Isolation of cDNA clones encoding two isoforms of nucleoside diphosphate kinase from bovine retina

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Abstract The cDNA encoding bovine retinal isoforms of nucleoside diphosphate kinase (NDP-kinase, EC 2.7.4.6) has been cloned and sequenced. Based on the partial amino acid sequence of the enzyme determined after trypsin digestion of purified NDP-kinase, primers were synthesized and used to isolate two different cDNA clones encoding the full length of two NDP-kinase isoforms. The nucleotide sequences of these clones contained open reading frames encoding 152-residue polypeptides with calculated molecular masses of 17.262 and 17.299 kDa, similar to that determined for the subunits of purified enzyme (17.5 and 18.5 kDa). The deduced NDP-kinase sequences showed high similarity with the known NDP-kinase sequences from other sources.

Key words: Nucleoside diphosphate kinase; Bovine retina; cDNA cloning

1. Introduction

Retinal rod outer segments embody several GTP-controlled pathways. In these cells, fast activation of the heterotrimeric G-protein by photoexcited rhodopsin and GTP accounts for two crucial features of light response: amplification and speed. It is widely accepted that the switch between GDP-bound (inactive) and GTP-bound (active) forms of G-protein is regulated by GDP-GTP exchange and GTP hydrolysis by the GTPase activity intrinsic to G-proteins. Activated G-protein stimulates the activity of the cyclic GMP phosphodiesterase (PDE) by removing its inhibitory γ-subunit, resulting in cGMP hydrolysis. The reduction in cGMP concentration causes the closure of plasma membrane sodium channels gated by cGMP and subsequent cell hyperpolarization [1]. Recovery of cytoplasmic cGMP levels and plasma membrane depolarization is believed to be regulated by a feedback mechanism controlled by calcium dependent guanylate cyclase [2,3].

The first step in the recovery of cGMP concentration is phosphorylation of GMP to GDP by guanylate kinase. GDP is further phosphorylated to GTP by nucleoside diphosphate kinase (NDP-kinase; ATP:NDP phosphotransferase, EC 2.7.4.6). These two consecutive phosphotransferase reactions regulate the supply of guanine nucleotides to signal transduction and other pathways [4].

NDP-kinase catalyzes the transfer of the terminal phosphate from nucleoside triphosphates to nucleoside diphosphates and exhibits broad specificity for the base [5]. Transfer of the terminal phosphate occurs by a ping-pong mechanism that involves the formation of the transient high energy phosphoprotein intermediate form of the enzyme due to phosphorylation of a histidine residue, followed by the transfer of this phosphate to an available substrate [5,6]. The interest in NDP-kinase, being long considered as a 'housekeeping' enzyme, has increased dramatically since the demonstration of its involvement in developmental processes, signal transduction, oncogenic transformation and tumor metastasis (for reviews see [7–9]).

Previously, we characterized bovine retinal NDP-kinase [10,11]. In this work we report on the isolation and characterization of cDNA species encoding two isoforms of bovine retinal NDP-kinase, designated as NBR-A and NBR-B.

2. Materials and methods

2.1. Protein purification and sequencing of peptides

NDP-kinase from bovine retina was purified as described [11]. Native enzyme and protein subunits separated by SDS-PAGE were resistant to Edman degradation. Protein subunits were therefore separated by SDS-PAGE and electrotransferred onto an Immobilon-P membrane (Millipore). The membrane was stained with Ponceau S (Sigma), NDP-kinase bands were excised, destained by 1% acetic acid and washed thoroughly with deionized water. Membrane strips were incubated in 3 ml of 0.5% polyvinylpyrrolidone-40/0.1 M acetic acid for 30 min at 37°C, whereupon they were cut into 1×1 mm pieces. Trypsin solution (enzyme:substrate = 1:20, 100 μl) in 0.05% acetonitrile/0.1 M Tris-HCl, pH 8.5, was added to the membrane pieces and incubated at 37°C for 8 h. The digest was applied to a reversed phase HPLC Zorbax C18 column after the addition of trifluoroacetic acid to a final concentration of 0.1%, and the peptides were eluted with a linear gradient of 0–70% acetonitrile containing 0.1% trifluoroacetic acid. Sequencing of peptides was performed by Edman degradation using a liquid-phase sequenator (Applied Biosystems, model 475 A).

