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Identification and characterization of human ribokinase and comparison of its properties with *E. coli* ribokinase and human adenosine kinase

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Abstract The gene responsible for ribokinase (RK) in human/eukaryotic cells has not yet been identified/characterized. Blast searches with *E. coli* RK have identified a human protein showing significant similarity to the bacterial RK. The cDNA for this protein was expressed in *E. coli* and the recombinant protein efficiently phosphorylated ribose to ribose-5-phosphate using ATP, confirming its identity as RK. In contrast to ribose, the enzyme exhibited very little to no phosphorylation of D-arabinose, D-xylose, D-fructose and D-galactose. The catalytic activity of human RK was dependent upon the presence of inorganic phosphate, as observed previously for *E. coli* RK and mammalian adenosine kinases (AK). A number of activators and inhibitors of human AK, produced very similar effects on the human and *E. coli* RKs, indicating that the catalytic mechanism of RK is very similar to that of the AKs.

Keywords: Ribokinase; Adenosine kinase; PfkB family of proteins; Phosphate dependency; Inhibitors and activators of adenosine kinase and ribokinase; Ribose metabolism

1. Introduction

Ribokinase is a carbohydrate kinase, which catalyzes the phosphorylation of ribose to ribose-5-phosphate (R-5-P). Exogenous ribose has shown beneficial effects in a number of studies. It has been reported to enhance cardiac and muscular performance and to accelerate the repletion of ATP in rat and canine myocardium as well as in isolated cardiomyocytes and endothelial cells [1–4]. Ribose addition also helps maintain higher levels of ATP in rat heart and dog kidney during transplantation experiments [5], and it led to improvement of neurological symptoms in a patient with adenylosuccinase deficiency [6]. In order for ribose to be incorporated into ATP or other high energy phosphorylated derivatives, ribose must first be converted into ribose-5-phosphate [1]. Hence, it is of much interest to identify and characterize the enzyme responsible for this critical first step.

The enzyme RK belongs to the PfkB family of carbohydrate kinases [7], which includes enzymes such as adenosine kinase (AK), inosine-guanosine kinase, fructokinase, 1-phosphofructokinase, and 6-phosphofructokinase minor. The overall sequence identity between PfkB family members is less than 30%, but remarkably high structural similarity is seen between RK [8] and AK [9]. Most of the biochemical and structural studies on RK have been carried out using enzyme from *E. coli* cells [10–12]. Although RK activity was first described in calf liver in 1956 [13], very little work on characterizing this activity in mammalian systems has been carried out. The identification of the gene encoding for human/eukaryotic RK, or detailed characterization of this activity in mammalian systems has not yet been reported. This communication describes, for the first time, definitive identification of the human RK gene and biochemical characterization of its encoded protein product. The substrate specificity of the enzyme as well as the effects of a number of activators and inhibitors of human AK on both human and *E. coli* RK were studied. Our results indicate that the catalytic mechanism of both human and *E. coli* RK is very similar to that of AK.

2. Methods and materials

2.1. Materials

D-[1-^{3}H] ribose (20 Ci/mmol), D-[1-^{3}H] arabinose (20 Ci/mmol), D-[^{14}C] fructose (300 mCi/mmol), D-[6-^{3}H] galactose (40 Ci/mmol), and D-[1-^{3}H] xylose (15 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). ATP was purchased from Pharmacia Biotech (Canada). All other chemicals were of analytical reagent grade.

2.2. Cloning, expression, and purification of E. coli and human RK

The *E. coli* RK gene (accession no. NC_000913) was amplified by PCR from the *E. coli* genomic DNA using specific oligonucleotide primers and cloned into pET22b expression vector. Human RK was identified by Blastp searches and its sequence was aligned with that of *E. coli* and other ribokinases. For expression of human RK cDNA, total mRNA from human HT1080 cells was prepared and reverse transcribed using reverse transcriptase. Using this cDNA, the cDNA for human RK was PCR amplified using specific primers based on the predicted start and end positions of the RK protein. After checking the sequence for its accuracy, it was cloned into the pET15b expression vector. For expression studies, the vector constructs were transformed into *E. coli* BL21 cells, which were induced with 0.1 mM IPTG for 6 h at room temperature. The $(His)_6$-tagged protein was purified to >90% purity by means of nickel affinity chromatography. Further purification was carried out by means of gel-filtration chromatography and the purified enzyme was stored at −20 °C in 10% glycerol.

2.3. Activity assay

RK activity was measured by a radiochemical method using D-[1-^{3}H] ribose, as described previously [11]. Assays were performed at 37 °C in 20 mM Tris–maleate buffer (pH 7.0) containing 125 mM KCl. The kinetic data were fitted to the equation:

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$v_0 = V_{max}/(K_m + A + (A^2/K_i))$

which describes the rate of a bisubstrate reaction where the concentration of an inhibitory substrate A is varied at a fixed concentration of the other substrate [14,15]. For the K_m determination of ribose, the concentration of ATP was held fixed at 5 mM, whereas the K_m determination of ATP was carried out in the presence of 2 mM ribose. Saturating concentrations of ribose were avoided in these experiments due to significant enzyme inhibition by the substrate at high concentration. In all cases, the concentration of free magnesium was fixed at 2 mM, which produced maximal enzyme activity. Magnesium also showed inhibitory effects on the enzyme activity at higher concentrations (not shown).

