Structural insight into the TFIIE–TFIIH interaction: TFIIE and p53 share the binding region on TFIIH

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RNA polymerase II and general transcription factors (GTFs) assemble on a promoter to form a transcription preinitiation complex (PIC). Among the GTFs, TFIIE recruits TFIIH to complete the PIC formation and regulates enzymatic activities of TFIIH. However, the mode of binding between TFIIE and TFIIH is poorly understood. Here, we demonstrate the specific binding of the C-terminal acidic domain (AC-D) of the human TFIIEξ subunit to the pleckstrin homology domain (PH-D) of the human TFIIH p62 subunit and describe the solution structures of the free and PH-D-bound forms of AC-D. Although the flexible N-terminal acidic tail from AC-D wraps around PH-D, the core domain of AC-D also interacts with PH-D. AC-D employs an entirely novel binding mode, which differs from the amphipathic helix method used by many transcriptional activators. So the binding surface between PH-D and AC-D is much broader than the specific binding surface between PH-D and the p53 acidic fragments. From our in vitro studies, we demonstrate that this interaction could be a switch to replace p53 with TFIIE on TFIIH in transcription. The EMBO Journal (2008) 27, 1161–1171. doi:10.1038/emboj.2008.47; Published online 20 March 2008 Subject Categories: chromatin & transcription; structural biology

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Introduction

In eukaryotes, transcription of protein-encoding genes is performed by RNA polymerase II (Pol II). Although it is a complex enzyme comprised of 12 subunits, Pol II alone is unable to accurately recognize promoters to initiate transcription. Transcription initiation by Pol II requires five proteins; TFIIB, TFIID, TFIIE, TFIIH and TFIIH collectively known as ‘general transcription factors (GTFs)’ (Orphanides et al, 1996; Roeder, 1996). Pol II and GTFs converge on a promoter in a highly ordered manner to form the preinitiation complex (PIC). After the binding of TFIIH to the TATA element located 30 nt upstream of the transcription initiation site (+1), TFIIB and TFIIH together with Pol II are recruited. TFIIE then joins the PIC, and finally TFIIE recruits TFIIH. After these steps, double-stranded DNA around the initiation site is melted to the single-stranded form by TFIIH (Dvir et al, 1996). TFIIH binds to the region between −10 and +2 (Douziech et al, 2000), where it is required to initiate melting and assist in the formation of the open complex (Holstege et al, 1996; Okamoto et al, 1998). Following extensive phosphorylation of the C-terminal domain (CTD) of the largest subunit Rpb1 of Pol II by TFIIH, activated Pol II releases all GTFs except for TFIIF and proceeds to transcription elongation (Lu et al, 1992). Upon promoter clearance, TFIIH increases the CTD kinase activity of TFIIH (Drapkin et al, 1994; Ohkuma and Roeder, 1994). Thus, it is now known that both TFIIE and TFIIH have significant functions in transcription initiation and the transition to elongation.

In relation to transcriptional machinery, so far the structures of TBP (TATA box-binding protein) subunit (Nikolov et al, 1992) from TFIIID, TBP–DNA (Kim et al, 1993a, b), TBP–DNA–TFIIB (Nikolov et al, 1995), Pol II (Cramer et al, 2000, 2001) and Pol II–TFIIH (Bushnell et al, 2004) have been determined. On the basis of these studies, we can see the detailed structural model for the TBP–DNA–TFIIB–Pol II complex. However, for TFIIF-, TFIIE- and TFIIH-associated complex, only their several domain structures have been determined and their interaction mode has not yet been available. For the structural modelling of the PIC, many structural insights into the interactions among Pol II and GTFs, in particular the interactions between the late entries, TFIIE and TFIIH, are required.

Human TFIIIE (hTFIIIE) is a heterodimer, consisting of an α subunit (hTFIIIEα, 57 kDa) and a β subunit (hTFIIIEβ, 34 kDa) (Ohkuma et al, 1990, 1991; Peterson et al, 1991; Sumimoto et al, 1991; Itoh et al, 2005; Jawhari et al, 2006). Both subunits possess several characteristic sequences and structural/functional domains; for example, a Ser, Thr, Asp and Glu-rich (STDE) sequence and an acidic amino-acid-rich sequence are found in the C-terminal region of hTFIIIEα, whereas a Ser-rich sequence is found in the N-terminal region of hTFIIIEβ. Furthermore, two tertiary structures of a zinc-finger domain of hTFIIIEα (Okuda et al, 2004) and a winged helix/forkhead domain of hTFIIIEβ (Okuda et al, 2000) have been solved by NMR spectroscopy.