2.2. Screening of the cDNA library

Taking into consideration the codon usage of known retinal proteins two degenerate oligonucleotides based on the sequences of the

Subunits of bovine retinal NDP-kinase

17.5 kDa	18.5 kDa
ASED	ASEDLLK
NIIXGS	NIIHGSDSVESAEK
FEQK	TFIAIKPDGXQR
LVAMK	

Fig. 1. NDP-kinase tryptic peptids. NDP-kinase subunits were separated by SDS-PAGE, transferred onto Immobilon-P membrane, cleaved by trypsin and purified by HPLC followed by sequencing by Edman degradation.

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tryptic peptides, as well as two additional probes corresponding to the most conservative sequences of NDP-kinases from different sources, were synthesized by an automatic DNA synthesizer (Applied Biosystems, model 381 A). Radiolabeled oligonucleotide probes were generated by T4 polynucleotide kinase with [γ-^{32}P]ATP [12]. Screening of the cDNA library of bovine retina in bacteriophage λ-ZAP kindly provided by Dr. M. Applebury (Department of Ophthalmology, University of Chicago) was performed using standard protocols [12]. Isolation of phage DNA and subcloning of cDNA inserts in the pBluescript M13 plasmid vectors (Stratagene) were conducted as described [13]. The plasmids were amplified in *Escherichia coli* XL1-Blue and plasmid DNA was prepared using Qiagen plasmid mini kit (100)

A

NBR-A

CCTGAGAGGA		AAGGGAAACG		AGTGGAGTCT		GGGAAGGAGG				40
AGGTGAAGAG		GACC								54
	ATG	GCC	AAC	AGC	GAG	CGC	ACC	TTC	ATT	GCC		84
1	Met	Ala	Asn	Ser	Glu	Arg	Thr	Phe	Ile	Ala	10	
	ATC	AAG	CCC	GAT	GGA	GTC	CAG	CGA	GGC	CTC		114
11	Ile	Lys	Pro	Asp	Gly	Val	Gln	Arg	Gly	Leu	20	
	ATT	GGA	GAA	ATA	ATC	AAG	CGT	TTT	GAG	CAA		144
21	Ile	Gly	Glu	Ile	Ile	Lys	Arg	Phe	Glu	Gln	30	
	AAG	GGA	TTC	CGT	CTT	GTT	GCC	ATG	AAA	TTC		174
31	Lys	Gly	Phe	Arg	Leu	Val	Ala	Met	Lys	Phe	40	
	ATG	CGG	GCT	TCT	GAA	GAC	CTT	CTC	AAG	GAG		204
41	Met	Arg	Ala	Ser	Glu	Asp	Leu	Leu	Lys	Glu	50	
	CAC	TAT	ATT	GAC	CTG	AAG	GAC	CGT	CCA	TTC		234
51	His	Tyr	Ile	Asp	Leu	Lys	Asp	Arg	Pro	Phe	60	
	TTT	GCT	GGC	CTG	GTG	AAA	TAC	ATG	CAC	TCA		264
61	Phe	Ala	Gly	Leu	Val	Lys	Tyr	Met	His	Ser	70	
	GGG	CCA	GTG	GTT	GCC	ATG	GTC	TGG	GAG	GGA		294
71	Gly	Pro	Val	Val	Ala	Met	Val	Trp	Glu	Gly	80	
	CTG	AAT	GTT	GTG	AAG	ACA	GGT	CGA	GTG	ATG		324
81	Leu	Asn	Val	Val	Lys	Thr	Gly	Arg	Val	Met	90	
	CTT	GGG	GAG	ACT	AAC	CCT	GCG	GAC	TCC	AAG		354
91	Leu	Gly	Glu	Thr	Asn	Pro	Ala	Asp	Ser	Lys	100	
	CCT	GGG	ACC	ATC	CGT	GGG	GAC	TTT	TGC	ATC		384
101	Pro	Gly	Thr	Ile	Arg	Gly	Asp	Phe	Cys	Ile	110	
	CAA	GTT	GGC	AGG	AAC	ATT	ATC	CAT	GGC	AGT		414
111	Gln	Val	Gly	Arg	Asn	Ile	Ile	His	Gly	Ser	120	
	GAT	TCC	GTG	GAG	AGT	GCA	GAG	AAG	GAG	ATT		444
121	Asp	Ser	Val	Glu	Ser	Ala	Glu	Lys	Glu	Ile	130	
	GCC	TTG	TGG	TTT	CAC	CCT	GAG	GAA	CTG	GTG		474
131	Ala	Leu	Trp	Phe	His	Pro	Glu	Glu	Leu	Val	140	
	AAT	TAC	AAG	AGC	TGT	GCT	CAG	AAC	TGG	ATC		504
141	Asn	Tyr	Lys	Ser	Cys	Ala	Gln	Asn	Trp	Ile	150	
	CAC	GAG	TGA									513
151	Tyr	Glu										
CTTGGAAGCA		GACCACAGTG		CTCGTCCCGT		GCTAGTTTCC				553
TTCCTTCCCG		CAGGCAAGGA		CCAGGCCATT		GCAAACCTGG				593
TTATTTCTGA		GAACTTGCTT		TTCATTTGGA		GGGAAACTCT				633
TGGAGCTGTG		GGTGCTCCAT		GTACAGTATT		AAATGCTATC				673
ATCAGATTAA		AATGTTCATC		TCC						696

