Cell-cycle-dependent phosphorylation of the basal transcription factor RAP74

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Abstract In this report, cell-cycle-dependent effects of TFIID on other basal transcription factors were investigated. We purified TFIID fractions from HeLa cells synchronized in the S/G2 phases and in early G1 phase, and show that RAP74 is phosphorylated more highly by the S/G2 phase TFIID fraction than by the early G1 phase TFIID fraction. Further analyses using deletion mutants of RAP74 revealed that amino acid residues 206–256 are phosphorylated by the TFIID fraction. Reconstitution of in vitro transcription activity indicates that the cell-cycle-dependent phosphorylation of RAP74 increases TFIIF transcription activity.

Key words: TFIIF; RAP74; Cell cycle; Phosphorylation

1. Introduction

The eukaryotic cell cycle is a highly regulated process that is dependent on the temporal expression of specific genes. The cloning of $TAF_{II}250$ [1,2], the largest subunit of TFIID, has provided a link between cell cycle regulation and the basal transcription machinery. $TAF_{II}250$ is encoded by CCG1, a gene that overcomes a G1 arrest in the temperature-sensitive hamster cell line ts13 [3,4]. Therefore $TAF_{II}250$ may be required for progression through the G1 phase and may be involved in cell cycle control.

We have previously investigated in vitro RNA polymerase II (pol II) basal transcription activity during the cell cycle by using nuclear extracts from synchronized HeLa cells and showed that the activity is low in the S/G2 phases and high in the early G1 phase [5]. Further analyses revealed that TFIID is the rate-limiting component for this difference and a qualitative change of TFIID and protein–protein interactions between TFIID and other transcription factors may play an important role in cell-cycle-dependent basal transcription activity. In this report, the cell-cycle-dependent effects of TFIID on other basal transcription factors were investigated. We prepared TFIID fractions from HeLa cells synchronized in the S/G2 phases and early G1 phase, and found that RAP74, a large subunit of TFIIF, is phosphorylated more highly by the S/G2 phase TFIID fraction than by the early G1 phase TFIID fraction. Interestingly, Dikstein et al. [6] reported recently that $TAF_{II}250$ is a protein serine kinase that selectively phosphorylated RAP74.

TFIIF [7,8], also termed FC [9], RAP30/74 [10], βγ in rat [11], or factor 5 in *Drosophila* [12], is a heterodimer composed of ~30 kDa (RAP30) and ~70 kDa (RAP74) subunits [8–10]. TFIIF can bind pol II directly [7,9,10] and recruits the enzyme to a preformed DNA-TFIID-TFIIB complex [7]. Furthermore, TFIIF also stimulates the elongation of nascent mRNA by pol II [7,12,13]. Native TFIIF subunits have a lower mobility than the bacterially expressed recombinant proteins on SDS-PAGE [14], indicating that TFIIF is post-translationally modified in vivo. In experiments in our laboratory, RAP74 was extensively phosphorylated in vivo and TFIIF transcription activity was up-regulated by protein phosphorylation of RAP74 [15]. Consistent with this, it is shown that the cell-cycle-dependent phosphorylation of RAP74 by TFIID fraction increases TFIIF transcription activity.

2. Materials and methods

2.1. Cell synchronization and extract preparation

HeLa S3 cells were grown at 37°C in RPMI medium with 10% fetal bovine serum in dishes (245×245×25 mm) at a density of $1–8\times10^5$ cells/ml. These cells were synchronized at the G1–S phase boundary by the double thymidine block protocol [16] with minor modifications [5]. Log phase cells were incubated in complete medium with addition of 2 mM thymidine for 16 h. After release in thymidine-free medium for 8 h the cells were incubated again with 2 mM thymidine for 16 h. After release in thymidine-free medium the cells were harvested at 8 and 12 h. Monitoring of the synchronization has been described before [5]. Nuclear extracts were prepared as described by Dignam et al. [17] with minor modifications as described before [5].

