdk9, cyclin T1 or T2, the abundant small nuclear RNA 7SK (8, 9), and the coupling protein Hexim1 (hexamethylene bisacetaamide-inducible protein 1) (10, 11). Hexim1 (also named MAQ1) has been identified in human vascular smooth muscle cells after treatment with hexamethylene bisacetamide (12) and was shown to suppress the transcriptional activity of cotransfected genes such as NF-κB or HIV-1 long terminal repeat (10, 13). Roughly half of nuclear P-TEFb in HeLa cells is sequestered in an inactive state; however, inactive and active P-TEFb complexes can rapidly interchange upon treatment with stress-inducing agents (10, 11). Cardiac hypertrophy inducers have been reported as physiological triggers of P-TEFb/7SK dissociation in cardiomyocytes (14). A transcriptional arrest triggers dissociation of 7SK and Hexim1 from Cdk9 and Cyclin T1 (or T2) and subsequent accumulation of kinase-active P-TEFb (8, 9). 7SK snRNA and Hexim1 thus form a kinase inhibitor that contributes to the regulation of gene transcription.

On a molecular level, several interaction sites that influence the formation of the large P-TEFb-Hexim1–7SK snRNA com-
Cyclin T-binding Domain of Hexim1

Hexim1 has been identified as a stable 12-kDa C-terminal cyclin T-binding domain of Hexim1 (TBD; residues 255–359) that is sufficient to bind cyclin T1 in vitro and is required for the binding to P-TEFb and inhibition of transcription in vivo. Fast fluorescence studies reveal a dissociation constant of both molecules around 1 μM. From size exclusion chromatography and isothermal titration calorimetry, full-length Hexim1 as well as the C-terminal domain are found to be dimeric, whereas the cyclin T1-Hexim1 complex is heterodimeric. Finally, HIV-1 Tat and Hexim1 bind mutually exclusively to cyclin T1, suggesting a competition between Tat and Hexim1 for a similar binding site on cyclin T1.

EXPERIMENTAL PROCEDURES

Protein Sequence Analysis—Computational investigations of the human Hexim1 sequence (BA931666) were done prior to protein fragmentation. For domain architecture analyses, the following open access programs were quoted: SMART (available on the World Wide Web at smart.embl-heidelberg.de/), Prosite (available on the World Wide Web at www.expasy.org/prosite/), AnDom (available on the World Wide Web at www.bork.embl-heidelberg.de/AnDom/), and Pfam (available on the World Wide Web at pfam.wustl.edu/). Coiled-coil predictions were run at EMBL computational services (available on the World Wide Web at www.ebi.ac.uk/services/coiled-coil-swrl/), and secondary structure predictions were calculated using PredictProtein (available on the World Wide Web at www.predictprotein.org/), the PSIPRED Protein Structure Prediction Server (available on the World Wide Web at bioinf.cs.ucl.ac.uk/psipred/), Scratch at the Institute for Genomics and Bioinformatics, University of California Irvine (available on the World Wide Web at www.igb.uci.edu/tools/scratch/), and Coils at the European Bioinformatics Institute (available on the World Wide Web at smart.embl-heidelberg.de/). Three-dimensional structures were calculated using the SWISS-MODEL (available on the World Wide Web at web.expasy.org/swissmod/). The coordinates of the homology model were deposited in the Protein Data Bank (pdb: 3HIE).

Protein Expression, Purification, and Fluorescence Labeling—Plasmids encoding Hexim1 (accession number AB021179) were generated by PCR-mediated amplification with a primer containing Neol and EcoRI restriction sites and cloned into the prokaryotic expression vectors pProExHTa (Invitrogen) or pGEX-TT-tv (Amersham Biosciences) for protein expression and purification. Site-directed mutagenesis was performed using the megaprimer method with both sense and antisense oligonucleotides. The plasmid coding for the CycT1-Tat fusion protein was described previously (20).

For Hexim1-TBD protein expression plasmids were transformed into Escherichia coli BL21 (DE3) cells (Novagen), expressed at 30 °C, and induced at an A600 of 0.6–1.0 with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h growth. Full-length Hexim1 and Hexim1 (residues 1–254) proteins were grown at 18 °C and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for overnight expression. Cells were fluidized in lysis buffer A (20 mM Tris/HCl, pH 7.8, 500 mM NaCl, 5 mM β-mercaptoethanol) with 20 mM imidazole and cleared by spinning for 30 min at 30,000 × g. The lysate was loaded onto 5 ml of Ni2+-nitrilotriacetic acid resin (Qiagen) that had been pre-equilibrated with lysis buffer. After washing with 10 volumes of lysis buffer A, the protein was eluted with 10 volumes of lysis buffer A using a linear gradient from 20 to 250 mM imidazole. The peak fractions were dialyzed in buffer A, and the histidine tag was cleaved off at 4 °C over 12 h with Tve protease. Hexim1 was depleted of the protease and of the uncleaved His-Hexim1 by affinity chromatography. The protein-containing flow-through was concentrated and then further purified by gel filtration on an S75 column in 20 mM Tris/HCl (pH 7.8), 50 mM NaCl. Fractions were analyzed by SDS-PAGE, and fractions containing Hexim1 (about 98% pure) were concentrated and stored at −80 °C. Protein concentrations were determined by extinction coefficient measurements.