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Human TFIIH (hTFIIH) is a much larger molecule (480 kDa) consisting of 10 subunits (Giglia-Mari et al., 2004). This is divided into two subcomplexes, a core complex (XPB, p34, p44, p52, p62 and p8/TTDA subunits) and a CDK-activating kinase complex (CAK) (CDK7, cyclin H and MAT1 subunits). The two subcomplexes are linked by the XPD subunit (Schultz et al., 2000). The intriguing feature is that this factor has three enzymatic activities: ATP-dependent DNA helicase, DNA-dependent ATPase and CTD kinase activities, and participates not only in transcription but also in DNA repair and cell cycle control. Of these activities of hTFIIH, hTFIIE stimulates the CTD kinase and ATPase activities, and represses the helicase activity (Lu et al., 1992; Drapkin et al., 1994; Ohkuma and Roeder, 1994).

With regard to the interaction between hTFIIE and hTFIIH, it has been shown that the C-terminal acidic region of hTFIIEx is necessary for native hTFIIH binding (Ohkuma et al., 1995) and hTFIIE strongly binds to the p62 subunit of hTFIIH (Yamamoto et al., 2001; Okuda et al., 2004). Besides these findings, however, little was known about this fundamental mechanism. Insight into the mechanism is gained from the work described here, which demonstrates that the C-terminal acidic domain (AC-D) of hTFIIEx containing the acidic region specifically binds to the N-terminal p62 subunit of hTFIIH. We have determined structures of both free hTFIIEx AC-D and its form bound to the PH-D of hTFIIH p62 by using NMR spectroscopy. The structures reveal that hTFIIEx AC-D recognizes p62 PH-D tightly through a combination of hydrophobic and electrostatic interactions. hTFIIEx AC-D is found to share its binding surface on p62 PH-D with the acidic transactivation domains (TADs) of tumour suppressor protein p53 (Di Lello et al., 2006) and herpes simplex virus protein VP16 (Di Lello et al., 2005). However, hTFIIEx AC-D employs an entirely novel binding mode, which differs from the amphipathic helix method used by many transcriptional activators. Our structural and functional studies are informative with regard to the roles of these proteins in the transcription initiation mechanism.

Results
Structure of hTFIIEx AC-D

It has been reported that the C-terminal acidic region of hTFIIEx is necessary for hTFIIH binding (Figure 1A) (Ohkuma et al., 1995). To characterize the precise interaction at the molecular level, we first solved a solution structure of the AC-D of hTFIIEx using NMR spectroscopy (Figure 1B and Table I). The protein has a globular structure with flexible and disordered tails, consisting of the 16 N-terminal residues (amino acids 378–393) and the 5 C-terminal residues (amino acids 435–439). The core region forms a compact structure;

Figure 1 The C-terminal acidic domain (AC-D) of hTFIIEx. (A) Sequence alignment of hTFIIEx AC-D and its homologues. H, human; M, mouse; X, Xenopus laevis; D, Drosophila melanogaster; C, Caenorhabditis elegans; Sp, Schizosaccharomyces pombe; Sc, Saccharomyces cerevisiae. Acidic amino acids, D and E are coloured in red. Conserved residues are shown in bold. The p62 PH-D-binding site of hTFIIEx and similar sequences are underlined. Secondary structures of hTFIIEx AC-D are depicted above human sequence. Arrows and cylinders represent β-strands and α-helices, respectively. Orange circles indicate residues participating in the formation of the hydrophobic core. Yellow circles indicate mutation sites. (B) Solution structure of hTFIIEx AC-D. Left, superposition of the backbone heavy atoms of the 20 lowest energy NMR structures. Right, ribbon representation of the average structure.
### Table 1 Structural statistics for the 20 best structures of hTFIIEz AC-D and its complex with hTFIIH p62 PH-D

|                      | Free hTFIIEz AC-D | Complex Htfieez AC-D | Complex HtfIIH p62 PH-D |
|----------------------|-----------------|---------------------|------------------------|
| **Experimental restraints** |                 |                     |                        |
| Total distance restraints | 1205            | 1296                | 2822                   |
| Intraresidue          | 101             | 145                 | 287                    |
| Sequential (i–j = 1)  | 372             | 372                 | 701                    |
| Medium-range (1 < i–j < 5) | 367            | 391                 | 536                    |
| Intramolecular long range (i–j ≥ 5) | 333 | 344                | 1222                   |
| Hydrogen bond         | 16 × 2          | 22 × 2              | 38 × 2                 |
| Number of dihedral restraints |        |                     |                        |
| ϕ                    | 35              | 35                  | 65                     |
| ψ                    | 32              | 35                  | 64                     |
| X1                   | 21              | 25                  | 46                     |
| X2                   | 3               | 2                   | 10                     |
| **Statistics for structure calculations** |                 |                     |                        |
| r.m.s. deviations from experimental restraints |         |                     |                        |
| Distance (Å)          | 0.010 ± 0.000   | 0.014 ± 0.000       |                        |
| Dihedral (deg)        | 0.080 ± 0.026   | 0.47 ± 0.03          |                        |
| r.m.s. deviations from idealized covalent geometry |     |                     |                        |
| Bonds (Å)             | 0.0020 ± 0.0001 | 0.0020 ± 0.0000     |                        |
| Angles (deg)          | 0.53 ± 0.00     | 0.55 ± 0.00         |                        |
| Improper (deg)        | 0.40 ± 0.01     | 0.44 ± 0.01         |                        |
| Final energies        |                 |                     |                        |
| E total (kcal mol⁻¹)  | 100.5 ± 0.6     | 347.8 ± 4.2         |                        |
| E bond (kcal mol⁻¹)   | 3.3 ± 0.3       | 11.1 ± 0.4          |                        |
| E angle (kcal mol⁻¹)  | 75.3 ± 0.6      | 230.4 ± 1.9         |                        |
| E van der Waals (kcal mol⁻¹) | 3.1 ± 0.4 | 20.6 ± 1.7          |                        |
| E NOE (kcal mol⁻¹)    | 6.5 ± 0.5       | 41.5 ± 2.8          |                        |
| E dihedral (kcal mol⁻¹) | 0.0 ± 0.0 | 3.6 ± 0.5          |                        |
| E improper (kcal mol⁻¹) | 12.4 ± 0.6 | 40.6 ± 1.1          |                        |
| Coordinate precision  |                 |                     |                        |
| Backbone atoms (Å)    | 0.24 ± 0.08b    | 0.20 ± 0.04b        | 0.44 ± 0.08c          |
| Heavy atoms (Å)       | 0.70 ± 0.07b    | 0.68 ± 0.05b        | 0.85 ± 0.10d          |
| Ramachandran plot statistics |     |                     |                        |
| Most favoured regions (%) | 82.9b   | 79.4d               |                        |
| Additional allowed regions (%) | 17.1b | 16.9d               |                        |
| Generously allowed regions (%) | 0.0b | 2.9d               |                        |
| Disallowed regions (%) | 0.0b | 0.7d               |                        |

