A Yeast Four-hybrid System Identifies Cdk-activating Kinase as a Regulator of the XPD Helicase, a Subunit of Transcription Factor IIH*

To understand the role of the various components of TFIIH, a DNA repair/transcription factor, a yeast four-hybrid system was designed. When the ternary Cdk-activating kinase (CAK) complex composed of Cdk7, cyclin H, and MAT1 was used as bait, the xeroderma pigmentosum (XP) D helicase of transcription factor IIH (TFIIH), among other proteins, was identified as an interacting partner. Deletion mutant analyses demonstrated that the coiled-coil and the hydrophobic domains of MAT1 interlink the CAK complex directly with the N-terminal domain of XPD. Using immunoprecipitates from cells coinfected with baculoviruses, we further validated the bridging function of XPD, which anchors CAK to the core TFIIH. In addition we show that upon interaction with MAT1, CAK inhibits the helicase activity of XPD. This inhibition is overcome upon binding to p44, a subunit of the core TFIIH. It is not surprising that under these conditions some XPD mutations affect interactions not only with p44, but also with MAT1, thus preventing either the CAK inhibitory function within CAK/XPD and/or the role of CAK within TFIIH and, consequently, explaining the variety of the XP phenotypes.

DNA replication, transcription, and translation are regulated through the ordered formation of large protein complexes, the activity of which is often modulated by protein-protein interactions as well as protein modifications such as phosphorylation, acetylation, methylation, etc. Several large complexes such as RNA polymerases (1), TFIIID (2), the transcription/DNA repair factor TFIIH (3, 4), or the mediator complexes (5) are involved in transcription initiation. To ensure that these complexes work properly, it seems likely that they might be regulated by other cellular components. These regulations may involve subtle and weak interactions between partners that are hardly detectable with classical methods such as the yeast two-hybrid system or immunoprecipitation using one partner as bait. To further understand the internal interactions within these large complexes and/or to identify the externally interacting partner(s), we theorize that the bait should be a multi-protein complex. In such a case, the target protein would recognize a specific structure displayed by the subunits of the complex used as bait. This multipoint surface will likely increase and stabilize the interaction between the target protein and the bait.

The transcription/DNA repair factor TFIIH is a complex enzyme composed of nine subunits, three of which (XPB, XPD, and Cdk7) have identified enzymatic activities (6). Once recruited either by the stable transcription preinitiation complex or by the damage recognition complex, the XPB and XPD helicases of TFIIH induce the opening of the DNA around the transcription start site and the lesion, respectively (6–9). Interestingly, mutations in these two proteins affect some of the enzymatic activities of TFIIH and give rise to three autosomal recessive genetic disorders, Xeroderma pigmentosum (XP), Cockayne syndrome, and trichothiodystrophy, the corresponding phenotypes of which are rather complex and diverse (10, 11). This prompted us to hypothesize that disruption of these enzymatic activities contributes to the observed phenotypes and to elucidate the molecular mechanisms.

We have focused our attention on the Cdk activating kinase complex (CAK), part of TFIIH. Several studies have detailed the role of Cdk7, part of CAK, which also contains cyclin H and MAT1 (12, 13). Indeed, Cdk7 phosphorylates both RNA polymerase II, likely favoring the transition from initiation to elongation (14, 15), and some DNA-binding proteins such as nuclear receptors, which activate their responsive genes (16, 17). Furthermore, free CAK phosphorylates and thus regulates proteins that are involved in the cell cycle (18–20). To investigate the role of CAK in transcription, DNA repair, and cell cycle, we developed a yeast four-hybrid system. Knowing that the ternary CAK complex can be stably assembled, we used CAK as bait for a fourth partner, which would be detected upon screening a cDNA library. We first validated this new assay by showing that the cDNA library screening identifies the XPD helicase and part of TFIIH as well as FIR (far upstream element-binding protein-interacting repressor), a protein involved in the regulation of c-Myc gene expression (21). In addition, we identified other proteins connected with transcription gene regulation, too, which will be further investigated. Coexpression experiments in baculovirus-infected cells demonstrated that XPD interlinks CAK mainly via the coiled-coil domain of MAT1 to the core TFIIH via the p44 subunit. We show further that CAK inhibits the helicase activity of XPD until incorporation into TFIIH, as illustrated by the release of the inhibition when p44 is present. This allowed us to understand TFIIH architecture, explain some XPD phenotypes, and design strategies for crystallographic studies.
Yeasts—All yeast methods such as media composition, transformation, and selection were performed as described (22). The L40 strain (MATa, trp1, his3, ade2, leu2,LYS3::(LexAop)pHIS3, URA3::(LexAop)p-lacZ) was used (23). Lift filter assay and liquid β-Gal assays were performed as described previously (24).