The surface-exposed cysteine at position 257 of the mutant protein Hexim1 (255–359, G257C, C297S) was labeled in thiol-free buffer (20 mM Tris/HCl (pH 7.8), 50 mM NaCl, 5% glycerol (v/v)) with a 10-fold excess of 5-iodoacetamidomethyliminophenazine-1-sulfonic acid (1,5-IAEDANS) (Molecular Probes, Inc., Eugene, OR). The reaction was run out at 4 °C for 12 h and subsequently stopped by buffer exchange via gel filtration. Labeling efficiency and specificity was confirmed by MALDI-TOF mass spectrometry, and removal of excess fluorophore was proved by SDS-PAGE using UV irradiation.

**GST Pull-down Assays**—For direct interaction assays between Hexim1, cyclin T1, and Tat, about 2 μg of GST or GST fusion proteins were immobilized on glutathione-Sepharose beads (Amersham Biosciences) and incubated with 10–20 μg of the respective target protein. Binding reactions were performed in 500 μl of buffer solution (20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM β-mercaptoethanol) for 2–4 h at 4 °C or for 0.5 h at room temperature, respectively. Beads were washed 3–5 times in the same buffer, and bound proteins were analyzed by Coomassie SDS-PAGE.

**Two Binding and Inhibition Assays**—Plasmid reporter pGSTAR was described previously (21). Plasmids coding for the FLAG-tagged full-length Hexim1 (pFLAG-CMV-2.Hexim1) and C-terminal deletion constructs were a kind gift from Dr. Zhou and Dr. Tanaka and were described previously (10, 13). To construct plasmids coding for the mutant Hexim1 (G257C/C297S) protein, the pFLAG-CMV-2.Hexim1 plasmid was subjected to site-directed mutagenesis with the QuikChange II XL site-directed mutagenesis kit (Stratagene). HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 μM t-glutamine, and 50 μg each of penicillin and streptomycin per ml. Cells were grown at 37 °C with 5% CO2. The goat polyclonal anti-CycT1 (sc-8127) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse monoclonal anti-FLAG M2 antibody and the anti-FLAG M2 beads were purchased from Sigma (product number M2) and Sigma (product number A9274), respectively. Total cell extracts were sonicated in 50-mm diameter Petri dishes ~12 h prior to transfection and transiently transfected with FuGen6 reagent according to the manufacturer’s instructions (Invitrogen). Chloramphenicol acetyltransferase enzymatic assays were performed as described (22). In all transfections, the amount of DNA was equilibrated with a corresponding empty vector. The activity of the reporter plasmid alone is given as 1. Data are representative of three independent transfections, which were performed in duplicate. The error bars give S.E.

HeLa cells were seeded into 100-mm diameter Petri dishes and transfected with the indicated plasmid vectors. About 36–48 h post-transfection, cells were lysed in 0.8 ml of lysis buffer (1% (v/v) Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1% (w/v) proteinase inhibitor) for 1 h on ice. Total cell extracts were sonicated and centrifuged at 10,000 × g for 10 min at 4 °C. Total cell extracts were then precipitated with the anti-FLAG M2 beads and examined for the presence of CycT1 and FLAG-tagged Hexim1 proteins by Western blotting with the anti-CycT1 and anti-FLAG antibodies according to standard protocols, respectively.

**Analytical Gel Filtration**—Analytical gel filtration was performed with a multicomponent Waters 626 LC system (Waters, MA) using a Superdex 75 column (10/30; column volume 25.7 ml; Amersham Biosciences). The column was first equilibrated with 20 mM Tris/HCl buffer (pH 7.8), 50 mM NaCl, 2 mM dithioerythritol following injection of the samples (30–50 μg). The flow rate was set to 0.5 ml/min. Elution profiles were monitored by UV absorption at 280 nm and calibrated to a low molecular weight standard (Amersham Biosciences). Equimolar concentrations of CycT1 and Hexim1 were mixed 30 min prior to the experiment and incubated at 25 °C. Gel filtration experiments were performed at room temperature.

**Isothermal Titration Calorimetry**—The thermodynamic parameter of Hexim1 multimerization and the CycT1/Hexim1-TBD interaction were determined by isothermal titration calorimetry (MCS-ITC; MicroCal, Amherst, MA). For Hexim1 dissociation experiments, 500 μM Hexim1 was dissolved in the titration buffer (100 mM Tris/HCl (pH 7.4), 150 mM NaCl) and injected into the cell, which was filled with the same buffer solution. For complex formation experiments, CycT1 was placed into the cell at concentrations varying between 5 and 70 μM, and a 10-fold higher concentration of Hexim1-TBD was filled in the syringe. Data evaluation was performed using the manufacturer’s analysis software, yielding ΔH° and ΔS° values with 0.5 kcal/mol errors each. All ITC experiments were carried out at 25 °C in 20 mM Tris/HCl buffer (pH 7.8) and 50 mM NaCl.