Note: aNone of the structures exhibited distance violations > 0.5 Å, dihedral angle violations > 5°.

bThe value was calculated over residues 393–433 of hTFIIEz AC-D in the free form or in the complex.

cThe value was calculated over residues 7–104 of hTFIIH p62 PH-D in the complex.

dThe value was calculated over residues 383–433 of hTFIIEz AC-D and residues 7–104 of hTFIIH p62 PH-D in the complex.

The β-turn (S1–S2) is followed by three α-helices (H1, H2 and H3). These structural elements interact with each other and are maintained by a small but rigid hydrophobic core formed by P394, V396, V398, F403, Y405, V408, L414, V415, M418, E422, K423, Y426, I427, M429 and M433 residues. As the hydrophobic core residues as well as consecutive acidic amino acids found in the N-terminal regions of the AC-Ds are highly conserved in metazoans, they would all be expected to have similar structural features to hTFIIEz AC-D (Figure 1A). The structure seems to be a novel fold; similar structures with a Z score over 2.0 could not be detected by the DALI server.

**hTFIIEz AC-D specifically binds to PH-D of hTFIIH p62**

Previous studies showed that hTFIIEz specifically bound to the p62 subunits of hTFIIH (Yamamoto et al., 2001; Okuda et al., 2004). Given that the C-terminal acidic region of hTFIIEz (residues 378–393) is essential for hTFIIH binding (Ohkuma et al., 1995), hTFIIEz AC-D is likely to be responsible for hTFIIH recognition. To confirm this and to identify the AC-D-binding region in p62, we performed a GST pull-down assay using hTFIIEz AC-D and GST-fused p62 deletion mutants (Figure 2A). After purification by glutathione-Sepharose column chromatography, all samples containing the C-terminal region, namely full-length GST–p62₁–₁₀₈, GST–p62₁₀₉–₁₅₄, GST–p62₁₅₅–₂₃₈ and GST–p62₁₃₃–₃₃₃ were considerably degraded or incompletely translated (data not shown). Though such instability of the C-terminal half of p62 has previously been reported (Jawhari et al., 2004), we found that full-length GST–p62₁–₁₅₄, GST–p62₁₀₉–₁₅₄, GST–p62₁₅₅–₂₃₈ and GST–p62₁₃₃–₃₃₃ bound to hTFIIEz AC-D, whereas no binding was observed with GST–p62₁₀₉–₁₅₄, GST–p62₁₅₅–₂₃₈ and GST alone (Figure 2B). p62 contains the structurally stable PH-D (residues 1–108) (Gervais et al., 2004) and double BSD domains (residues 109–232) (Doersk et al., 2002) within the N-terminal half. The truncation variants with hTFIIEz-binding ability all possess the PH-D. We therefore asked whether p62 PH-D could interact with full-length hTFIIEz (Figure 2B). The results showed that GST-fused p62₁–₁₅₄, p62₁₀₉–₁₅₄, p62₁₅₅–₂₃₈...
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and p62\textsubscript{1–333}, all of which contain N-terminal 108 residues, bound to hTFIIEx, whereas the mutants p62\textsubscript{109–548}, p62\textsubscript{238–548} and p62\textsubscript{333–548}, which lack those 108 residues, could not bind to hTFIIEx (Figure 2B, lanes 2–5 versus lanes 6–8). Thus, we concluded that hTFIIEx binds specifically to the PH-D of hTFIIH p62 (p62 PH-D) through its AC-D.

