Interactions of Cdk7 and Kin28 with Hint/PKCI-1 and Hnt1 Histidine Triad Proteins*

Received for publication, July 28, 2000, and in revised form, August 21, 2000
Published, JBC Papers in Press, August 24, 2000
DOI 10.1074/jbc.C000505200

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Cyclin-dependent kinase 7 (Cdk7) forms a trimeric complex with cyclin H and Mat1 to form the mammalian Cdk-activating kinase, CAK, as well as a part of the basal transcription factor TFIIH, where Cdk7 phosphorylates the C-terminal domain (CTD) of the large subunit of RNA polymerase II. Here, we report a novel interaction between Cdk7 and a histidine triad (HIT) family protein, Hint/PKCI-1. This interaction was initially observed in a yeast two-hybrid study and subsequently verified by co-immunoprecipitation and subcellular localization studies, where overexpression of Cdk7 leads to partial relocation of Hint to the nucleus. The physical association is independent of cyclin H binding or Cdk7 kinase activity and is conserved between the related S. cerevisiae and H. sapiens. The CTD of the large subunit of RNA polymerase II is phosphorylated by a Hint-PKCI-1 complex with cyclin H and Mat1 and is sufficient to activate Cdk7, whereas CTD phosphorylation is mediated more efficiently by Cdk7 within TFIIH.

* This study was supported by grants from the Academy of Finland, University of Helsinki, Helsinki University Central Hospital EVO funds, Finnish Cancer Society, Finnish Cancer Institute, and Sigrid Juselius Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Cdk, cyclin-dependent kinase; CAK, Cdk-activating kinase; Pol II, polymerase II; CTD, C-terminal domain; HIT, histidine triad; FHIT, fragile histidine triad; HINT, histidine triad nucleotide-binding protein; PKCI-1, protein kinase C inhibitor-1; PCR, polymerase chain reaction; GST, glutathione S-transferase; HA, hemagglutinin.

through phosphorylation of its C-terminal domain (CTD) as a part of the basal transcription factor TFIIH. The two functions of Cdk7 may be mediated via specific protein complexes, because a trimeric complex containing Cdk7 together with cyclin H and Mat1 is efficient as a CAK, whereas CTD phosphorylation is mediated more efficiently by Cdk7 within TFIIH.

In Saccharomyces cerevisiae, the CAK and the TFIIH-associated CTD kinase activities reside in two distinct proteins (reviewed in Ref. 1). The Cdk7 kinase forms a complex with a cyclin H homologue, Ccl1, and a Mat1 homologue, Tfb3/Rig2, and functions as a TFIIH kinase phosphorylating CTD. In contrast to the mammalian complex, Kin28-Cycl1/Thr3/Rig2 does not possess CAK activity, but instead, a monomeric CAK Cdk1/Civl1 phosphorylates and activates budding yeast Cdc28 and Kin28 Cdk5.

The role of Cdk7/Cin28 in TFIIH appears to be involved in facilitating the processing of nascent transcripts (capping, splicing, and polyadenylation) through CTD phosphorylation (2–4). In addition to Cdk7/cyclin H-Mat1, TFIIH contains at least six subunits (5) of which the XPD/ERCC2 helicase is involved in anchoring Cdk7-cyclin H-Mat1 to TFIIH (5–7) via association with Mat1 (8).

The Cdk7/cyclin H-Mat1 complex has also been reported to associate directly with p53 (9), RARα (10), RARγ (11), Oct-1 (12), and HIV Tat (13). Here, we identify a novel interaction between the Cdk7 and Kin28 kinases and the Hint/PKCI-1 and Hnt1 histidine triad (HIT) proteins, respectively.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—S. cerevisiae yeast strains used in this study were EGY48 (MATa, ura3–52, trp1–Δ3, his3–Δ200, leu2–Δ1, 6LexAop-LEU2) (14), YPH499 (MATa, ade2–101, lys2–801, ura3–52, trp1–Δ3, his3–Δ200, leu2–Δ1), YPH498–500 (MATa/a, ade2–101, lys2–801, ura3–52, trp1–Δ3, his3–Δ200, leu2–Δ1) (a kind gift from V. Van Mullem), JG4 (MATa, ura3–52, trp1–Δ3, his3–Δ200, leu2–Δ1, 5′-AGT GAG CCA TTG ATG TCG GCA CCG TAC TCA ACA ACA ACC ATC TTA GAG GCS-3′, HNT1 homologous sequence shown in italics) and hnt1::HIS3 (5′-ATG GAG CCA TTG ATG TCG GCA CCG TAC TCA ACA ACA ACC ATC TTA GAG GCS-3′, HNT1 homologous sequence shown in italics) and hnt1::HIS5 (5′-ATG GAG CCA TTG ATG TCG GCA CCG TAC TCA ACA ACA ACC ATC TTA GAG GCS-3′, HNT1 homologous sequence shown in italics) were used in polymerase chain reaction (PCR) to amplify a HIS3 selection cassette. The PCR product was subsequently transformed into a haploid and a diploid S. cerevisiae strain, YPH499 and YPH499–500, respectively, and correct integration to the HNT1 locus was confirmed by using appropriate external primers to HNT1 (nucleotides 848–867 and 1988–2008 in GenBank™ accession no. X56956). Because the haploid HNT1 disruptant strain, termed NKK1, was viable, it was used in subsequent studies. Mating, sporulation, and random spore analysis were performed according to standard procedures to produce the hnt1::HIS3; hnt1::HIS3; hnt1::HIS5; hnt1::HIS5 double mutant haploid strain termed NKK2, identified by auxotrophy analysis, genotyping, and temperature sensitivity.