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Fluorescence Spectroscopy Measurements and Data Analysis—All fluorescence measurements were performed at 20 °C in a buffer containing 20 mM Tris/HCl (pH 7.8), 50 mM NaCl, 5% (v/v) glycerol, and 2 mM dithioerythritol. Equilibrium measurements were carried out on a SPEX FluoroMax III spectrofluorometer (JobinYvon, Longjumeau, France) at an excitation wavelength of 337 nm and a band pass of ±1 nm. For emission spectra, fluorescence emission was monitored with a 420-nm cut-off filter. Binding of CycT1 to Hexim1-TBD was detected by changes in the fluorescence of the EDANS fluorophore coupled to the protein. The resulting inverse time constant corresponds to the observed rate constant $k_{on}$ within an experimental error of 2–10%. Unless indicated otherwise, all measurements were done in buffer containing 20 mM Tris/HCl buffer (pH 7.8) and 50 mM NaCl at 20 °C.

RESULTS

Indication of a Highly Charged C-terminal Domain in Hexim1—We started to analyze the human Hexim1 protein (accession number BAA36166) by computational methods, including modular domain searches and secondary structure prediction analyses (Fig. 1). As was found in SMART, Prosite, AnDom, and PFam investigations, the 41-kDa Hexim1 protein does not contain any known functional or structural domain but is proposed to carry a central bipartite nuclear localization signal, which has already been confirmed in cellular studies (11, 13, 15). Furthermore, a C-terminal coiled-coil region from position 283 to 349 or from position 284 to 314 and position 322 to 342 was suggested (coiled-coil prediction). Overall, the protein is highly hydrophilic, with 125 charged residues of 359 amino acids (Fig. 1B). These charges are distributed almost homogeneously over the entire protein but accumulate in one positive cluster (residues 150–165; nuclear localization signal) and two negative clusters (residues 211–219 and 234–253). On average, nontransmembrane human proteins contain only 23% charged residues, whereas Hexim1 contains 35%. Similarly, bulky hydrophobic residues such as Leu, Ile, Val, Met, and Phe are spread over the whole protein with slightly higher appearance of the C-terminal to position 250 (Fig. 1B). In contrast, numerous proline residues that often induce nonstructured regions occur particularly frequently in the N-terminal part of Hexim1 up to position 133 followed by another five prolines until position 218, whereas only one is found in the C-terminal 141 residues.

Binding curves, titration experiments, and dissociation experiments were analyzed with Origin 7.0 (OriginLab Corp.).

Stopped Flow Measurements—Measurements of association rates were done using an SX.18MV stopped flow apparatus (Applied Photophysics, Surrey, UK) by rapid mixing of 3 μM Hexim1-TBD-EDANS and 1.5–30 μM GST-CycT1 (1–292). The dansyl-based fluorophore was excited at 337 nm, and the fluorescence emission was recorded through a 420-nm cut-off filter. Binding of CycT1 to Hexim1-TBD was detected by a change in the fluorescence of the EDANS fluorophore coupled to cysteine 257. An exponential equation was fitted to the fluorescence traces according to pseudo-first-order kinetics. The resulting inverse time constant corresponds to the observed rate constant $k_{on}$ within an experimental error of 2–10%. Unless indicated otherwise, all measurements were done in buffer containing 20 mM Tris/HCl buffer (pH 7.8) and 50 mM NaCl at 20 °C.

The protein sequence analysis was complemented by secondary structure predictions from four independent calculations (Fig. 1C). Overall, only a small part of Hexim1 is predicted to adopt a $\alpha$-helical fold, whereas large segments, particularly of the N-terminal region, are proposed to be unfolded. The suggested helical parts converge into two clusters from position 170 to 195 and for the proposed coiled-coil region from position 260 to 350. The prediction of unfolded regions in the N-terminal region may be most likely due to the high degree of polarity and the numerous glutamic acid (13.0%), proline (9.6%), serine (7.5%), glutamine (7.5%), and lysine (7.1%) residues. These residues often indicate intrinsically unstructured protein domains (23). The detailed reliability index of the prediction for $\alpha$-helical and extended secondary structure elements as derived from the PredictProtein calculation is shown in Fig. 1D. In summary, the C terminus of Hexim1 is proposed to form a folded region, whereas the N-terminal part appears rather unstructured.

The C-terminal Domain of Hexim1 Interacts with Cyclin T1 Directly in Vitro—To investigate the proposed domain modules in Hexim1, we cloned four different constructs as His epitope-tagged fusion proteins, expressed these in *E. coli*, and used...
affinity tag chromatography for purification. High protein yields could be obtained using standard protocols; however, full-length Hexim1 as well as the N-terminal fragment 1–254 appeared less stable during purification and were degraded, whereas the C-terminal fragments 255–359 and 266–359 remained stable and soluble. Interestingly, the 41-kDa Hexim1 protein migrated through the SDS-PAGE at an apparent size of 70 kDa, as observed before (11, 13). Similarly, the 28.1-kDa N-terminal fragment 1–254 migrated very slowly at 50 kDa, whereas the two C-terminal 12.5- and 11.4-kDa fragments run at their expected sizes (Fig. 2, lanes 3, 6, 9, and 12). Molecular masses of the expressed proteins were confirmed for control by MALDI-TOF mass spectrometry (data not shown). These migration patterns may indicate that the N-terminal region of Hexim1 is largely unfolded, whereas the C terminus represents a structured domain.