3. Results

3.1. Identification of the human RK gene and expression of the corresponding cDNA

The gene responsible for RK activity and the corresponding protein has not yet been identified or biochemically characterized in human and other eukaryotic cells. Our Blastp searches of the NCBI nr database with bacterial (*E. coli*) RK sequence identified a protein in the human genome that has been annotated as ribokinase (protein length 322 aa, accession number NP_071411){Lander, 2001 276 /id}. Significant blast hits with low E values ($<1e^{-25}$) were also observed for a variety of other eukaryotic organisms including rat, mouse, dog, chicken, frog, *Drosophila*, nematode, *Leishmania*, *Trypanosoma*, *Entamoeba*, *Giardia*, various plants (e.g. *Arabidopsis thaliana*) and fungi (e.g. *Saccharomyces cerevisiae*), etc. Interestingly, when Blast searches were performed with either the human (or mouse) RK sequence (instead of the *E. coli* RK sequence), the blast hits with the lowest E values were for the animal homologs followed by those of the bacterial homologs. The hits from plants and fungi had much higher E values in comparison to the bacterial homologs indicating that they were more distantly related to the human or animal homologs in comparison to the bacterial proteins.

Table 1 presents a pair-wise sequence identity/similarity matrix of RK sequences from representative prokaryotic and eukaryotic species. As seen, the human RK shows a much higher degree of sequence identity to the RK sequences from protist species (columns F and G) and various bacteria (columns H and I) as compared to that observed for the homologs from plants and fungi (columns D and E). These results are unexpected as the fungi and plants are more closely related to the animals than the bacteria [16]. The human RK also shows significant sequence similarity to the human as well as other eukaryotic adenosine kinases. However, this sequence similarity is lower than that seen for any of the ribokinases. In Fig. 1A, a multiple sequence alignment of RK from some representative prokaryotic and eukaryotic species is presented. There are several short stretches that are conserved in all of the ribokinases, and overall about 12–15% of the residues in divergent species are completely conserved. The human RK gene, which is located on chromosome 2 (locus NM_022184), is approximately 110 kb in length and is comprised of 8 exons of varying lengths (Fig. 1B).

The PfkB family of proteins include many carbohydrate kinases including RK and AK [7]. Based upon the observed sequence similarity between *E. coli* RK and the human protein (Table 1), one cannot be certain that the human protein in question corresponds to ribokinase and not some other sugar kinase. The lower similarity of the human protein to fungi and plant homologs as compared to bacteria highlights this problem. To determine whether the protein product of this putative human gene indeed corresponds to RK, the cDNA for this gene was PCR amplified from human HT1080 cells and expressed in *E. coli* cells. In cells transformed with the recombinant plasmid, a protein of the expected molecular mass (~30 kDa) was expressed at high level upon induction with IPTG. This protein was purified to near homogeneity by nickel-affinity and gel-filtration chromatography.

3.2. Biochemical characterization of human ribokinase

The protein purified as outlined above was shown to carry out efficient phosphorylation of ribose to ribose-5-phosphate in presence of ATP (under standard conditions containing 2 mM ribose, 5 mM ATP and 10 mM Pi) confirming its identity as RK. Although ribose metabolism and transport has

Table 1
Pair-wise identity/similarity between RK and AK sequences from different species

Source of sequence	% Identity or similarity in amino acid sequences[a]											
	A	B	C	D	E	F	G	H	I	J	K	L
RK												
(A) *H. sapiens*		69.6	42.8	26.1	29.1	41.9	37.1	39.1	35.5	21.0	23.5	24.1
(B) *X. tropicalis*	81.4		44.1	25.9	27.9	39.8	37.1	39.0	31.9	21.7	23.4	22.5
(C) *D. melano.*	60.9	60.3		26.9	30.6	36.2	31.5	39.4	36.0	24.1	26.1	22.9
(D) *A. thaliana*	41.6	39.3	37.7		28.3	24.0	26.4	27.4	29.7	21.0	23.3	22.4
(E) *S. cerevisiae*	43.4	42.6	44.6	41.5		28.0	27.4	31.9	30.7	26.9	25.3	22.1
(F) *T. cruzi*	56.9	54.5	52.3	39.2	45.8		38.7	39.4	36.1	23.5	26.9	23.7
(G) *En. histolytica*	55.3	54.7	48.3	40.7	43.5	55.0		30.3	31.3	20.7	19.3	21.8
(H) *B. cepacia*	50.9	52.7	51.4	41.4	46.4	51.5	49.5		42.5	22.0	21.7	27.5
(I) *E. coli*	53.2	48.0	48.8	45.7	48.9	51.8	49.5	55.3		23.2	22.6	20.3
(J) *H. sapiens*	37.8	40.0	40.1	37.4	39.5	35.9	36.6	34.6	40.1		57.2	39.9
AK												
(K) *A. thaliana*	41.1	40.0	41.6	38.1	40.8	40.2	35.9	37.4	41.2	74.6		39.4
(L) *S. cerevisiae*	41.0	38.7	37.1	40.8	38.0	39.4	38.7	40.9	36.9	59.8	55.7	

[a]Sequence alignment and identity/similarity between different pairs of sequences were carried out by using the BioEdit program. Upper and lower triangles indicate the percent identity and percent similarity, respectively, between pairs of sequences. Abbreviations in the sequence names are: A., *Arabidopsis*; B, *Burkholderia*; *D. melano.*, *Drosophila melangaster*; *En.*, *Entamoeba*; *E.*, *Escherichia*; *H.*, *Homo*; *S.*, *Saccharomyces*; *T.*, *Trypanosoma*; *X.*, *Xenopus*. RK, ribokinase; AK, adenosine kinase.