Yeast Two-hybrid Screening and Expression Plasmids—The yeast two-hybrid screening system was performed as described (16) using LexA DNA binding domain fusion in pE202 and B42 activation domain fusion in pJG4–5 (14) using HaLa (14) and human keratinocyte (a kind gift from A. Zervos (17)) cDNA libraries in pJG4–5. The plasmids used were: CDK7/pEG202, CDK7–K41M/pEG202, CDK7–T170E/pEG202, CDK7–K41M/pEG202, CDK7–T170E/pEG202, CDK7–K41M/pEG202, CDK7–T170E/pEG202, CDK7–K41M/pEG202, CDK7–T170E/pEG202, and cyclin H/pEG202 (16). CDK7/pJG4–5 was subcloned from CDK7/pEG202. The KIN28/pEG202 and KIN28/pJG4–5 were PCR subcloned with appropriate primers, creating an EcoRI-Xhol fragment from KIN28/pYES (a kind gift from D. Hermand, and HNT1/pJG4–5 was PCR subcloned from HNT1/pYF.
Cdk7 and Kin28 Interactions with Hint/PKCI-1 and Hnt1

RESULTS AND DISCUSSION

Cdk7 and Kin28 Interact with Hint and Hnt1—We were interested in characterizing novel proteins interacting with the Cdk7 kinase. For this purpose, we performed a yeast two-hybrid screen using LexA-Cdk7 bait with the indicated proteins and the subsequent kinase assays and Western blotting analysis were performed as described (20) using monoclonal α-HA (12CA5, Babco Inc., Berkeley, CA) or α-FLAG (M5, Kodak) antibodies.

Production of GST Proteins and Solution Binding Assay—For bacterial expression, an EcoRI-XhoI insert from HINT/pJG4–5 was cloned into pGEX-4T-1 (Amersham Pharmacia Biotech) to generate recombinant protein as described (16). Subsequently 1.5 mg of U2OS human osteosarcoma cell extracts in ELB lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 5 mM EDTA, 0.1% Nonidet P-40 with 1 mM diethyrltol, 2.5 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerol phosphate, 1 μg/ml leupeptin) were incubated with 5 μg of GST or GST-Hint for 1 h at 4 °C, bound to glutathione-Sepharose (Amersham Pharmacia Biotech) for 2 h, and washed four times with ELB. Bound proteins were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with polyclonal α-Cdk7 antibody (21).

Mammalian Cell Culture, Transformation, and Immunofluorescence—U2OS osteosarcoma cells were cultured using standard protocols and transfected with the calcium phosphate transfection method (22) using plasmid EcoRI-XhoI containing HINT cDNAs in pCINeo (Promega). Transfected cells were fixed on coverslips with 3.5% (w/v) paraformaldehyde and permeabilized with 0.1% Triton X-100 for 5 min. Double immunofluorescence was performed using rabbit polyclonal α-Cdk7 or mouse monoclonal α-HA (12CA5) followed by rhodamine-conjugated goat α-rabbit and fluorescein-conjugated goat α-mouse secondary antibodies (Roche Molecular Biochemicals). The nuclei were visualized with Hoechst 33342 (Sigma; 0.5 μg/ml), and the immunostainings were viewed and documented with a Zeiss Axiopt microscope.