2.2. Purification of TFIID fraction and recombinant TFIIF

Nuclear extracts were applied to a phosphocellulose (P11, Whatman) column equilibrated with buffer D. After washing the column with buffer D containing 0.5 M KCl, TFIID was eluted stepwise by buffer D containing 0.7 M KCl. Recombinant RAP30, recombinant RAP74, and deletion mutants of RAP74 were expressed in *E. coli* and purified as described before [14]. Recombinant RAP74 was expressed with *E. coli* chaperon GroESL to increase solubility of RAP74 [18].

2.3. In vitro kinase assay

TFIID fraction was incubated with 50 ng of recombinant RAP74 at 30°C for 30 min in 10 μl of kinase buffer containing 25 mM HEPES (pH 7.9), 12.5 mM $MgCl_2$, 0.1 M KCl, 0.1 mM EDTA, 0.1% NP-40, and 3 μCi [γ-^{32}P]ATP. The reactions were stopped by the addition of protein sample buffer followed by SDS-PAGE and autoradiography for 3–15 min.

2.4. In vitro transcription assay

In vitro transcription activity of phosphorylated recombinant RAP74 was assayed as before [9,19] with minor modifications. 50 ng of recombinant RAP74 was phosphorylated by 5 ng of the S/G2 phase TFIID fraction and by 5 ng of the early G1 phase TFIID fraction. A mixture of 50 ng of phosphorylated recombinant RAP74

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Abbreviations: TF, transcription factor; TAF, TBP-associated factor

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and 50 ng of recombinant RAP30 was first incubated at 30°C for 30 min in 20 μl. The reaction was performed in a total of 20 μl containing 0.1 ng of preincubated TFIIF, 10 ng of TATA box binding protein (TBP), 20 ng of TFIIB, 0.4 μg of FE (a mixture of TFIIE and TFIIH), 0.8 μg of the D1P2 fraction (a crude fraction containing FA and FB according to our nomenclature; see Ref. [20]), and 0.5 μg of RNA polymerase II using 2 μg of pMLC2AT-Δ50 [21] which contains the region −50 to +10 of Ad2MLP and a downstream G-less cassette. The above mixture was incubated for the assembly of the initiation complex at 30°C for 30 min, and nucleoside triphosphates were added to a final concentration of 600 μM for ATP and CTP and 25 μM for UTP (5 μCi). After 60 min, the reaction was stopped and radioactive RNA was isolated. RNA transcripts were analyzed on 6% polyacrylamide-7M urea gel.