Plasmids—pLex9-Cdk7/Met-25-cyclin H, and pVP16-XPD were described previously (25). For pPD-VP16, the cDNA of XPD was amplified by polymerase chain reaction using the sense primer 5′-GC-GAAGCTTATGAGCTCACTGGGAC-3′ (primer 1) and the antisense primer 5′-GATGCTGTACCTTGACCTGACTG-3′ (primer 2), digested with HindIII and Xhol and cloned in the corresponding sites of pVP16. pXPD-VP16 was made by using primer 1 and the antisense primer 5′-GATGCTGTACCTTGACCTGACTG-3′. For pXPD-VP16 we used the sense primer 5′-GATACAGGAATTCTTAACT-3′ and primer 2. The XPD mutants R112H, D234N, and G602D were constructed by polymerase chain reaction amplification using primers 1 and 2 of the clones pACter-R112H, pACter-D234N, and pACter-G602D, used for baculovirus transfection,2 pYEA was made by replacing the Saccharomyces cerevisiae HIS3 gene from YEg00 (26) through the Saccharomyces cerevisiae ADE2 gene. The cDNA of human MAT1 was amplified by polymerase chain reaction using the sense primer 5′-CGAAGTATCTGAGCTCACTGGGAC-3′ and the antisense primer 5′-GATGAGCTGACCTTGACCTGACTG-3′. After digestion with EcoRI, the MAT1 fragment was cloned in the EcoRI site of pYEA to obtain pYEA-MAT1. The pYEA-MAT1 mutants MAT1-1–66, MAT1-67–189, MAT1-191–309, and MAT1-67–309 were derived by insertion of the corresponding fragments obtained through EcoRI digestion from the pVP16 clones described elsewhere (27). All constructs described above were sequenced. pLexA-derived constructs allow selection on tryptophan-lacking medium, pYEA constructs on adenine-lacking medium, and pVP16 constructs on leucine-lacking medium.

cDNA Library Screening—For library screening a Matchmaker cDNA library derived from HeLa cell mRNA (CLONTECH) was used. The transformation of the library was performed according to the manufacturer's protocol. Cells were grown on medium lacking tryptophan, adenine, leucine and histidine (TALH medium) containing 20 μM 3-amino-1,2,4-triazole, a suppressor of nonspecific HIS3 expression. After 8–10 days, colonies larger than 2 mm in diameter were collected (~500) and restreaked on the same medium. 109 clones that re-grew were analyzed in liquid β-Gal assays.

Overexpression of Recombinant Proteins—XPD, Cdk7, cyclin H, MAT1, and p44 were overexpressed in S9 cells, and protein extracts were prepared as described elsewhere (6, 8, 27). 150 μg of protein extract were incubated in buffer W (20 mM Tris, 10% glycerol, 1 mM EDTA, 150 mM KCl, and 0.1% Nonidet P-40) for 4 h with 10 μl of protein A-Sepharose-conjugated antibody at 4 °C and then extensively washed with buffer W. After equilibration to 50 mM KCl, one-half of the beads was used for the helicase assay and the other for polyacrylamide gel electrophoresis followed by Western blot analysis as described elsewhere (8).

RESULTS

Design of the Yeast Four-hybrid System—The reconstitution of a quaternary complex in a yeast four-hybrid system should lead, as in the yeast two- or three-hybrid systems (25, 28), to the activation of reporter genes such as the HIS3 gene or the lacZ gene (Fig. 1). We took advantage of the fact that Cdk7 is a very stable ternary complex containing Cdk7, cyclin H, and MAT1 (29, 30), which exist in the cell either as part of TFIIH or as an element of the cell cycle cascade. As such, Cdk7 interacts with subunits of TFIIH (12, 27, 31, 32) and other cyclin-dependent kinases (Cdc2 (33), Cdk2 (34), Cdk4 (35)) as well as substrates like nuclear receptors or p53 (Ref. 19, 20, and 36 and references therein).