Fig. 2. The nucleotide sequences and deduced amino acid sequences of NBR-A (A) and NBR-B (B). A: The peptides from bovine retinal NDP-kinase, amino acid sequences which were determined by Edman degradation, are underlined. B: The codons of NBR-B different from those of NBR-A are indicated by circles.

B

NBR-B

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CCCTTCCATT    TGCATCTTTC    ACTTCAAAGG    TATAAAAGCT          40
GTTGATTTTA    GGCTTTAGGA    ATTTGAGTTG    TAGGAGGAAT          80
AGCATTACTG    TGCACAGAAC    CATAGACAGC    AAGACTGCAA         120
ATGCCTTTCT    TTGGGCCCTG    AACATCTCAA    AGGGTAGGCT         160
GTGAGAGGCT    CCATTCGGAA    TTCCGTCCAC    CTGCCTATCC         200
CCAGGACC                                                   208

    ATG  GCC  AAC  AGC  GAG  CGC  ACC  TTC  ATT  GCC          238
1   Met  Ala  Asn  Ser  Glu  Arg  Thr  Phe  Ile  Ala  10

    ATC  AAG  CCC  GAT  GGA  GTC  CAG  CGA  GGC  CTC          268
11  Ile  Lys  Pro  Asp  Gly  Val  Gln  Arg  Gly  Leu  20

    ATG  GGA  GAA  ATA  ATC  AAG  CGT  TTT  GAG  CAA          298
21  Met  Gly  Glu  Ile  Ile  Lys  Arg  Phe  Glu  Gln  30

    AAG  GGA  TTC  CGT  CTT  GTT  GCC  ATG  AAA  TTC          328
31  Lys  Gly  Phe  Arg  Leu  Val  Ala  Met  Lys  Phe  40

    ATG  CGG  GCT  TCT  GAA  GAC  CTT  CTC  AAG  GAG          358
41  Met  Arg  Ala  Ser  Glu  Asp  Leu  Leu  Lys  Glu  50

    CAC  TAT  ATT  GAC  CTG  AAG  GAC  CGT  CCA  TTC          388
51  His  Tyr  Ile  Asp  Leu  Lys  Asp  Arg  Pro  Phe  60

    TTT  GCT  GGC  CTG  GTG  AAA  TAC  ATG  CAC  TCA          418
61  Phe  Ala  Gly  Leu  Val  Lys  Tyr  Met  His  Ser  70