Structure of complex between hTFIIEx AC-D and hTFIIH p62 PH-D
To obtain the p62 PH-D-bound structure of hTFIIEx AC-D using NMR, we performed NMR titration experiments in buffers both with and without 100 mM NaCl for both domains (Supplementary Figures 1–3). Although in the 100 mM NaCl buffer the dissociation constant (K\textsubscript{D}) between AC-D and p62 PH-D was estimated from the titration plots as 376 ± 81 nM (Supplementary Figure 1C) or 237 ± 82 nM (Supplementary Figure 2C), the NaCl-free buffer NMR titration experiment showed much stronger binding affinity between AC-D and p62 PH-D because of the slow exchange timescale with a K\textsubscript{D} below about 150 nM (Supplementary Figure 3). On the basis of these results, we determined the complex structure in 20 mM potassium phosphate buffer without NaCl.

In total, 4489 NOE-derived distance restraints, including 371 intermolecular NOEs, 120 hydrogen bond restraints and 282 dihedral angle restraints were used to determine the complex structure (Table I). The complex structure is shown in Figure 3A and B. The occluded solvent-accessible surface between hTFIIEx AC-D and p62 PH-D was ~2300 Å\textsuperscript{2}.

The core structure of hTFIIEx AC-D and p62 PH-D in the complex was essentially the same as seen in each isolated free structure except for the extended and highly acidic N-terminal tail of hTFIIEx AC-D. In the free form, it is disordered (Figure 1B) but upon complex formation becomes fixed, forming a new β strand that extensively overlays the positively charged surface of p62 PH-D formed by K18, K19, K54, K60, K62 and K93 (Figure 3C). Nine consecutive acidic residues from the N-terminal tail of hTFIIEx AC-D run across the second β-sheet (S5, S6 and S7) of p62 PH-D, electrostatically interacting with K18 and K19 on the loop between the S1 and S2 strands and K60 and K62 on the loop between S5 and S6 strands. The extended tail of hTFIIEx AC-D curves at E386 (Figure 3B). Polypeptides from F387 to A391 of hTFIIEx AC-D align along the S5 strand of p62 PH-D, forming an antiparallel β-sheets structure with it. It is noteworthy that F387 in the sequence of acidic residues is accommodated in a shallow pocket on the second β-sheet of p62 PH-D formed by K54, I55, S56, K60, Q64, L65, Q66 and N76 (Figure 3D). In the pocket, hydrophobic interactions between the aromatic side chain of F387 (hTFIIEx) and the aliphatic portions of K54 and K60 (p62) and amino-aromatic interactions (Burley and Petsko, 1986) between the side chains of F387 and Q64, Q66 and N76 (p62) were observed. E388 (hTFIIEx) interacts through van der Waals contacts with I55 and P57 (p62), and E389 (hTFIIEx) forms a salt bridge with K54 (p62). Similar to as seen for F387 (hTFIIEx), V390 was inserted into a shallow pocket between the S5 strand and the C-terminal H1 helix of p62 PH-D, formed by Q53 and I55 on the S5 strand and K93 and Q97 on the H1 helix (Figure 3E). V390 makes extensive hydrophobic contacts with I55 and the aliphatic regions of Q53, K93 and Q97. The N-terminal tail of hTFIIEx AC-D bends further at D392 (Figure 3B), which causes van der Waals contacts between L100 and P101 (p62) (Figure 3F). D393 (hTFIIEx) at the end of the tail lies in close proximity to Q97 (p62) making van der Waals contact with it.

These interacting amino acids were also observed in the NMR titration experiments. In hTFIIEx AC-D, the NMR signals of E386, F387, E388, E389, V390, A391 and D392 were changed significantly upon addition of p62 PH-D (Supplementary Figure 1B) and also in p62 PH-D the NMR signals of K19, Q53, K54, I55, S56, E58, K60, A61, I63, Q64, L65, Q66, T74, T75 and F77 were changed by adding hTFIIEx AC-D (Supplementary Figure 2B).

It is remarkable that in addition to the interaction involving the N-terminal flexible tail of hTFIIEx AC-D, its core structure also participates in the binding to p62 PH-D (Figure 3F). Several residues in the N- and C-terminal regions of the hTFIIEx AC-D core structure interact with residues located in the C-terminal region of p62 PH-D. P394 (hTFIIEx) at the N terminus of the core structure contributes to the formation of the hydrophobic core of hTFIIEx AC-D and simultaneously makes intimate van der Waals contacts with P101 (p62) and also with the aliphatic portion of Q98 (p62). I395 (hTFIIEx), which is exposed to the surface in the free form, now makes hydrophobic contact with the aliphatic segment of Q98 (p62). R432 (hTFIIEx), which is at the end of the H3 helix, makes van der Waals contacts with P101 and the hydrophobic portion of K102 (p62). M433 (TFIIH) makes van der Waals contact with the aliphatic region of K104 (p62), which is also able to make an electrostatic interaction with D392 or D436. These interactions allow the core structure of
hTFIIE α AC-D to take up a position to the side of p62 PH-D, such that the whole complex structure is well defined as shown in Figure 3A.