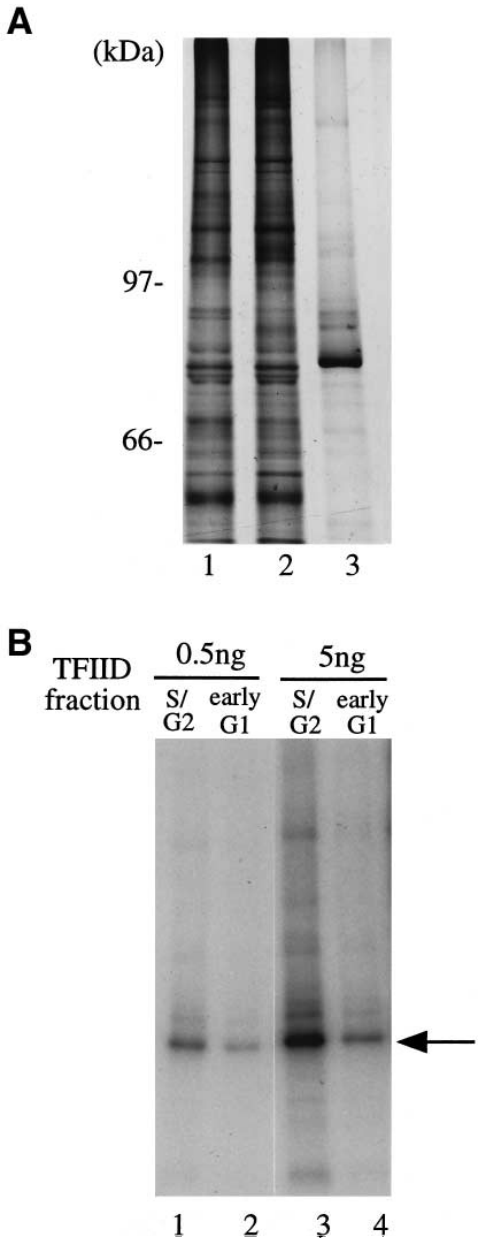

Fig. 1. In vitro kinase assays with recombinant RAP74 in the S/G2 phase TFIID fraction and in the early G1 phase TFIID fraction. A: 2 μg of the S/G2 phase TFIID fraction (lane 1), 2 μg of the early G1 phase TFIID fraction (lane 2), and 50 ng of recombinant RAP74 (lane 3) were analyzed on 7% SDS-PAGE and stained by silver staining. B: In vitro kinase assays were performed with 50 ng of recombinant RAP74 in 0.5 ng (lane 1) and 5 ng (lane 3) of the S/G2 phase TFIID fraction, and in 0.5 ng (lane 2) and 5 ng (lane 4) of the early G1 phase TFIID fraction. Arrow indicates RAP74.