Therefore one protein partner is fused to a DNA binding domain (here, LexA-Cdk7), which recognizes a specific upstream sequence in the promoter of the reporter gene. Another partner is fused to the activation domain (here, X-VP16), which will interact with the basal transcription machinery. The direct (two-hybrid) or the mediated (three- or four-hybrid) interaction between these two fusion proteins then allows the growth of the yeast on histidine-lacking medium and the detection of β-galactosidase activity. Thus, the two other partners of Cdk7 within CAK, cyclin H and MAT1, should be expressed independently from the two fusion proteins. Cyclin H was from the conditional expression vector pLex9-Cdk7/Met-25-cyclin H. MAT1 was under the control of the phosphoglycerate kinase promoter in the plasmid pYEA-MAT1. It has to be pointed out that the Met-25 promoter regulates the expression of cyclin H; therefore, the two other partners of Cdk7 as a control for checking the specificity of the quaternary complex assembly. Indeed, the Met-25 promoter, which is active under normal medium conditions, can be repressed in the presence of methionine, thus preventing the formation of the quaternary complex and consequently, the reporter gene expression (Fig. 1B).

We have sequentially transformed the L40 yeast strain with the plasmids pLex9-Cdk7/Met-25-cyclin H and pYEA-MAT1. Using the ternary CAK complex (including Cdk7, cyclin H, and MAT1) as bait, we then screened a cDNA library derived from HeLa cell mRNA fused to the VP16 activation domain. Double screening of the yeast transformants (1.5 million) by plating on TALH medium gave 109 clones that were positive in the liquid β-Gal assay. Eighteen of the clones with the highest β-Gal activities have been sequenced. To our surprise we identified the XPD helicase, a subunit of the core-TFIIH (6, 12, 27), as an interacting partner with one of the highest β-Gal activities. As listed in Table I, we also found some other proteins involved in the regulation of gene expression (HSP60, hoxC10, pRB-binding protein II, polypeptide elongation factor 1a (eEF-1), FIR, or SR splicing factor). In addition, we have identified two different proteasome subunits and the calcium-dependent protease as members of diverse degradation pathways as well as two still unknown proteins. Up to now only FIR has been shown to interact with TFIIH, resulting in the repression of promoter escape of mRNA polymerase II, which was activated before by FUSE-binding protein (21). These findings fit with previous reports and validate our assay in which a ternary complex can be used as bait to screen for interacting partners.

XPD Interacts with CAK—To further validate our assay, we investigated how the XPD helicase interacts with CAK. Therefore expression vectors in which the activating VP16 domain was fused either to the C terminus (XPD-VP16) or to the N terminus (VP16-XPD) of XPD were constructed (Fig. 2B). Yeast

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2 S. Dubaele, unpublished results.
CAK Regulates XPD

The names of the cDNAs identified in the yeast four-hybrid screen are listed together with the number of clones and the β-Gal activity (XPD = 100%).

| cDNA        | No. of clones | β-Gal |
|-------------|---------------|-------|
| XPD         | 1             | 100   |
| HoxC10      | 1             | 120   |
| FIR         | 3             | 79    |
| pRB-binding protein II | 3 | 55    |
| Proteasome subunit | 1 | 52    |
| Polypropylene elongation factor 1a (eEF-1) | 2 | 47    |
| Calcium-dependent protease | 1 | 47    |
| SR splicing factor | 1 | 29    |
| Proteasome subunit | 2 | 28    |
| HSP60 (heat shock protein) | 1 | 25    |
| Unknown A5  | 1             | 39    |
| Unknown B21 | 1             | 22    |
| VP16        | 2             |       |

