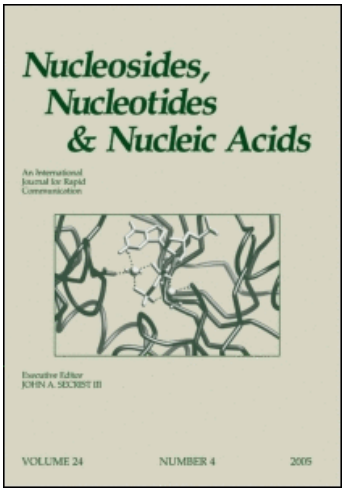

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Investigation of the Substrate Recognition of *Drosophila melanogaster* Nucleoside Kinase by Site Directed Mutagenesis

N. Solaroli[ab]; M. Bjerke[a]; M. Johansson[a]; A. Karlsson[a]
[a] Division of Clinical Virology F68, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden [b] Department of Pharmaceutical Science, Ferrara University, Ferrara, Italy

Online publication date: 27 October 2004

To cite this Article Solaroli, N. , Bjerke, M. , Johansson, M. and Karlsson, A.(2004) 'Investigation of the Substrate Recognition of *Drosophila melanogaster* Nucleoside Kinase by Site Directed Mutagenesis', Nucleosides, Nucleotides and Nucleic Acids, 23: 8, 1527 — 1529
To link to this Article: DOI: 10.1081/NCN-200027744
URL: http://dx.doi.org/10.1081/NCN-200027744

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 8 & 9, pp. 1527–1529, 2004

Investigation of the Substrate Recognition of *Drosophila melanogaster* Nucleoside Kinase by Site Directed Mutagenesis

N. Solaroli,[1,2,*] M. Bjerke,[1] M. Johansson,[1] and A. Karlsson[1]

[1]Division of Clinical Virology F68, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden
[2]Department of Pharmaceutical Science, Ferrara University, Ferrara, Italy

ABSTRACT

The deoxyribonucleoside kinase of *Drosophila melanogaster* (*Dm*-dNK) has a broad substrate specificity and a higher catalytic rate than other known deoxyribonucleoside kinases. Therefore it is a natural candidate for possible use as a suicide gene in combined gene/chemotherapy of cancer. We have performed site directed mutagenesis and tested different truncated forms of the enzyme in order to increase the affinity for ganciclovir.

Key Words: Gene therapy; Nucleoside analog; Suicide gene.

INTRODUCTION

Expression of herpes simplex virus thymidine kinase (HSV-TK) in tumor cells and subsequent systemic chemotherapy with the nucleoside analog ganciclovir (GCV) is the prototype for combined gene/chemotherapy of malignant tumors. In search for an

*Correspondence: N. Solaroli, Division of Clinical Virology F68, Karolinska Institute, Huddinge University Hospital, Stockholm S-14186, Sweden.

DOI: 10.1081/NCN-200027744
1525-7770 (Print); 1532-2335 (Online)

alternative suicide gene to HSV-TK, we have cloned and characterized a deoxyribonucleoside kinase of *Drosophila melanogaster* (*Dm*-dNK). The enzyme efficiently phosphorylates several anti-viral and anti-cancer nucleoside analogs with a higher catalytic rate than other known deoxyribonucleoside kinases. Despite this broad substrate specificity, *Dm*-dNK does not phosphorylate GCV or other guanosine nucleoside analogs. Recently it has been shown that the *Dm*-dNK with 20 amino acids deletion of the C-terminal[1] has even higher catalytic rates for deoxyribonucleosides compared to the wild type, and it was also reported that only a few amino acids are important to change the substrate specificity from pyrimidines to purines.[2] Based on these previous studies we have constructed the mutated enzymes, with and without the C-terminal deletion, and performed determinations of the kinetic properties regarding GCV phosphorylation. The most efficient enzyme phosphorylating GCV will be expressed in cancer cells for further studies as a novel suicide gene candidate.

MATERIALS AND METHODS

Site-directed mutagenesis: Site-directed mutagenesis of the *Dm*-dNK cDNA was performed using the QuikChange site-directed mutagenesis kit (Stratagene).

Expression and purification: We expressed the *Dm*-dNK cDNA in E. coli as a fusion protein to glutathione S-transferase (GST). The expressed protein was purified using glutathione-sepharose 4B (Amersham Pharmacia Biotech).