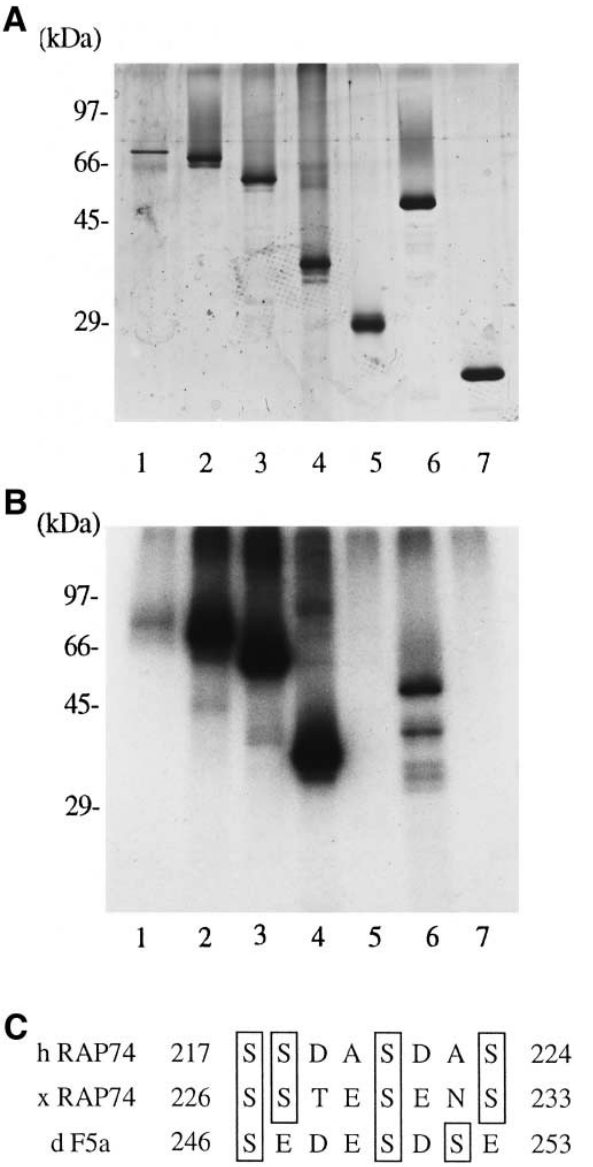

Fig. 2. In vitro kinase assays with deletion mutants of RAP74. A: Various deletion mutants of RAP74 were analyzed on 10% SDS-PAGE and stained by silver stain. Lane 1, wild type; lane 2, r74(1–435); lane 3, r74(1–356); lane 4, r74(1–256); lane 5, r74(1–205); lane 6, r74(205–517); lane 7, r74(356–517). B: In vitro kinase assays with various deletion mutants of RAP74 in the S/G2 phase TFIID fraction. Lanes 1–7 are the same as (A). C: Evolutionary conservation of serine residues in amino acids 217–224 acids of RAP74. Human RAP74 (h RAP74) [19,22] is compared with *Xenopus* RAP74 (x RAP74) [23] and *Drosophila* Factor 5a (d F5a) [24,25]. Serine residues are boxed.

3. Results

HeLa S3 cells were synchronized at the G1–S phase boundary by double thymidine block. The effectiveness of this synchronization was monitored before [5] and the cells were found to be predominantly in the S/G2 phases and early G1 phase at 8 and 12 h after release from the thymidine block, respectively. Nuclear extracts were prepared from cells

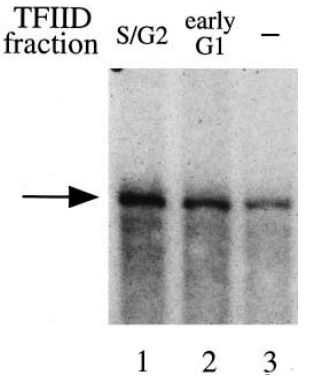

Fig. 3. In vitro transcription activity of phosphorylated RAP74. Reconstitution of in vitro transcription using phosphorylated RAP74 was assayed as described in Section 2. Lane 1, phosphorylated RAP74 by the S/G2 phases TFIID fraction; lane 2, phosphorylated RAP74 by the early G1 phase TFIID fraction; lane 3, unphosphorylated RAP74.

synchronized at 8 and 12 h after release and TFIID fractions were subjected to partial purification at each time point. Fig. 1A shows the S/G2 phase TFIID fraction (lane 1) and the early G1 phase fraction (lane 2). Although we performed in vitro kinase assays with recombinant TBP, TFIIB, and RAP30, the TFIID fractions did not phosphorylate these proteins (data not shown). In contrast, recombinant RAP74 was phosphorylated by the TFIID fractions and the kinase activity was significantly higher in the S/G2 phase fraction than in the early G1 phase fraction upon the use of the same concentration of the TFIID fractions (Fig. 1B).

To investigate the phosphorylated region of RAP74, C-terminal deletion mutants containing the C-terminal sequence deleted down to amino acids 435, 356, 256 and 205, and N-terminal deletion mutants containing N-terminal sequence deleted down to residues 205 and 356 were prepared (Fig. 2A). Fig. 2B shows in vitro kinase assay with these deletion mutants in the S/G2 phase TFIID fraction. Although r74(1–435), r74(1–356), r74(1–256), and r74(205–517) were phosphorylated, r74(1–205) and r74(356–517) were not. Therefore the TFIID fraction phosphorylates amino acid residues 206–256 of RAP74. Amino acid residues 206–256 contain 5 serine residues and do not contain any threonine or tyrosine residues. Particularly amino acid residues 217–224 have a cluster of serine residues (Fig. 2C). Comparison with human RAP74 [19,22], *Xenopus* RAP74 [23], and *Drosophila* Factor 5a [24,25] shows that these serine residues may be highly conserved during evolution. Therefore it is highly likely that the serine residues in amino acids 217–224 are phosphorylated by TFIID in a cell-cycle-dependent manner.