Direct binding of Hexim1 to the cyclin T1 subunit of P-TEFb was analyzed by GST pull-down experiments, using the cyclin box repeats of CyclT1 (residues 1–292) fused to GST and coupled to GSH-Sepharose beads. We found that full-length Hexim1 as well as the two C-terminal fragments bind CyclT1 directly but not the N-terminal fragment 1–254 (Fig. 2, lanes 2, 5, 8, and 11). Incubation and washing conditions were chosen so that Hexim1 proteins do not bind arbitrarily to GST-loaded beads (Fig. 2, lanes 1, 4, 7, and 10). Since the C-terminal fragment 255–359 appeared easier to handle in terms of expression yields, affinity tag removal, and solubility than the shorter fragment 266–359, we consider this 12.5-kDa fragment the TBD of Hexim1.

The C-terminal Domain of Hexim1 Is Required for Binding to P-TEFb and the Inhibition of Transcription in Cells—To extend the findings from the binding experiment in vitro and to address the requirement for the C-terminal domain of Hexim1 for its function, we performed functional studies in HeLa cells (Fig. 3). For these studies, we used a wild-type and two mutant FLAG-epitope-tagged Hexim1 proteins, in which their C termini were shortened progressively, resulting in the construction of Hexim1 from position 1 to 314 and from position 1 to 286, respectively.

The expression yields could be obtained using standard protocols; however, full-length Hexim1 as well as the N-terminal fragment 1–254 appeared less stable during purification and were degraded, whereas the C-terminal fragments 255–359 and 266–359 remained stable and soluble. Interestingly, the 41-kDa Hexim1 protein migrated through the SDS-PAGE at an apparent size of 70 kDa, as observed before (11, 13). Similarly, the 28.1-kDa N-terminal fragment 1–254 migrated very slowly at 50 kDa, whereas the two C-terminal 12.5- and 11.4-kDa fragments run at their expected sizes (Fig. 2, lanes 3, 6, 9, and 12). Molecular masses of the expressed proteins were confirmed for control by MALDI-TOF mass spectrometry (data not shown). These migration patterns may indicate that the N-terminal region of Hexim1 is largely unfolded, whereas the C terminus represents a structured domain.

FIG. 2. A 12-kDa C-terminal domain of Hexim1 binds cyclin T1. In vitro pull-down experiments with GST-fused CyclT1-(1–292) and full-length Hexim1 or various fragments thereof reveal a C-terminal domain in Hexim1 required for the interaction. Full-length Hexim1-(1–359) and fragments 255–359 and 266–359 are precipitated from solution by GST-CyclT1 coupled to GSH-beads (lanes 2, 5, and 8), whereas the alternate fragment Hexim1-(1–253) is not (lane 11). Shown is an SDS-PAGE Coomassie gel with typically 2 μg of input of the GST-CyclT1 fusion protein and the bound precipitate, incubation of GST alone with the Hexim1 fragments (lanes 1, 4, 7, and 10) as control, and 20% of the Hexim1 protein input (lanes 3, 6, 9, and 12).

First, to define the importance of the C-terminal domain of Hexim1 for the binding between Hexim1 and P-TEFb, we performed immunoprecipitation assays (Fig. 3A). We expressed the FLAG-tagged Hexim1 proteins by using transient expression assays in HeLa cells and immunoprecipitated them from total cell lysates using anti-FLAG-agarose beads. Their binding to endogenous P-TEFb was inspected by Western blotting using antibodies directed against CyclT1. As expected, the wild-type Hexim1 protein bound CyclT1 in cells. Likewise, the mutant Hexim1-(1–286) protein failed to bind CyclT1 (Fig. 3A, lanes 1–3). Levels of the FLAG-tagged Hexim1 proteins in the immunoprecipitations were comparable (Fig. 3A, lower panel). Thus, the C-terminal region in Hexim1 from position 286 to 314 is necessary for its binding to CyclT1 in cells.

To examine the functional importance of the C-terminal domain of Hexim1, we next tested the abilities of the FLAG-tagged Hexim1 proteins to inhibit transcriptional activation by P-TEFb in cells (Fig. 3B). We took advantage of the system, which consists of a plasmid reporter pG6TAR that contains six Gal4 DNA-binding sites positioned upstream of the HIV-1 long terminal repeat, followed by the chloramphenicol acetyltransferase reporter gene and the hybrid Gal4.CyclT1 protein. The recruitment of the latter to the pG6TAR promoter via its Gal4 DNA-binding domain activates transcription, which can be measured by the chloramphenicol acetyltransferase reporter assays (21). Indeed, when we expressed the Gal4.CyclT1 chimera together with the pG6TAR in HeLa cells, chloramphenicol acetyltransferase activity increased 27-fold over basal levels. Consistent with its inhibitory function, the coexpression of the wild-type Hexim1 protein decreased this activity 4-fold. Furthermore, the mutant Hexim1-(1–314) protein decreased...
transcription similarly. In contrast, the mutant Hexim1-(1–286) protein lost this inhibitory activity (Fig. 3B, bars 1–3). Expression levels of the FLAG-tagged Hexim1 proteins were comparable (Fig. 3B, lower panel). Thus, residues from position 286 to 314 in the C-terminal domain of Hexim1 are required for the inhibition of P-TEFb in cells. Taken together, we conclude that the binding to P-TEFb and the inhibition of transcriptional activation by P-TEFb in cells depend on the integrity of the C-terminal domain in Hexim1.