Fig. 1. Cdk7 and Kin28 interact with Hint and Hnt1 histidine triad proteins. A, EGY48 yeast strains expressing the indicated DNA-binding domain (LexA-), and transactivation domain (B42-) fusion proteins were streaked on synthetic complete medium containing bromo-4-chloro-3-indolyl β-D-galactoside to detect lacZ marker gene expression. B, α-FLAG Western blotting analysis of α-HA immunoprecipitates (IP) from yeast lysates expressing the indicated HA-tagged and FLAG-tagged proteins. C, α-Cdk7 Western blotting analysis of proteins purified from U2OS osteosarcoma cell extracts with 5 μg of GST or GST-Hint as indicated.

As S. cerevisiae genes KIN28 and HNT1 represent apparent homologues of CDK7 and HINT, respectively, we subsequently tested whether the two-hybrid interaction was conserved from man to yeast. To this end, we used LexA-Kin28 and B42-Hnt1 proteins in the yeast two-hybrid system, noting that expression of these proteins also activated the lacZ reporter (Fig. 1A).

We next investigated whether the HIT proteins could be detected in Cdk7 or Kin28 immunocomplexes (Fig. 1B). We expressed HA epitope-tagged HA-Cdk7, HA-Kin28, HA-Hint, or HA-Hnt1 together with FLAG epitope-tagged FLAG-Hint or FLAG-Hnt1 in yeast cells in the combinations indicated in Fig. 1B. Complex formation was subsequently assessed by immunoprecipitation of HA-tagged proteins followed by Western blotting with α-FLAG antibodies. The results demonstrate that FLAG-Hint and FLAG-Hnt1 co-immunoprecipitate with HA-Cdk7 (Fig. 1B, lane 5) and HA-Kin28 (Fig. 1B, lane 6), respectively. Structural studies have demonstrated that Hint forms homodimers (24, 35). This was also noted in our co-immunoprecipitation studies, where both FLAG-Hint and FLAG-Hnt1 efficiently co-precipitated with the corresponding HA-tagged proteins (Fig. 1B, lanes 3 and 4). The yeast and human HIT orthologues were also able to form a complex with each other (data not shown), indicating structural conservation between Hint and Hnt1 with 34% identity at the amino acid level. An association between Hint and endogenous mammalian Cdk7 could also be demonstrated, as Cdk7 specifically co-purified with...
**Functional Characterization of the Cdk7-Hint Interaction**

The evolutionary conservation of the interactions on one hand between human Cdk7 (a CAK and a CTD kinase) and Hint and on the other hand between yeast Kin28 (exclusively a CTD kinase) and Hnt1 suggests that the interaction is functionally significant. To address this possibility, we initially characterized the ability of Hint to associate with Cdk7 mutants using the two-hybrid assay (Fig. 2A). Using the well-characterized interaction between Cdk7 and cyclin H as a comparison, it was first noted that the Cdk7-Hint interaction appeared to be stronger based on lacZ expression (Fig. 2A, top panels). As previously noted for cyclin H (16), Hint also efficiently associated with a kinase-deficient Cdk7 (Cdk7-K41M), although Hint did not associate with an N-terminally deleted Cdk7 (Cdk7Δ1–132). Hint association was also observed with a Cdk7 in which the T-loop activation site threonine had been mutated to glutamic acid (Cdk7-T170E) in an attempt to mimic phosphorylation of this site. As cyclin H was previously demonstrated not to associate with the T170E mutant (16), the results indicate that the Hint-Cdk7 interaction is independent of cyclin H binding. On the other hand, Hint-associated Cdk7 retained its kinase activity (Fig. 2B), suggesting that the association of Hint with Cdk7 does not exclude cyclin binding, which is required for Cdk7's kinase activity. This was further supported by the demonstration of Hint in cyclin H immunocomplexes in yeast cells also expressing Cdk7 (Fig. 2C). Thus, Hint apparently does not represent a Cdk inhibitor; rather, it may be associated with modifying Cdk7 substrate specificity, as suggested by the preferential phosphorylation of a RNA Pol II CTD substrate by Hint-associated Cdk7 (Fig. 2B).