    GGG  CCC  GTG  GTT  GCC  ATG  GTC  TGG  GAG  GGA          448
71  Gly  Pro  Val  Val  Ala  Met  Val  Trp  Glu  Gly  80

    CTG  AAT  GTT  GTG  AAG  ACA  GGT  CGA  GTG  ATG          478
81  Leu  Asn  Val  Val  Lys  Thr  Gly  Arg  Val  Met  90

    CTT  GGG  GAG  ACT  AAC  CCT  GCG  GAC  TCC  AAG          508
91  Leu  Gly  Glu  Thr  Asn  Pro  Ala  Asp  Ser  Lys  100

    CCT  GGG  ACC  ATC  CGT  GGT  GAC  TTT  TGC  ATC          538
101 Pro  Gly  Thr  Ile  Arg  Gly  Asp  Phe  Cys  Ile  110

    CAA  GTT  GGC  AGG  AAC  ATT  ATC  CAT  GGC  AGT          568
111 Gln  Val  Gly  Arg  Asn  Ile  Ile  His  Gly  Ser  120

    GAT  TCC  GTG  GAG  AGT  GCA  GAG  AAG  GAG  ATT          598
121 Asp  Ser  Val  Glu  Ser  Ala  Glu  Lys  Glu  Ile  130

    GCC  TTG  TGG  TTT  CGC  CCT  GAG  GAA  CTG  GTG          628
131 Ala  Leu  Trp  Phe  Arg  Pro  Glu  Glu  Leu  Val  140

    AAT  TAC  AAG  AGC  TGT  GCT  CAG  AAC  TGG  ATC          658
141 Asn  Tyr  Lys  Ser  Cys  Ala  Gln  Asn  Trp  Ile  150

    CAC  GAG  TGA                                          667
151 Tyr  Glu

CTTGGAAGCA    GACCGACAGT    GCTCGGTCCC    ATGCTAGTTT          707
CCTTCCTTCC    CGCAGGCAAG    GACCAGGCTA    TTGCAAACCT          747
GGTTATTTCT    GAGAACTTGC    TTTTCATTTG    GAGGGAAACT          787
CTTGGAGCTG    TGGGTGCTCC    ATGTACAGTA    TTAAATGCTA          827
TCATCAGATT    AAAATGTTCA    TCTCAAGGAA    AAAAAAAA            865
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Table 1
Amino acid sequence identity between NDP-kinases from various sources

	1	2	3	4	5	6	7	8	9
1. awd, *Drosophila melanogaster* [19]									
2. NDP-kinase, *Dictyostelium discoideum* [20]	60.0								
3. NDP-kinase, *Myxococcus xanthus* [21]	48.8	43.1							
4. NBR-A, bovine retina	74.4	58.1	47.2						
5. NBR-B, bovine retina	74.4	58.1	47.2	98.8					
6. NDP-kinase α, rat liver [15]	74.4	55.6	44.1	85.8	85.8				
7. NDP-kinase β, rat liver [16]	73.8	58.1	44.1	88.9	88.9	90.1			
8. p18, rat mucosal mast cells [22]	76.9	58.1	47.2	87.0	87.0	91.9	84.5		
9. nm23-H1, human [19]	78.1	61.9	46.0	90.1	89.5	86.4	92.0	87.0	
10. nm23-H2, human [23]	78.1	59.4	46.0	87.0	87.0	93.2	86.4	95.0	88.9

according to the recommendation of the manufacturer. The inserts cDNA was sequenced completely in both directions by the Sanger chain termination method [14] using Sequenase 2.0 sequencing kit (USB). EMBL accession numbers: NBR A-X92956, NBR B-X92957.