Analytical Gel Filtration of the Complex Formation between Cyclin T1 and Hexim1—We studied the molecular dispersion of cyclin T1 and Hexim1 protein fragments and their complex formation by size exclusion chromatography. To this end, several independent measurement cycles using different batches of proteins were performed on a Superdex 75 column that was equilibrated in low salt Tris buffer and calibrated to the elution profiles of a low molecular weight standard. A representative chromatogram is shown in Fig. 4. We first applied the Cyclin T-binding domain (residues 255–359) of Hexim1 and full-length Hexim1 to the column. To our surprise, the Hexim1-TBD with a molecular mass of 12.5 kDa elutes at a retention time of 19.10 min (at a flow rate of 0.5 ml/min), corresponding to a size with an apparent mass of 45 kDa. The elution profile, however, was very well defined, showing a single symmetrical sharp absorption line. A similar course was observed for the full-length protein of 43.7 kDa (including the poly-His tag) that elutes almost at the exclusion limit of the separating volume after 15.8 min, corresponding to an apparent mass of more than 75 kDa. These elution properties suggested that Hexim1 is either in an oligomeric state, forming at least homomeric dimers, or that it is strongly shaped elliptically.

The cyclin box repeats of Cyclin T1-(1–292) with a molecular mass of 33.7 kDa are supposed to form a globular helical structure (25). The elution profile at 20.47 min or approximately 36 kDa is indeed in agreement with a monomeric state of this domain that exhibits some tendency to form oligomers (see left slope). Finally, the preformed complex of Cyclin T1-(1–292) and Hexim1-(255–359) coelutes at 17.7 min, indicating clearly the direct interaction of the two molecules during migration. The flanking shoulder at 21-min retention time is due to excess CycT1, whereas the peak at 32.4 min represents a polyhistidine fragment of 2.8-kDa size that remained after Tev protease cleavage from the affinity tag. This coelution profile may indicate the formation of a heterodimeric 1:2 complex between CycT1 and Hexim1-TBD, since the unequal ratio may result in excess CycT1.

Dimer Formation of Hexim1 Is Mediated in Part by the Cyclin T-Binding Domain—To analyze further the proposed dimerization of Hexim1, we performed GST pull-down experiments and isothermal titration calorimetry (Fig. 5). Full-length GST-Hexim1 coupled to GSH beads precipitated efficiently nontagged Hexim1-(1–359) but also the N-terminal fragment (residues 1–254). Next, using GST-Hexim1-TBD as binding anchor, only small amounts of the TBD-(255–359) were pulled down, indicating the formation of a weak TBD dimer (Fig. 5A, lanes 2, 5, and 8). These bindings were specific, since GST alone did not bind to any of the target proteins under the conditions chosen (lanes 1, 4, and 7). Purified target proteins are shown in the input lanes.

Next, a protein solution of 0.5 mM Hexim1-TBD in 50 mM NaCl low salt buffer was placed in the syringe and injected stepwise in portions of 8 μl to the measurement cell of the calorimeter that was filled with the same buffer. As expected, an increase in heating power was detected that decreased exponentially with time after each injection resulting from the liberation of heat upon dissociation of the dimeric molecules into monomers (Fig. 5B). The best fit of a dissociation curve to the experimental data were received, assuming a discrete stoichiometry factor n = 2 for the aggregation state of the protein, which suggests, for example, the transition from a tetramer to a dimer upon dilution. The fit yield an equilibrium dissociation constant of multimerization $K_d$ (multimer) = 790 μM and an enthalpy of multimerization $\Delta H^\circ = -10.4$ kcal/mol. These data indicate the formation of a weak homomer for the T1-binding domain of Hexim1.

Equilibrium Fluorescence Titrations Reveal a Medium Binding Affinity for Hexim1-TBD to Cyclin T1—Fluorescence spectroscopy is a very sensitive method to determine kinetic interaction parameters. To use molecular concentrations well below the dissociation constant for Hexim1 multimerization, we applied this technique for the analysis of CycT1 binding. We first tested the wild type cysteine at position 297 of the Hexim1-TBD for fluorophore labeling. This cysteine was not expected to be accessible for covalent coupling, due to a neighboring leucine 298, which should form the core in a putative coiled-coil structure. However, according to mass spectrometry, the TBD was
trum is shown in red, and the emission wavelength recorded to observe equilibrium titration fluorescence changes at 490 ± 7.5 nm is hatched. The excitation wavelength was set to 337 nm. We next measured fluorescence intensity changes upon binding association by the addition of increasing concentrations of CycT1-(1–292) to 0.5 μM TBD-EDANS (Fig. 6D). The fluorescence intensity was normalized to the starting value and corrected for the dilution effect. A fit to the binding association curve yielded a binding affinity $K_d = 2.6 \mu M$, which suggested a medium binding affinity between the two protein domains.