Since the XPD helicase is one of the major players in NER, and XPD mutations lead to XP, Cockayne syndrome, and trichothiodystrophy diseases, we investigated which part of XPD targets CAK. Three-dimensional models of the XPD structure designed from the structure of UVRB (37) show that XPD can be divided into two domains, the N-terminal domain, which includes three helicase elements (Fig. 3A), and the C-terminal domain, in which 80% of the mutations seen in XPD patients are located (11, 38). Some of them weaken the interaction with p44, thereby inhibiting the XPD helicase activity of the holotFIIH (8). Therefore, we constructed two XPD deletion mutants (Fig. 2B), one containing the N-terminal half with the central part (amino acids 1–436, XPDN-VP16) and the other containing the C-terminal half of XPD (amino acids 431–760, XPDC-VP16). XPDN-VP16 and XPDC-VP16 proteins were then expressed together with the CAK subunits in the L40 yeast strain. XPDC-VP16, which lacks the 1–430 N-terminal moiety of XPD, prevents the formation of the quaternary complex, whereas XPDN-VP16 allows β-Gal synthesis (Fig. 2A, lanes 1 and 11, respectively). Nevertheless, it has to be pointed out that truncation of the C-terminal part of XPD partially affects the optimal gene expression induction (around 70% compared with XPD-wild type), underlying the importance of a well preserved XPD architecture.

**XPD Mutations Weaken CAK-XPD Interaction**—We then wondered if some mutations found within XPD patients (38) would affect the formation of the CAK-XPD and, therefore, the TFIIH transcription and/or DNA repair activity. Fused expression vectors bearing XPD mutations identical to those found in three patients (R112H (39), G602D (40), and D234N (41)) were designed (Fig. 3A). These hybrid mutants were expressed in yeast together with Cdk7, cyclin H, and MAT1. Filter-leaf assays combined with liquid β-Gal assays demonstrated that XPD-R112H as well as XPD-D234N weaken the binding capacity of XPD for the CAK complex (Fig. 3B, lanes 2 and 4). Together these data suggest that the N-terminal domain of XPD is targeted by CAK and that some XPD phenotypes could be due to a defect in these interactions. It is, however, interesting to note that the XPD-G602D mutation results in a 40% drop of β-Gal activity, meaning that this mutation, which is not involved in the XPD/p44-interacting domain, affects CAK-XPD interaction (8, 42).

**MAT1 Bridges CAK to XPD**—Having demonstrated (Fig. 2A, lane 7) that the expression of MAT1 is crucial for the formation of the CAK-XPD, we asked which parts of MAT1 were critical interaction domains. MAT1 can be subdivided into three regions (27), the N-terminal ring finger (MAT1(1–66)) domain, the coiled-coil (MAT1(67–189)) domain, and the C-terminal hydrophobic (MAT1(191–309)) domain. None of these three domains, when transfected with those expressing Cdk7, cyclin H, and XPD, allow synthesis of β-Gal (Fig. 4, lanes 1–5) and, therefore, are not sufficient to promote the formation of the quaternary complex. On the other hand, when fused together, the coiled-coil domain and the hydrophobic C terminus of MAT1 (MAT1(67–309)) lead to β-Gal activity comparable with that of the entire MAT1 (lanes 1 and 6). It should be noted that MAT1(67–309) and the truncated XPDN-VP16 together with LexA-Cdk7 and cyclin H are still able to partially reconstitute the transcriptional activator (lane 7). This demonstrates that

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**TABLE I**

| cDNA        | No. of clones | β-Gal |
|-------------|---------------|-------|
| XPD         | 1             | 100   |
| HoxC10      | 1             | 120   |
| FIR         | 3             | 79    |
| pRB-binding protein II | 3 | 55    |
| Proteasome subunit | 1 | 52    |
| Polypropylene elongation factor 1a (eEF-1) | 2 | 47    |
| Calcium-dependent protease | 1 | 47    |
| SR splicing factor | 1 | 29    |
| Proteasome subunit | 2 | 28    |
| HSP60 (heat shock protein) | 1 | 25    |
| Unknown A5  | 1             | 39    |
| Unknown B21 | 1             | 22    |
| VP16        | 2             |       |

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3. R. Bienstock and B. van Houtten, personal communication.
4. B. Sandrock, and J.-M. Egly, unpublished data.
some motifs in the C-terminal part of XPD are also necessary for the CAK complex assembly, a point already suggested by transfection using XPD-G602D mutant (Fig. 3, lane 3).