**Effects of hTFIIE α AC-D mutations on binding to p62 PH-D**

In functional studies of hTFIIE α, we made several mutants of hTFIIE α by changing S365, V372, D380, E383, F387, V390

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**Figure 3** Structure of complex of hTFIIE α AC-D and hTFIIH p62 PH-D. (A) Superposition of the backbone heavy atoms of the 20 lowest energy NMR structures. Structures are superimposed over residues 383–433 of hTFIIE α AC-D shown in green, and residues 7–104 of p62 PH-D shown in orange (left), residues 393–433 of TFIIez AC-D (middle) and residues 7–104 of p62 PH-D (right), respectively. (B) Ribbon representation of the average structure. (C) Electrostatic interaction. Positive and negative potentials on the molecular surface are coloured in blue and red, respectively. (D) Binding pocket of p62 PH-D for F387 of hTFIIE α AC-D. (E) Binding pocket of p62 PH-D for V390 of hTFIIE α AC-D. In (C–E), hTFIIE α AC-D is represented as a stick model, and p62 PH-D is shown as a molecular surface. (F) Interface between the core structure of hTFIIE α AC-D and p62 PH-D. Residues that participate in the binding are shown with the side chains.
and D393 to alanine (S365A, V372A, D380A, E383A, F387A, V390A and D393A) as well as S365E, V372D, F387E and V390K. These mutants were expressed in Escherichia coli with hexa-histidine (6H) at the N terminus. All were soluble and could therefore be easily purified using a Ni-nitrilotriacetic acid (NTA) agarose column (Figure 4A).

The ability of hTFIIEα mutants to bind to GST-tagged p62 PH-D was examined by in vitro binding assay (Figure 4B). The p62 PH-D-binding activity of the hTFIIEα mutants was severely reduced when the AC-D residues, F387 and V390, which fit into shallow pockets of p62 PH-D, were changed to F387A, F387E, V390A and V390K (Figure 4B, second column p62 PH-D, lanes 9–12). We have shown previously that the N terminus of hTFIIEα is essential for binding to hTFIIEβ (Ohkuma et al., 1995). Consistent with these observations is the fact that none of the hTFIIEα mutations affected the binding of hTFIIEα to hTFIIEβ (Figure 4B, third column IIEαβ, lanes 3–13).

Functional roles of hTFIIEα AC-D during transcription
To further investigate the functional roles of hTFIIEα AC-D, we first checked the effects of the hTFIIEα mutants on basal transcription using the adenovirus major late pML(C2AT) promoter. hTFIIEαAC-D, lanes 3–13). We next tested the effects on CTD phosphorylation. Each hTFIIEα mutant was mixed with hTFIIH and Pol II. The mixture was analysed by SDS–PAGE and phosphorylated Rpb1 from Pol II was detected by autoradiography (Figure 4D). All mutants failed to stimulate CTD phosphorylation properly compared with wild type (Figure 4D, lanes 3–13 versus lane 2). Phosphorylation profiles of the above-described mutants (S365A, S365E, V372A, V372D, D380A and E383A) phosphorylated CTD but most of Rpb1 was detected at the hypo-phosphorylated IIa position (Figure 4D, lanes 3–8). In contrast, the p62 PH-D-binding defective mutants phosphorylated CTD only weakly (Figure 4D, lanes 9–12).

A part of the hTFIIE-binding surface on hTFIIH p62 is shared with transcriptional activator p53
Recently, the structure of a complex of the PH-D of Saccharomyces cerevisiae Tfb1, a homologue of human p62, with a TAD2 of activator p53 was determined by NMR spectroscopy (Di Lello et al., 2006). The structure of the Tfb1 PH-D closely resembles that of p62, except for its shorter connecting loop between S6 and S7 (Figure 5A and B). Furthermore, the herpes simplex virus protein 16 (VP16) TAD also interacts with virtually identical sites of Tfb1 and p62 PH-D (Di Lello et al., 2005). Interestingly, the binding sites
of p62 for p53 TAD2 and VP16 TAD significantly overlap with a part of the binding site for hTFIIEα AC-D. However, their binding mode is entirely different. The binding site of p53 TAD2 peptide is disordered in an unbound state, but it forms a nine-residue amphipathic α-helix upon binding to Tfb1 PH-D and p62 PH-D. The p53 helix contacts the second β-sheet of Tfb1 PH-D through the interactions of I50 (p53)—M59, M88 (Tfb1); E51 (p53)—R61 or R86 (Tfb1); L60/L65 (Tfb1); and E56 (p53)—K57 (Tfb1). Although the N-terminal tail of hTFIIEα AC-D also becomes ordered upon binding to p62 PH-D, it forms a bent extended structure containing a S0 strand, but not α-helix. In spite of such great structural differences, both hTFIIEα AC-D and p53 TAD2 peptide insert phenylalanine residues, F387 of hTFIIEα AC-D and F54 of p53 TAD2, into the equivalent pocket on the second β-sheet of p62 PH-D.