We have previously reported that RAP74 is extensively phosphorylated in vivo and that TFIIF transcription activity is up-regulated by protein phosphorylation of RAP74 [15]. To analyze the effect of the cell-cycle-dependent phosphorylation of RAP74 on pol II basal transcription activity, reconstitution of in vitro transcription activity using the phosphorylated recombinant RAP74 was performed. Fig. 3 shows that phosphorylated RAP74 by the early G1 phase TFIID fraction (lane 2) had stronger activity than unphosphorylated RAP74 (lane 3) and that high-phosphorylated RAP74 by the S/G2 phase fraction (lane 1) had the strongest activity. These data indicate that the cell-cycle-dependent phosphorylation of RAP74 increases the in vitro transcription activity.

4. Discussion

In this report, cell-cycle-dependent effects of TFIID on other basal transcription factors were investigated. We prepared TFIID fractions from HeLa cells synchronized in the S/G2 phases and early G1 phase, and found that RAP74 is phosphorylated more highly by the S/G2 phases TFIID fraction than by the early G1 phase TFIID fraction. In experiments in our laboratory, RAP74 was extensively phosphorylated in vivo but RAP30 was not and TFIIF transcription initiation and elongation activities were up-regulated by protein phosphorylation of RAP74 [15]. Consistent with this, it was shown that the cell-cycle-dependent phosphorylation of RAP74 increases the in vitro transcription activity. Also, we have previously reported that in vitro pol II basal transcription activity is low in the S/G2 phases and high in the early G1 phase [5]. The changes in TFIIF activity and basal transcription activity during the cell cycle are quite opposite. In our former report [5], post-translationally modified recombinant TFIIF is not the rate-limiting step for the change of transcription activity, indicating that phosphorylated TFIIF activity does not have a dominant effect on pol II basal transcription activity during the cell cycle.

RAP74 is proposed to be structurally separated into three regions—a globular N-terminal domain (1–179), a charged domain (180–356), and a globular C-terminal domain (357–517) [19,22]. The N-terminal region is important for RAP30 binding [14,26] and the C-terminal region contributes to transcription activity [14,26,27]. The internal region of RAP74 carries three multiple charge clusters and three hyper-charge runs [28]. Multiple charge clusters and hyper-charge runs are rare sequence features occurring in $<4\%$ of all eukaryotic proteins. It was proposed that the distinguishing charge features are related to interactions with nucleic acids and other proteins in the initiation and processing of the transcription apparatus. Also several possible phosphorylation sites for many protein kinases including casein kinase II, protein kinase C, and protein kinase A have been found along with the charge clusters [19]. In this report, it was shown that the TFIID fraction phosphorylates amino acid residues 206–256 which is in the central charged domain of RAP74. This result suggests that cell-cycle-dependent phosphorylation affects the assembly and function of the pol II initiation and/or elongation complex. Next, amino acid residues 206–256 contain 5 serine residues and do not contain any threonine or tyrosine residues. Particularly amino acid residues 217–224 have a cluster of serine residues and the serine residues may be highly conserved during evolution (Fig. 2C). Therefore it is highly likely that the serine residues in amino acids 217–224 are phosphorylated by TFIID in a cell-cycle-dependent manner.

$TAF_{II}250$ [1,2], the largest subunit of TFIID, rescues the temperature-sensitive hamster cell line, ts13, and overcomes a G1 arrest. Therefore $TAF_{II}250$ may be required for progression through the G1 phase and may be involved in cell cycle control. Recently Dikstein et al. [6] reported that $TAF_{II}250$ is a protein serine kinase that selectively phosphorylates RAP74 but not other basal transcription factors and that N- and C-terminal domains of $TAF_{II}250$ are required for efficient transphosphorylation of RAP74. It is possible that $TAF_{II}250$ phos-

phorylates the serine residues in amino acids 217–224 of RAP74 in a cell-cycle-dependent manner. Furthermore, Ruppert and Tjian [29] reported that human $TAF_{II}250$ interacts with RAP74 and that the RAP74 binding region of $TAF_{II}250$ is required to rescue ts13 cells. Therefore the cell-cycle-dependent phosphorylation of RAP74 may play an important role in cell cycle progression.

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