**Fig. 3.** Mutations in XPD derived from XP/XP-Cockayne syndrome or trichothiodystrophy patients have a reduced interaction capacity to the CAK complex. A, illustration of the XPD structure (38). White boxes represent the DNA/DNA helicase elements derived from sequence homology studies. R112H, D234N, and G602D represent mutations found in trichothiodystrophy, XP, and XP/Cockayne syndrome patients, respectively. B, yeast cells are transformed with pLexA-Cdk7/Met-25-cyclin H and pYEA-MAT1 together with either pXPD-VP16, pXPD(R112H)-VP16, pXPD(D234N)-VP16, pXPD(G602D)-VP16, or the empty control VP16 as indicated. Lift filter assay and liquid β-Gal assays are performed in the absence (black columns) or in the presence of 1 mM methionine (gray columns). The values for the liquid β-Gal assays (in %) represent at least four independent experiments and are shown with S.D. bars, where CAK-XPD = 100%.

**Fig. 4.** MAT1 links CAK to XPD. Combinatorial expression of wild type or mutated XPD, Cdk7, cyclin H, and MAT1 as indicated allows synthesis of β-Gal. Wild type or truncated forms of MAT1 are designated at the top of the figure. The values (in %) represent at least four independent experiments and are shown with S.D. bars, where CAK-XPD = 100%.

**DISCUSSION**

A Highly Specific Assay for Screening—In the course of this study we have designed a yeast four-hybrid system using a ternary complex as bait to screen any cDNA library. Under these conditions, the target protein recognizes the ternary complex more specifically through multipoint attachment. Our system, which uses the CAK complex as bait, also possesses its

CAK Regulates XPD
own internal control since, when one of the partner (cyclin H) is not expressed, the ternary complex cannot be formed. In addition to the improvement of both the specificity and the strength of the interaction, this assay is also flexible for additional approaches; it can be used to analyze structural proteins (MAT1) or to screen for either bridging proteins of a complex or inhibitors, which hinder complex formation (25).

This assay, which could be adapted once a ternary protein complex has been identified, was in fact set up to investigate the role of the Cdk-activating kinase CAK and its putative target proteins. A cDNA screening for CAK-interacting partners with the four-hybrid system has identified two proteins among others that have already been described as targets for CAK, the FIR protein (21) and the XPD helicase (12, 32, 43). This validates this highly specific method to screen and analyze multiprotein complexes. Because only the expression of VP16 fused to the C terminus of the target protein (XPD-VP16) allows formation of the activator (Fig. 2A), our system further points out how each library screening can fail to identify all possible interacting partners.

Switching Off and On XPD Helicase—It was demonstrated that in HeLa cell protein extracts the CAK as well as CAK/H18528XPD complexes exist in addition to TFIIH (12, 31, 32). Together with previous work (27) we have now definitively established that XPD bridges p44, a subunit of the core TFIIH to the MAT1 subunit of CAK. However it is unclear what the biological significance of CAKXPD complexes is in DNA repair and transcription. Our study now shows how XPD, which possesses a helicase activity, is switched off upon interaction with MAT1, thus rendering the CAKXPD complex unable to unwind DNA. The ability to switch off XPD avoids the deregulated helicase activity of XPD apart from its activity in TFIIH, which could
lead to the unwinding of any DNA not linked to proper transcription and repair reactions. Upon binding to the XPD sub-unit of CAXPD, p44 not only counteracts the repressive effect of MAT1 but also stimulates the XPD helicase activity. Moreover, our data allow us to hypothesize that whether or not CAXPD exists free in the cell, it recovers its helicase activity only once it associates with the core TFIIH. The inhibition, which can be due to some conformational changes induced by MAT1, which may mask either the DNA and/or the ATP binding site of XPD, can be circumvented by p44. It is thus likely that some mutations in XPD might affect not only its binding to p44, leading to the appearance of XP phenotype, but also to MAT1. Indeed, a weaker interaction between XPD and MAT1 (and consequently CAX) will not disturb the XPD activity within TFIIH but rather the kinase activity of CAX toward substrates such as RNA polymerase II and/or nuclear receptors.

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