3. Results and discussion

3.1. Isolation of peptide fragments and construction of oligonucleotide probes

Native NDP-kinase was resistant to Edman degradation, indicating that the N-terminus of protein was blocked. The nature of this blocking group was not determined. NDP-kinase subunits were therefore separated by SDS-PAGE and cleaved with trypsin. Sequencing the peptides separated by HPLC on Zorbax C18 column resulted in obtaining about 27% of the polypeptide chain sequence (see Fig. 1). From the peptide sequences and the codon usage of several known rod outer segments proteins, two oligonucleotides were synthesized to screen the retinal cDNA library: 5′-ACCTTC-ATCGCCATCAAGCCCGACGG-3′ and 5′-TC(G/T)(C/G)-(T/A)(T/G)CCGTG(T/G)AT(T/G)ATGTT-3′. The third and fourth probes were prepared on the basis of the most conserved amino acid sequences of known nucleoside NDP-kinases and used as 'control' probes: 5′-ATGGCCAAC-(A/T)G(C/T)GAGCGTACCTTCAT-3′ and 5′-CTGGCCT-GGTGAA(A/G)TACATGCACTCAGG-3′.

3.2. Isolation and sequencing of NDP-kinase cDNA clones

Screening the cDNA library of bovine retina with the oligonucleotide probes yielded several positive clones and among them, one containing the whole sequence of NDP-kinase. NDP-kinases from other mammalian organisms (rat liver [15,16], bovine brain [17] and human erythrocytes [18]) contain at least two isoforms, therefore a search for further clones was attempted. The cDNA containing whole sequence of NDP-kinase was sequenced and four unique probes based on the nucleotide sequence of this clone were synthesized (probes not shown). The cDNA library was screened again using these probes. This yielded ten positive clones from 2×10^6 recombinants screened. 7 and 2 represent the whole sequences for the NBR-A and NBR-B, respectively. The sizes of the cDNA inserts in the plasmids were determined after digestion with *Eco*RI to be 696 and 863 bp for the clones encoding NBR-A and NBR-B, respectively (Fig. 2). The amino acid sequences used to synthesize oligonucleotide probes for the first screening were found in the open reading frames of both inserts. The open reading frames contained all the peptide sequences determined, including those from N- and C-terminal regions. We concluded that these clones contained the entire coding sequences of NDP-kinase with the reading frame starting from ATG (nucleotides 55–57) and ending at TGA (nucleotides 511–513) for NBR-A and starting from ATG (nucleotides 209–211) and ending at TGA (nucleotides 665–667) for NBR-B. The nucleotide sequence of 3′-untranslated region including the polyadenylation site was 201 bp long for NBR-B. The 3′-untranslated region of NBR-A included 186 bp. The cDNA clones differed from each other in four codons shown in Fig. 2B in circles. Two nucleotide substitutions are responsible for the differences in the isoforms: Ile-21 and Arg-135 in NBR-A are replaced by Met and His residues in NBR-B, respectively. Two other differences in nucleotide content do not result in the amino acid substitutions. The deduced amino acid sequence of NDP-kinase consists of 152 amino acid residues. The molecular masses calculated from the amino acid sequences were 17.262 kDa for NBR-A and 17.299 kDa for NBR-B.

Bovine retinal NDP-kinase exhibited a doublet of protein polypeptides on SDS-PAGE with the apparent molecular masses of 17.5 kDa and 18.5 kDa, and we found two clones encoding different gene isoforms. But in this case the correspondence between the separated NDP-kinase polypeptides and the products of the gene isoforms could not be established exactly because of the absence of tryptic peptides which specifically belong to NBR-A or NBR-B, close molecular masses of the gene products and high similarity between NBR-A and NBR-B. It is possible that a third form of NDP-kinase exists in bovine retina.

The results of a comparison between bovine retinal NDP-kinase and NDP-kinases from other sources are given in Table 1. Both NBR-A and NBR-B sequences can be well aligned with those of the human Nm23-H1 protein (90.1% and 89.5% identity, respectively) and rat β-isoform of NDP-kinase (88.9% identity for both isoforms). Almost all sequences are similar to the sequence of bovine retinal NDP-kinase without any internal gaps. The exceptions are the proteins from *Dictyostelium discoideum* and *Myxococcus xanthus*.

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