Although the p53 TAD2 peptide consisting of residues 20–73 forms no contacts beside this limited area, hTFIIEα AC-D further interacts with p62 PH-D as mentioned above. The binding surface area of hTFIIEα AC-D and p62 PH-D is calculated as ~2300 Å², which is much larger than the binding area of p53 TAD2 for Tfb1 calculated as ~800 Å².

To analyse this interaction biochemically, several point mutants of p62 PH-D were created, bacterially expressed and used in binding studies with hTFIIEα AC-D and p53 TAD2 (Figure 5C). As controls, AC-D-containing hTFIIEα wild type (IIEα wt) and hTFIIEα351–439 (IIEα351–439) were also examined in parallel. As shown, K54, which forms the shallow pocket for F387 of hTFIIEα AC-D with its side chain interacting electrostatically with E389 of hTFIIEα AC-D, was the residue for which mutation to alanine had the largest effect as it prevented binding of all three hTFIIEα proteins tested (Figure 5C, lane 5). In addition, Q66, which also forms the same shallow pocket for F387 through amino-aromatic interaction, was shown to be essential for binding.
to hTFIIEz AC-D (Figure 5C, lane 6). The adjacent residues, V68, T74 and N76 of p62 PH-D as well as the N-terminal basic residues, K18 and K19, also affected binding but to a lesser extent (Figure 5C, lanes 3, 4, 7–9). A similar but distinct inhibition profile was observed for the N-terminal TAD of p53 (p531–73). In this case, Q66 was also central to the interaction but the essential binding residues were more widespread (Figure 5C, the bottom column, lanes 3 and 6–9).

Replacement of p53 bound to hTFIIE p62 with hTFIIEz
As the overlap of the p62 PH-D-binding region of hTFIIEz AC-D with that of p53 TAD2 was observed and judging from the functional context that p53 may recruit TFIIEH at transcriptional activation but at some point TFIIE should take over to recruit TFIIE into the PIC, we then asked whether p53 can be replaced on p62 with hTFIIEz. As shown in Figure 5D, p62 PH-D bound to GST-p53 TAD (p531–73) was removed upon addition of 6H-hTFIIEz wild type (Figure 5D, lanes 2–4). Unbound fractions were then mixed with Ni-NTA resin and FLAG-p62 PH-D bound to 6H-hTFIIEz was detected by western blotting (Figure 5D, lanes 7–9). This clearly demonstrates that p53 binding to p62 can be replaced with hTFIIEz.

Discussion
Interaction between hTFIIEz AC-D and hTFIIH p62 PH-D and its evolutionary conservation
In the present study, the specific interaction between hTFIIEz AC-D and hTFIIH p62 PH-D was explored, and structures of both the free and PH-D-bound forms of hTFIIEz AC-D were determined. This is the first report of the structural determination of the complex describing the interaction between TFIIE and TFIIFH at the molecular level. In the case of hTFIIEz AC-D, its binding site as identified here (residues 378–395) is consistent with the previous report that a deletion mutant of hTFIIEz, A377–393 could no longer bind to hTFIIFH, whereas a mutant with residues 351–439 could bind (Okhuma et al., 1995). hTFIIEz possesses another acidic region, the STDE (Ser, Thr, Asp and Glu-rich) region (residues 352–365) immediately before the hTFIIEz AC-D. We also examined the binding ability of peptide possessing only an acidic region, STDE, of hTFIIEz AC-D. Although the residues of p62 PH-D whose signals changed significantly were mostly consistent with the binding of AC-D and AC-D381–394, the extents of signal changes in the C-terminal region, to I395, R432 and M433 of AC-D binding region, were reduced. These results clearly indicate that hTFIIEz AC-D, which contains both the core structure and the flexible tail, is necessary and sufficient for the specific binding. This is an entirely new binding mode compared with the canonical binding modes found in some transcriptional activators or repressors, in which an intrinsically disordered region (Dyson and Wright, 2002) of each activation or repression domain binds to a target protein with part of the flexible region forming an ordered structure upon binding to the target. The core structure of hTFIIEz AC-D is essential for its binding to p62 PH-D in addition to its flexible arm.

The binding site of hTFIIH p62 PH-D was localized to the second β-sheet (S5, S6 and S7), the loops between S1 and S2 and between S5 and S6 and the C-terminal H1 helix, where a substantial positive cluster is formed. Therefore, it is reasonable to speculate that the N-terminal highly acidic tail of hTFIIEz AC-D strongly binds to the positively charged surface of hTFIIH p62 PH-D. This is supported by the result that the binding is strengthened by removing NaCl from the buffer in the NMR titration experiments. However, given that the first acidic region, STDE, of hTFIIEz has no effect on the binding, the interaction is not merely based on electrostatic interactions. As seen in the complex structure, the highly conserved F387 and V390 residues in the acidic region of hTFIIEz AC-D, not in the STDE region (Figure 1A) make a large contribution to binding. Thus, the combination of electrostatic and hydrophobic interactions is essential for specific binding.