For control, we tested if full-length Hexim1 (G257C/C297S) mutant protein is similarly active in cells as the wild type protein (Fig. 6C). Hexim1 proteins were expressed in HeLa cells and immunoprecipitated by anti-FLAG beads as before. As can be seen from precipitation data, both wild type and mutant Hexim1 bound similarly to CycT1 and also inhibited the transactivation function of Gal4.CycT1-activated transcription to a similar degree. Thus, we conclude that the mutations introduced in Hexim1 to establish the fluorescence assay do not alter the cellular activity.

**Analysis of the Binding Kinetics by Stopped Flow Measurements**—To further investigate the dynamic nature of the interaction between Hexim1 and CycT1, stopped flow experiments were performed. Equal volumes of 1.5 μM TBD-EDANS and varying concentrations (0.75–15 μM) of GST-CycT1-(1–292) were rapidly mixed at a 1:1 ratio (v/v) in the measurement cell, and fluorescence changes were detected for the first 2 s (Fig. 7A). Fluorescence excitation was set at a 337-nm wavelength, and the change of intensity was detected in a range from 420 to 600 nm. The observed association courses could be fit perfectly with a single exponential curve, indicating a direct binding interaction. Next, the dissociation rate for the TBD-CycT1 complex was determined by competition experiments. The complex of 1.5 μM TBD-EDANS with 1.5 μM GST-CycT1 was mixed with 45 μM unlabeled TBD in the stopped flow apparatus (Fig. 7B). Fitting a single exponential curve to the spectra yields a dissociation rate $k_{off}$ of 1.42 s$^{-1}$. From these data, the association kinetics of CycT1 and Hexim1-TBD was analyzed, assuming pseudo-first-order conditions (Fig. 7C). The apparent rate constants $k_{obs}$ that varied between 3.2 and 40 s$^{-1}$ were plotted against the final concentration of CycT1 and yielded a linear curve with a slope $k_{on} = 2.59 \text{ M}^{-1} \text{s}^{-1}$ and an axis intercept $k_{off} = 1.47 \text{ s}^{-1}$. The molecular affinity that results from these calculations reveals a dissociation constant $K_d = 0.56 \mu M$ for the CycT1-Hexim1-TBD interaction.

**Hexim1 and HIV-1 Tat Bind Cyclin T1 in a Mutually Exclusive Fashion**—Finally, we sought to study effects of the transcription activator HIV-1 Tat on the formation of the CycT1-Hexim1 complex. Tat stimulates the transcription of viral mRNA via the interaction with CycT1 of P-TEFb (26). We first applied GST pull-down assays for the analysis of protein binding and incubated with highly purified proteins that were resolved by SDS-PAGE Coomassie staining (Fig. 8A). In a first series of experiments, we used GST-CycT1-(1–292) immobilized on GSH-beads to pull down either Hexim1-TBD, Tat, or both simultaneously from solution (lanes 1–7). In a second series, the GST anchor was attached to Tat, and either CycT1, Hexim1-TBD, or the complex of both were subjected to coprecipitation (lanes 8–12). As shown before, GST-CycT1 binds each Hexim1-TBD and Tat in separate assays (lanes 2 and 4). Amounts of both binding effectors equal to the amount of incubation solution resulted in pull-down of both molecules by GST-CycT1 with a slight preference for Tat (lane 5). However, when we used a fusion chimera of CycT1 and Tat that is transcriptionally active (20), we could not pull down any Hexim1-TBD from solution (lane 3).
To further analyze if a ternary complex between Cyclin T1, Tat, and Hexim1 can be formed or if Hexim1 and Tat bind cyclin T1 mutually exclusively, we used GST-Tat to pull down either cyclin T1, Hexim1, or both from solution. As shown clearly, GST-Tat binds CyclinT1 (lane 9), but the addition of Hexim1 does not lead to the simultaneous interaction of all three proteins, since again only CycT1 is precipitated (lane 10). Therefore, we concluded that Hexim-TBD and Tat either compete for a similar binding surface on CyclinT1-(1–292) or that binding of Tat to cyclin T1 prevents simultaneous binding of Hexim1 due to steric hindrance or induced structural changes.

Next, we analyzed the complex formation between cyclin T1, Tat, and Hexim1-TBD by size exclusion chromatography (Fig. 8B). Elution profiles of CyclinT1-(1–292), TBD-(255–359), and the complex of CyclinT1-TBD on a Superdex 75 column were observed similarly as before. The addition of Tat-(1–86) to CyclinT1 led to complex formation as indicated by a shift to an earlier retention time at 19.3 min. Interestingly, although Tat (86 amino acids) and TBD (105 amino acids) are of similar molecular weight, the individual complex with CyclinT1 is of a very different size as indicated by the different retention times. Finally, all three proteins (CyclinT1, Tat, and Hexim1-TBD) were incubated in equal concentrations for 30 min before subjection to the gel filtration column. As can be seen, no indication for a ternary complex formation could be observed that would be expected to elute at earlier retention times than the CyclinT1-TBD complex. We rather found formation of the CyclinT1-Tat complex predominating as well as a minor partition of the CyclinT1-TBD complex (Fig. 8B, light blue line). These observations suggest that the tighter binding Tat dominates over Hexim1 for a similar binding site on cyclin T1, meaning that only one or the other complex can be formed. Thus, HIV-1 Tat might reverse the inhibitory effect of Hexim1 on P-TEFb by disturbing their interaction, leading to a release of active P-TEFb and the activation of transcription.