**Redistribution of Hint in Cells Overexpressing Cdk7**—Previous reports on the subcellular localization of Hint in mammalian cells have indicated either a cytoskeletal localization in LM217 human fibroblast cells (36) or a nuclear and cytoplasmic localization in rat basophilic leukemia cells (37), whereas Cdk7 is exclusively a nuclear protein (38). To investigate whether Hint and Cdk7 could be detected in the same subcellular compartments, U2OS human osteosarcoma cells were transfected with an HA-Hint-expressing plasmid alone or together with a Cdk7-expressing plasmid and subjected to immunofluorescence analysis (Fig. 3). In both cases, HA-Hint protein was detected throughout the cell including nuclei with a prominent perinuclear staining (Fig. 3A). However, in some cells also overexpressing Cdk7, HA-Hint staining was more prominent in the nuclei, where it co-localized with Cdk7 (Fig. 3D). This result demonstrates that overexpression of Cdk7 leads to redistribution of Hint in mammalian cells, perhaps because of the formation of direct complexes between these proteins. The results also demonstrate that in addition to the co-localization of Hint with Cdk7 in the nucleus, a significant fraction of Hint is cytoplasmic, suggesting thatHint is likely to be involved in additional functions not related to Cdk7. This suggestion is in line with previous studies identifying interactions between Hint and the ATDC (ataxia telangiectasia group D-complementing) gene product (18) and the mi transcription factor (37).

**Genetic Interaction between KIN28 and HNT1**—The extension of the association of Cdk7 and Hint to the S. cerevisiae Kin28 and Hnt1 proteins, together with the availability of temperature-sensitive alleles of KIN28 (15), prompted us to investigate whether KIN28 and HNT1 displayed genetic interactions. This analysis was initiated by generating yeast strain NKK1 in which a HIS3 selection cassette replaced amino acids 14–146 (of 158) of HNT1 (Fig. 4A). The haploid disruptant strain proved to be viable and exhibited no apparent phenotype, indicating that HNT1 is not an essential gene. Subsequently, a double mutant haploid strain, NKK2, was generated.
The identification of physical and genetic interactions between the Cdk7 and Kin28 CTD kinases with the Hint and Hnt1 HIT proteins is interesting in the light of a recent report indicating that Hint associates with and negatively regulates the microphthalmia transcription factor mi (37). It raises the possibility that HIT proteins may bridge the interactions between basal transcription factors and transcription activators.

Acknowledgments—We thank Gerard Faye (Institut Curie, Orsay, France), Vincent Van Mullem (Laboratoire de Génétique Moléculaire, Namur, Belgique), Antonis Zervos (Cutaneous Biology Research Center, Charlestown, MA), and Damien Hermand (Laboratoire de Génétique Moléculaire, Namur, Belgique) for reagents. Per Ljungdahl (Ludwig Institute for Cancer Research, Stockholm, Sweden) is acknowledged for helpful suggestions and members of the Makela laboratory for discussions and help.

REFERENCES

1. Kaldis, P. (1999) Cell. Mol. Life Sci. 55, 284–296
2. McCracken, S., Feng, N., Rossinina, E., Yankulov, K., Brothers, G., Siderovski, D., Hassel, A., Foster, S., Shuman, S., and Bentley, D. L. (1997) Genes Dev. 11, 3306–3318
3. Cho, E. J., Takagi, T., Moore, C. R., and Buratowski, S. (1997) Genes Dev. 11, 3319–3326
4. Rodriguez, C. R., Cho, E. J., Keogh, M. C. L., Moore, C. L., Greenleaf, A. L., and Buratowski, S. (2000) Mol. Cell. Biol. 20, 104–112
5. Rossignol, M., Kolb-Cheney, L., and Egly, J. M. (1997) EMBO J. 16, 1628–1637
6. Drakakis, R., Le Roy, G., Cho, H., Aktulutchev, S., and Reinberg, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6488–6493
7. Reardon, J. T., Ge, H., Gibbs, E., Sancar, A., Hurwitz, J., and Pan, Z. Q. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6482–6487
8. Bussel, D., Kerieli, A., Sandrock, B., Petersman, A., Gileadi, O., and Egly, J. M. (2000) J. Biol. Chem. 275, 22815–22823
9. Ko, L. J., Shieh, S. Y., Chen, X., Jayaraman, L., Tama, K., Taya, Y., Prives, C., and Pan, Z. Q. (1997) Mol. Cell. Biol. 17, 7220–7229
10. Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J. M., and Chambon, P. (1997) Cell 90, 97–107
11. Bastien, J., Adam-Stitah, S., Biedl, T., Egly, J. M., Chambon, P., and Rochette-Egly, C. (2000) J. Biol. Chem. 275, 21896–21904
12. Inamoto, S., Segil, N., Pan, Z. Q., Kimura, M., and Roeder, R. G. (1997) EMBO J. 16, 7529–7538