Enzyme assays: The activity of the purified recombinant enzymes was assayed in a 50 μl reaction mixture containing: 50 mM Tris-HCl pH 7.6, 0.1 mg/ml BSA, 2.5 mM ATP, 5 mM $MgCl_2$, 5mM dithiothreitol, and 0.15 μM [methyl-^{3}H]dThd or [8-3H]-GCV

Table 1. a) Kinetics of dThd and GCV phosphorylation by recombinant *Dm*-dNK mutants. b) Nucleoside and nucleoside analog phosphorylation by recombinant *Dm*-dNK mutant enzymes.

	Deoxythymidine			Ganciclovir		
a	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Dm-dNK	2.5	4350	1740	n.d.	n.d.	
M88R wt	930	1200	1.29	920	31	0.033
M88R Δ20	790	1000	1.26	1200	20	0.016
V84A + M88R wt	5020	1150	0.23	2680	38	0.014
V84A + M88R Δ20	3300	1860	0.56	1750	23	0.013
V84A + M88R + A110D				5060	23	0.004

	Substrates 500 μM								
b	dThd	dAdo	dGuo	BVDU	AraA	AraG	dFdC	dFdG	GCV
Dm-dNK	100	55	<1	59	<1	<1	24	<1	<1
M88R Δ20	100	160	114	136	122	46	200	69	20
V84A + M88R Δ20	100	167	118	132	105	24	124	63	30

(BVDU = (E)-5-(2-bromovinyl)-2′-deoxyuridine; AraA = 9-β-D-arabinofuranosyladenine; AraG = 1-β-D-arabinofuranosylguanine; dFdC = 2′,2′-difluorodeoxycytidine; dFdG = 2′2′-difluorodeoxyguanosine; n.d. = not detectable)

(Moravek Biochemicals, Inc.). The samples were incubated 30 min at 37°C and every 10 min 10 μl aliquots were spotted on Whatman DE-81 filter paper disks. The filter bound nucleoside monophosphates were eluted in 500 μl of 0.1 N HCl and 0.1 N KCl and the radioactivity quantified by scintillation counting. The substrate specificity of the purified enzymes was assayed by thin layer chromatography.

RESULTS

We have performed site-directed mutagenesis of three active site amino acid residues of *Dm*-dNK. The residues are V84A, M88R, A110D, and we have created three different mutated proteins: the previously published M88R, V84A + M88R + A110D and the novel mutant V84A + M88R.

The enzymes were expressed as fusion proteins, with the GST part located in the N-terminal of *Dm*-dNK not to interfere with the C-terminal truncation.

The kinetics properties of the three proteins fused with the GST were measured as K_m and V_{max} using GCV as substrate (Table 1a). We further decided to investigate the Δ20 truncated form of the two most active proteins. The kinetics properties were measured using deoxythymidine (dThd) and GCV as substrates (Table 1a). We also determined the relative phosphorylation of several nucleoside analogs using dThd as reference (Table 1b).

DISCUSSION

We have performed site-directed mutagenesis with the aim to generate mutant enzymes with better kinetic properties for possible use in suicide gene therapy. In particular the investigation was focused on the evaluation of improved enzymes able to efficiently phosphorylate the nucleoside analog GCV.

The triple mutant showed very poor capacity to phosphorylate GCV. The comparison between M88R wt and M88R Δ20 showed for the C-terminal truncated mutant a decrease in GCV phosphorylation and a small increase in dThd phosphorylation. The V84A + M88R Δ20 mutant showed a lower K_m than the untruncated form both for dThd and GCV phosphorylation. No major differences between the two mutants were visible in the TLC assays. Both truncated enzymes have approximately the same specificity as the untruncated form (data not shown).

The future perspectives of this study will be to test the truncated forms of the enzyme carrying these mutations in a cell culture system to evaluate the possibility of an improved suicide gene.

REFERENCES

1. Munch-Petersen, B.; Knecht, W.; Lenz, C.; Sondergaard, L.; Piskur, J. Functional expression of a multisubstrate deoxyribonucleoside kinase from Drosophila melanogaster and its C-terminal deletion mutants. J. Biol. Chem. **2000**, *275*, 6673–6679.
2. Knecht, W.; Sandrini, M.P.; Johansson, K.; Eklund, H.; Munch-Petersen, B.; Piskur, J. A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. EMBO J. **2002**, *21*, 1873–1880.