It is interesting that hTFIIEz AC-D partially shares the binding surface of hTFIIEz p62 PH-D with acidic transcriptional activators, p53 and VP16 TADs (Di Lello et al., 2005, 2006). As p53 and VP16 TADs are able to bind to the PH-Ds of both p62 and Tfb1, their interactions are likely to be evolutionally conserved. For yeast TFIIEz (Tfa1), functional significance of the C-terminal region and specific interaction between Tfa1 and Tfb1 has been reported (Bushnell et al., 1996; Kuldell and Buratowski, 1997). We aligned sequences of TFIIEz AC-D from other species to ascertain whether the interaction observed in human is evolutionally conserved (Figure 1A). As described above, the residues forming the hydrophobic core are well conserved in metazoans, but not in yeast, S. cerevisiae and Schizosaccharomyces pombe. Thus, yeast homologues are unlikely to have a similar core structure to that of human AC-D. However, in yeast similar sequences to the binding site are found in the equivalent positions. Furthermore, in contrast to metazoans, only fungal homologues possess the third acidic region at the C termini. Surprisingly, similar sequences to the binding site are found in the third acidic regions of both S. cerevisiae and S. pombe. Considering that the main binding site of TFIIEz AC-D is located on the N-terminal tail outside the core structure, Tfa1 does not seem to have a similar core AC-D structure, but the interaction with PH-D is likely to be conserved. This suggests that the interplay between hTFIIEz AC-D, hTFIIH p62 PH-D and p53 TAD2 (and VP16 TAD as well) is evolutionally conserved.

Functional roles of hTFIIEz AC-D
In hTFIIEz AC-D, F387 and V390 are the centre of the p62 PH-D-binding region (Figure 4B). As expected, the p62 PH-D-binding defective mutants (F387A, F387E, V390A and V390K) showed defects in in vitro transcription (20–40% reduction from the wild type, Figure 4C, lane 2 versus lanes 9–12). The reason why transcription did not correlate well with the binding and CTD phosphorylation defects might be because there are more than 25 proteins involved in transcription, whereas only limited factors were used for both binding and phosphorylation studies (hTFIIEz AC-D mutants and p62 PH-D were used for binding studies and hTFIIEz
AC-D mutants, hTFIIH and Pol II were used for CTD phosphorylation. As a result, other GTFs will be able to support the recruitment of hTFIIH to the correct position in the PIC even in the absence of hTFIIEx AC-D. In addition, bigger defects were observed for mutations of the STDE and in the N-terminal half of hTFIIEx AC-D (S365, V372, D380 and E383) (Figure 4C, lanes 3–8). This effect maybe a result of the action of GTFs with the possibility that this region of hTFIIEx exerts an effect as a binding site for one or more of the general factors. We will test this possibility in the immediate future.

Functional implication of the interplay between TFIIE, TFIIH and acidic transcriptional activators

In many cases, acidic TADs are disordered in an unbound form, but form an amphipathic helix upon binding to target proteins, for example, p53 TAD2–RPA70 (replication protein A 70) (Bochkareva et al, 2005), p53 TAD1–MDM2 (ubiquitin ligase) (Kussie et al, 1996), VP16 TAD–hTAF63 (human TBP-associated factor) (Usugi et al, 1997) complexes as well as the recently determined Tfb1 PH-D–p53 TAD2 complex (Di Lello et al, 2006). In contrast, hTFIIEx AC-D uniquely binds to p62 PH-D through its core structure together with its flexible N-terminal tail. The $K_d$ values for the binding of p53 TAD2 to p62 and Tfb1 PH-Ds determined by isothermal titration calorimetry (ITC) are 3175 ± 570 and 391 ± 74 nM, respectively (Di Lello et al, 2006). In the binding of VP16 TAD to Tfb1 PH-D, the $K_d$ value estimated by NMR titration experiment was ~4000–7000 nM (Di Lello et al, 2005). Compared with these $K_d$ values, the binding of hTFIIEx AC-D to p62 PH-D is rather strong. One of the well-known functions of transcriptional activators is to promote transcription initiation by increasing recruitment efficiency of Pol II and GTFs (Ptashne and Gann, 1997). TFIIE is recruited through many activator-mediated routes. p62 has been shown to interact with the TADs of not only VP16 and p53 (Xiao et al, 1994) but also E2F-1 (Pearson and Greenblatt, 1997), and the oestrogen receptor (ER) (Chen et al, 2000). Of these, it was demonstrated that p53, VP16 and ERz target p62 PH-D. The notable finding from the present study is that hTFIIEx also targets p62 PH-D (Figure 2). To our knowledge, this is the first GTF demonstrated to possess a functional TAD-like motif. As shown in Figure 5D, hTFIIEx could replace p53 TAD and then bind to p62 PH-D. It can be imagined that when p53 TAD2 is unphosphorylated, the delivery of p62 from p53 TAD2 to hTFIIEx would be inefficient and they would function cooperatively in transcription initiation. However, when p53 TAD2 is doubly phosphorylated at S46 and T55, the affinity of p53 TAD2 for p62 PH-D would be comparable to that for hTFIIEx resulting in p53 and p62 (hTFIIH) functioning in DNA repair or in processes other than transactivation. Further studies are required to verify these possibilities.