**DISCUSSION**

The results presented here suggest the identification of a globular and stable domain in Hexim1 that directly interacts with the cyclin box repeats of cyclin T1. This TBD of 12 kDa binds to cyclin T1 in vitro with a dissociation constant of 1.2 µM, whereas functional assays confirmed that the TBD is required for the binding of Hexim1 to P-TEFb and inhibition of transcriptional activity in vivo. HIV-1 Tat was found to compete with Hexim1 for the interaction with P-TEFb, since both the transcription activator and the transcription regulator bind mutually exclusively to cyclin T1.

Based on computational analysis and previous studies (16), two C-terminal protein fragments of Hexim1 were generated (residues 255–359 and 266–359) that appeared to be highly soluble and stable. Both fragments were found to bind directly to cyclin T1-(1–292); however, from GST pull-down assays, it appeared that the longer fragment bound significantly better than the shorter one (Fig. 2). Since we found that the 255–359 protein fragment was much better to handle in terms of protein expression and protease cleavage, we cannot differentiate if the stronger interaction indicates a
measurements and normalized to the initial fluorescence signal at Hexim1-TBD. The complex of TBD-EDANS with GST-CycT1 (1.5 B spectra yields a dissociation rate in the stopped flow apparatus. Fitting a single exponential curve to the mixed at a 1:1 ratio (v/v) with a 30-fold excess of unlabeled Hexim1-TBD concentration of CycT1. A fit to a linear curve yields the results exponential fit to the spectra shown in and 15

The concentration of the ligand GST-CycT1-(1–292) varied between 0.75

266 to the binding interface or if only the integrity of the longer fragment is improved. A higher grade of oligomeriza-

tion for the 266–359 fragment could for instance affect the binding affinity to cyclin T1. C-terminal truncations 1–314 and 1–286 in functional assays confirmed the importance of this region for the interaction with CycT1 (Fig. 3). Whereas both full-length Hexim1 and fragment 1–314 precipitated CycT1 from HeLa cells, the fragment 1–286 did not. Since CycT1 has never been detected in its free form (19) but always minimally in the complex with Cdk9, which results in its activation and nuclear translocation (27), this fragment should act on P-TEFb similarly. Indeed, transcription activation was not any more inhibited by the shorter fragment but only with the two longer Hexim1 fragments. We therefore conclude that the protein fragment 255–359 of Hexim1 forms the cyclin T-binding domain and that residues between positions 255 and 314 must significantly contribute to the binding interface.

We next used biochemical assays to examine in greater detail the C-terminal domain of Hexim1 and its binding properties to cyclin T1. Analytical gel filtration experiments showed that CycT1 and Hexim1-TBD elute together from the column, indicating a stable complex formation (Fig. 4). However, the migration of Hexim1 proteins was surprising, since already the 12-kDa TBD eluted at a retention time indicating an even larger molecule size than the 34-kDa cyclin domain. We diluted the concentration of the protein subjected to the column by factor 4 and factor 10 but always found the TBD to be running monodispersers at a size corresponding to 45 kDa (data not shown). This indicates either a structural shape of the TBD that largely differs from an ideal spherical form or the formation of stable homomeric oligomers, such as a dimers, trimers, or tetratomers, or a combination of both. We further addressed this question by direct in vitro binding assays using GST fusion proteins and isothermal titration calorimetry (Fig. 5). Indeed, GST-Hexim1 proteins were able to pull down nontagged full-length Hexim1 and the N-terminal fragment Hexim1-(1–254) but also the Hexim1-TBD-(255–359).

The mutual interaction between the TBD confirmed the direct binding, suggesting a specific dimer formation. In line with this observation, ITC revealed a weak multimer dissociation constant of 790 µM and a transition between oligomeric states with a stoichiometry factor 2, corresponding, for example, to a tetramer to dimer dilution. Such a weak affinity as determined for the TBD multimerization is usually not sufficient to keep molecules together on a gel filtration column. This implies that the elution profile of the TBD may correspond to an elliptically shaped molecule rather than to a high order multimer. Therefore, a helical coiled-coil structure of the TBD, as already proposed by computational methods, would be well in line with the observed gel filtration data.

