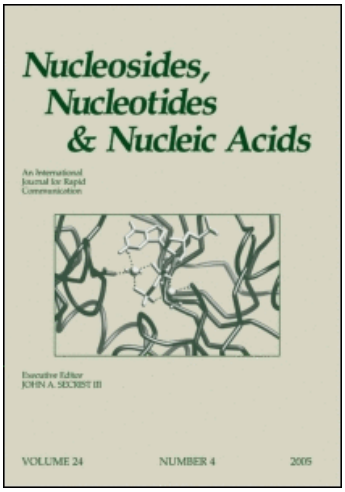

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IDENTIFICATION AND CHARACTERIZATION OF MITOCHONDRIAL FACTORS MODULATING THYMIDINE KINASE 2 ACTIVITY

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□ *Thymidine kinase 2 (TK2) is one of four deoxynucleoside kinases (dNKs) in humans and plays a crucial role in the initial phosphorylation of pyrimidine nucleosides in the salvage pathway in mitochondria. Nucleoside analogues, like AZT, are substrates of TK2 and induced mitochondrial toxicity in long-term therapy. We found that AZT and FLT inhibited dThd phosphorylation but stimulated dCyd phosphorylation catalyzed by TK2. However, mitochondrial phosphorylation of both dThd and dCyd was inhibited by AZT and FLT. Here a preliminary identification and characterization of mitochondrial factors is reported.*

Keywords Mitochondrial thymidine kinase 2; AZT; FLT; mitochondrial factors

INTRODUCTION

Mitochondrial thymidine kinase (TK2) catalyses the phosphorylation of thymidine (dThd) and deoxycytidine (dCyd) to the corresponding nucleoside monophosphates. TK2 is constitutively expressed and plays an important role in providing precursors for the replication and maintenance of mitochondrial DNA (mtDNA), especially in nonproliferating cells where the S-phase specific de novo pathway of dTMP synthesis is downregulated.[1] Some nucleoside analogues, such as 3′-azido-3′- deoxythmidine (AZT) used in anti-HIV therapy, are also substrates of TK2 and caused side effects after prolonged treatment, namely mitochondrial toxicity with symptoms similar to those of mtDNA depletion syndrome (MDS) patients with TK2 deficiency.[2]

We investigated the effects of the thymidine analogues AZT and 3′-fluorothymidine (FLT) on the activities of both partial purified rat TK2 and human recombinant TK2 (hTK2). In reconstitution experiment, a mitochondrial factors in crude rat liver mitochondrial extract was found to

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be able to stimulate human recombinant TK2 by inducing high molecular weight complexes. The TK2 modifying factors were purified from rat liver mitochondrial extracts and some initial characterization was done.

MATERIALS AND METHODS

Human recombinant TK2 was expressed and purified as the previously reported.[3] Rat TK2 was partially purified from rat liver mitochondrial total extracts by anion exchange chromatography on a DEAE-Sephadex column. The flow through (abbreviated as FT in this article) fraction from anion exchange chromatography was collected and used for further experiments. Protein concentrations were measured by Bio-Rad protein assay using BSA as standard. The TK2 activity was determined by a radiochemical method as described[4] using tritium labeled dThd and dCyd as substrates.

Purified hTK2 was mixed with FT and applied to a Suprose 12 column and eluted with a buffer containing 25 mM Tris/HCl (pH 7.6), 150 mM KCl, and 2 mM $MgCl_2$. The fractions were assayed for TK2 activity with dThd as substrate, and analyzed by SDS-PAGE and Western blots using a TK2-antibody produced against a C-terminal peptide.

In order to isolate the mitochondrial factors, a TK2-Sepharose column was made by coupling hTK2 to CNBr-actived Sepharose 4 Fast Flow. FT was applied to the column and eluted with 1M Glycine/HCl (pH 2.8), and neutralized with 1M Tris/HCl (pH 9.4). The eluate was saved for further analysis.

RESULTS AND DISCUSSION

Effects of Nucleoside Analogues on dThd and dCyd Phosophorylation by TK2

Similar to hTK2, purified rat liver TK2 phosphorylated dThd and dCyd with K_m values for dThd and dCyd of 18.8 and 41.4 μM, respectively, and V_{max} values of 69.4 and 88.5 nmol/min/mg, respectively. AZT and FLT inhibited the phosphorylation of dThd by both hTK2 and rat liver TK2 with K_i values 3.9 and 1.4 μM, respectively. However, at physiological relevant concentration dCyd phosphorylation was not inhibited by AZT or FLT. Instead, approximate 30% stimulation was observed.

In rat liver mitochondrial total extracts, the phosphorylation of dThd and dCyd by TK2 was inhibited by AZT or FLT, indicating that there must be some components in mitochondria involved in the regulation of TK2 activity and these components could be separated from TK2 by anion exchange chromatography.