Materials and methods

Construction of mutants of hTFIIEx and hTFIIH p62 subunits

By using the Multi Site-Directed Mutagenesis Kit (Medical Biology Laboratory) as a template with wild-type hTFIIEx cDNA plasmid or a hTFIIH p62 cDNA mutant in which a NdeI site was disrupted by changing the third nucleotide of the 45th histidine codon of wild-type hTFIIH p62 cDNA, T to C, various oligonucleotide-mediated point mutants were created (Kunkel et al, 1987). The mutants were checked by sequencing using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The oligonucleotides used for mutation are listed in Supplementary Table 1. The NdeI–BamHI fragment of mutated hTFIIEx cDNA was subcloned into the pET11d vector (Novagen) making the N-terminal hexa histidine-tagged hTFIIEx (6His–hTFIIEx) expression plasmid. The NdeI–BamHI fragment of mutant hTFIIH p62 cDNA was subcloned into the pET vector making the N-terminal FLAG-tagged hTFIIH p62 PH-D (FLAG–p62 PH) expression plasmid.

Purification of hTFIIEx AC-D and hTFIIH p62 PH-D

hTFIIEx AC-D (residues 378–439) was expressed as an hexa histidine-tagged product in pET3a vectors (Novagen) in E. coli BL21(DE3)pLysS (Novagen). Lysed supernatant was loaded onto the Ni-NTA-agarose (Qiagen) column. The eluate was then applied to the Ni-NTA-agarose (Qiagen) column. The eluate was then applied to the Ni-NTA-agarose (Qiagen) column. The eluate was then applied to the Ni-NTA-agarose (Qiagen) column. Fractions passing through the column were concentrated and applied onto Superdex30 (GE Healthcare). hTFIIH p62 PH-D was purified as described (Gervais et al, 2004).

NMR spectroscopy

Measurements of NMR spectra, structural calculations and NMR titration experiments are described in Supplementary data.

Purification of recombinant proteins

Recombinant point mutant hTFIIEx proteins were expressed in E. coli RosettaTM(DE3)pLysS (Novagen), and recombinant hTFIIH p62 point mutants were expressed in BL21(DE3)pLysS by induction with isopropyl-β-D-thiogalactopyranoside (Studier et al, 1990). The purification method of these recombinant proteins was as described previously (Watanabe et al, 2003). Typical preparations were >90% pure, judging by Coomassie blue staining of an SDS–polyacrylamide gel.
GST pull-down assay

GST fusion proteins were used for protein interaction assays (Okamoto et al., 1998). The bound proteins were released by boiling in SDS-PAGE loading buffer, separated by SDS-PAGE and detected by western blotting with anti-hTFIIIE rabbit antisera (1:3000 dilution), anti-FLAG M2 monoclonal antibody (Sigma) and anti-tp53 (DO-1) (Santa Cruz) using the enhanced chemiluminescence detection system (GE Healthcare).

In vitro transcription assay

Recombinant GSTs as well as native Pol II and hTFIIH were purified as described previously (Watanabe et al., 2003). In vitro transcription was performed as described (Ohkuma et al., 1995). The plasmid pML(CAT)A-50 containing the adenovirus 2 major late promoter, which gives a 390-nt transcript, was used as either a supercoiled or a linearized template for basal transcription assay (Yamamoto et al., 2001). To prepare the linearized template, pML(CAT)A-50 was digested with SmaI. After transcription, radiolabeled transcripts were subjected to urea-PAGE and detected by autoradiography. The transcripts were quantified by a Fuji BAS5000 Bio-Imaging Analyzer. Relative transcription activities of the mutant hTFIIIE proteins were calculated by defining the transcription activity of the wild-type hTFIIIEa as 100%.

Kinase assay

In addition to transcription factors added as described in the legend of Figure 4D, the kinase reaction mixture (25 µl) contained 5 mM MgCl2, 60 mM KCl, 12% (v/v) glycerol, 2% (w/v) polyethylene glycol 8000, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 240 µg/ml of bovine serum albumin, 5 µM ATP and 3 µCi of [γ-32P]ATP. Phosphorylation reactions were carried out at 30°C for 1 h and stopped by addition of 75 µl of phosphorylation stop solution (10 mM EDTA, 0.1% NP40 and 0.05% SDS). Phosphorylated proteins were precipitated with TCA, analyzed by SDS-PAGE (5.5% acrylamide), and detected by autoradiography with Fuji RX-U X-ray film.

Accession numbers

Coordinates of hTFIIIEa AC-D free and in complex with hTFIIH p62 PH-D have been deposited in the Protein Data Bank (www.rcsb.org) under accession codes 2RNQ and 2RNR, respectively.

Supplementary data

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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