Two independent fluorescence spectroscopy measurement series determined the binding affinity between the cyclin domain of cyclin T1 and Hexim1-TBD. The geometric mean between the results of the equilibrium titration and the stopped flow kinetics yielded a dissociation constant of 1.2 µM and on and off rates of 2.59 µM⁻¹ s⁻¹ and 1.45 s⁻¹, respectively. This indicates a medium tight direct binding interaction between the two domains. However, in context with the full-length proteins and with the obligate partners Cdk9 for Cyclin T1 and 7SK snRNA for Hexim1, the affinity of this two subcomplexes may increase. The fluorescence label 1,5-IAEDANS that was covalently attached to a mutant cysteine G257C turned out to be a very sensitive and suitable indicator to measure the protein interaction kinetics. Of note, the clear single exponential behavior in the stopped flow association curves suggests a well defined domain-domain interaction that does not require an additional step as would be, for

![Fig. 7. Binding kinetics of the interaction between cyclin T1 and Hexim1. A, stopped flow measurements for the determination of the observed association rates. The final protein concentration of C257EDANS-labeled Hexim1-TBD was kept in all experiments at 1.5 µM. The concentration of the ligand GST-CycT1-(1–292) varied between 0.75 and 15 µM. Each fluorescence spectrum is averaged from 8 individual measurements and normalized to the initial fluorescence signal at t = 0 s. B, dissociation of the Hexim1-CycT1 complex after the addition of excess Hexim1-TBD. The complex of TBD-EDANS with GST-CycT1 (1.5 µM) was mixed at a 1:1 ratio (v/v) with a 30-fold excess of unlabeled Hexim1-TBD in the stopped flow apparatus. Fitting a single exponential curve to the spectra yields a dissociation rate k off of 1.42 s⁻¹. C, association of CycT1 and Hexim1-TBD. Kinetics were analyzed assuming pseudo-first-order conditions. The apparent rate constants k app were derived from a single exponential fit to the spectra shown in A and plotted against the final concentration of CycT1. A fit to a linear curve yields the results k app = 2.59 µM⁻¹ s⁻¹ (slope) and k on = 1.47 s⁻¹ (intercept).](http://www.jbc.org/)

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example, an induced fit mechanism due to conformational transitions in either domain.

The formation of the cyclin T1-Hexim1 complex was proved in the presence of HIV-1 Tat. It has been shown before that optimal binding of Tat to cyclin T1 requires a critical cysteine at position 261 of cyclin T1 that is suggested to form an intermolecular zinc finger with Tat (28) and that residues at the C-terminal edge of the cyclin domain from the Tat-TAR recognition motif (28, 29). Here we find that both regulator proteins Hexim1-TBD and Tat are precipitated simultaneously from solution with GST-fused cyclin T1 (residues 1–292). This can be explained either by the formation of a ternary complex or with comparable binding affinities for a similar binding site on cyclin T1, respectively. However, a fusion chimera of cyclin T1 and Tat did not pull down Hexim1-TBD, and similarly GST-Tat only interacted with cyclin T1, which was itself not able to bind the TBD at the same time. This strongly suggests a mutually exclusive interaction of the two molecules with cyclin T1, either by binding to the same surface or by a mutually steric hindrance.

In previous experiments, dissociation constants of 9.1 and 0.4 nM between the complex of Tat and CycT1-(1–303) in the absence and presence of TAR RNA were reported, respectively (30). Since these affinities to CycT1 are more than 100 times tighter compared with the dissociation constant between CycT1 and Hexim1-TBD identified here, it is surprising that Tat is not precipitated significantly better from solution than the TBD. This could be due to less active Tat (e.g. if only a minor fraction of the recombinantly expressed protein is folded correctly). The pull down performed with GST-Tat and CycT1 and TBD in solution is therefore much more meaningful, since only properly folded Tat binds CycT1. The observation that the GST-Tat/CycT1 complex fails to bind the TBD simultaneously might be a starting point to analyze in functional assays how transcription inhibition of P-TEFb by Hexim1–7SK is transferred to transcription activation by competition with Tat-TAR.

The identification of a cyclin T-binding domain in Hexim1 is the first step toward a biochemical understanding of P-TEFb regulation but does not explain how the kinase activity of Cdk9 is inhibited. Two different mechanisms of cyclin-dependent
kinase inhibition have been structurally elucidated (for a review, see Ref. 31). The crystal structure of the p27Cip inhibitor bound to the cyclin A-Cdk9 complex revealed an extended structure that interacts with both subunits and inserts a tyrosine side chain to block the ATP-binding site (32). In contrast, proteins of the INK4 family of kinase inhibitors prevent cyclin D binding to Cdk4 or Cdk6 by distorting the ATP-binding site (33, 34). Since Hexim1 and 7SK precipitate both subunits CycT1 and Cdk9 of P-TEFb (10, 11, 16) the latter mechanism does not serve as a model for P-TEFb transcription regulation. However, unstructured regions N-terminal to the TBD in Hexim1 might be the primary recognition domain that serves to assemble the two subcomplexes of P-TEFb and Hexim1/7SK, whereas intrinsically unstructured regions close to the TBD then wrap around the cyclin-kinase pair and inhibit the kinase activity. The functional role of 7SK snRNA for the Cdk inhibitor is yet to be defined, and phosphorylation events on Cdk9 or the C-terminal domain may additionally contribute to the binding specificity (17, 24, 36). Future biochemical and structural studies will be directed to analyze this mechanism of regulation.

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