Stimulation of hTK2 Activity by Mitochondrial Factors

Upon size exclusion chromatography on a Suprose 12 column, hTK2 was eluted as single peak (fraction 20–30; Figure 1A). However, when the same amount of hTK2 was mixed with FT in which rat liver TK2 has been removed by anion exchange chromatography, the hTK2 activity was eluted in two broad peaks (fraction 10 to 25). Furthermore, the activity level with dThd

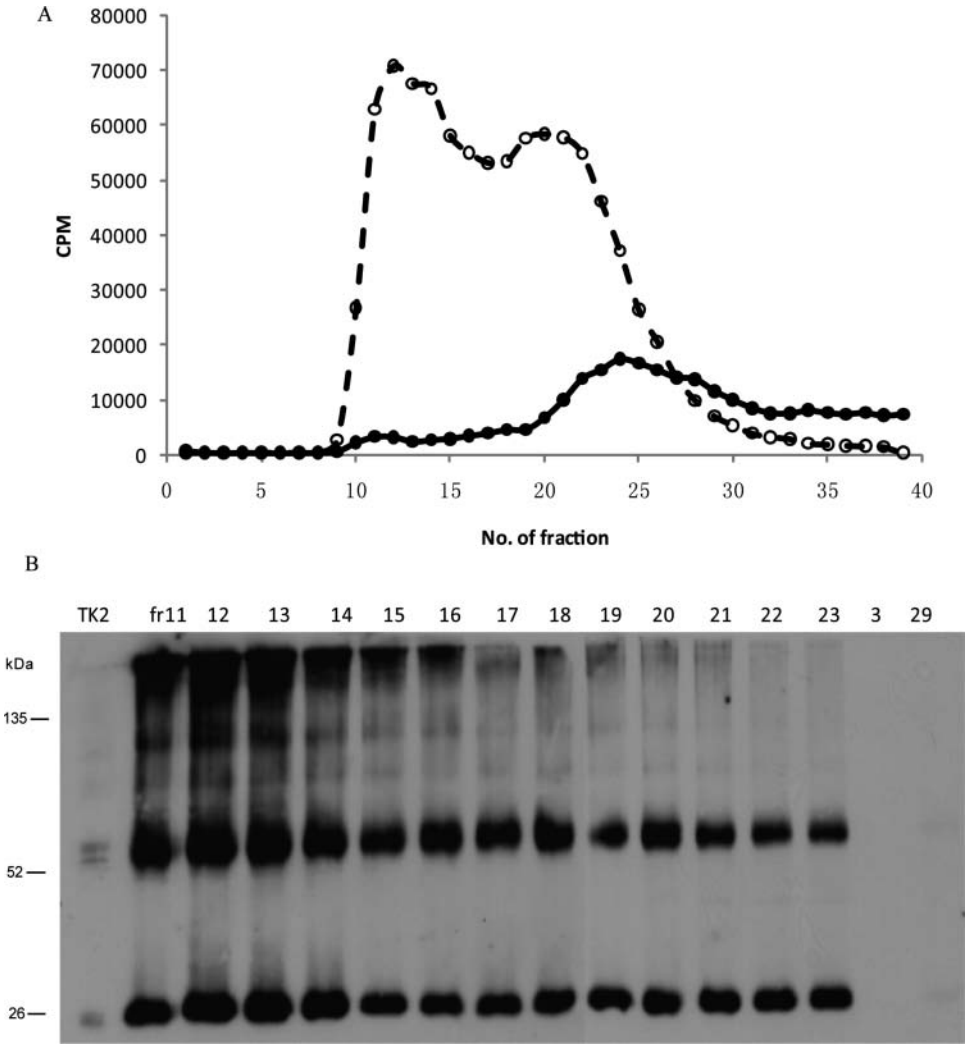

FIGURE 1 Stimulation of human recombinant TK2 by mitochondrial extract. A) The hTK2 alone (solid line) or mixed with FT (dashed line) was applied to a Suprose 12 column and eluted. Fractions were collected and assayed for the TK2 activity with dThd as substrate. B) Nonreducing SDS-PAGE and Western-blotting analysis of fractions from the latter group. Lane 1, hTK2; Lanes 2–14 correspond to fraction 11–23; Lanes 15 and 16 were fraction 3 and 29, respectively.

as substrate was ~3-fold as compared with when hTK2 was alone applied to the column (Figure 1A).

Fractions with hTK2 activity were analyzed by nonreducing SDS-PAGE and Western-blot using a peptide antibody against hTK2. As shown in Figure 1B, fraction 11–16 contained hTK2 molecules in monomer, dimer, and even oligomer forms while only monomers and dimers were observed in fraction 17–23. These results suggested that in FT fraction there are some factors which bind to hTK2 and form a high molecular weight complex, or alternatively induce oligomerization of hTK2.

In order to isolate the factors from the FT fraction, a TK2-Sepharose column was made by coupling hTK2 to the activated CNBr-actived Sepharose. FT was loaded to the column and eluted. The eluate was then mixed with hTK2, applied to the Suprose 12 column and eluted with 1M Glycine/HCl (pH 2.8). Fractions were assayed for TK2 activity. The activity profile was similar to the results with the mixture of hTK2 and FT. However, we were unable to identify the molecular nature of these factors due to the limited amount of material. A scale up preparation is needed to identify these factors.

Our results showed that mitochondria contain factors, which can stimulate the activity of TK2 by noncovalent interactions. The functional consequence of this regulation of TK2 remains to be determined.

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