Volume 336, number 1, 163–167 FEBS 13400 December 1993

High-level expression of functional cGMP-dependent protein kinase using the baculovirus system

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Received 18 October 1993; revised version received 2 November 1993

The understanding of the structure and function of cGMP-dependent protein kinase (cGMP kinase) has been hindered by the difficulty to obtain large quantities of functional enzyme. A recombinant baculovirus encoding bovine cGMP kinase Iα was constructed and purified. Infected insect cells synthesized large amounts of soluble and biologically active cGMP kinase Iα representing up to 10% of the total cell extract protein. The recombinant enzyme had an identical apparent molecular mass, cGMP affinity and kinase activity as the native bovine lung enzyme. The high-level expression of functional cGMP kinase Iα should provide an excellent tool to study further the structure and function of cGMP kinase.

cGMP-dependent protein kinase; Baculovirus expression

1. INTRODUCTION

Serine/threonine protein kinases are involved in signal transducing pathways and play an important role in the regulation of eukaryotic cell functions [1]. Cyclic GMP-dependent protein kinase (cGMP kinase) is a member of this large protein kinase family [2–4]. Two types of cGMP kinase have been identified and cloned from mammalian tissues [5–7]. The bovine type I enzyme is a homodimer of 75 kDa subunits and is present at low concentrations in most tissues. Traditional purification of the enzyme is difficult and tedious. This has hampered detailed biochemical and functional studies.

Biologically active enzyme could be expressed in mammalian cell lines [8,9], but only at low levels, e.g. up to 0.5% of the soluble cell protein in transiently transfected COS cells [8]. Bacterial expression of cGMP kinase led to the synthesis of large amounts of cGMP kinase reaching about 30% of the total cell protein [10]. However, the recombinant protein was catalytically inactive and mainly present in an insoluble form, which could not be refolded to a functional enzyme [10].

Recently, expression of recombinant proteins in insect cells using vectors based on a baculovirus has emerged as an efficient method for the high-level expression of biologically active eukaryotic proteins [11,12]. This expression system has been used successfully for the overexpression of several serine/threonine protein kinases [12–15]. However, like in other expression systems, levels of recombinant proteins may vary substantially (by factor of 500) and synthesis of a fully functional protein is not guaranteed [12,16]. Here we report the generation of milligramme amounts of biologically active bovine cGMP kinase Iα using the baculovirus/insect cell system, thus overcoming the problems inherent to mammalian and bacterial expression systems.

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2. MATERIALS AND METHODS

2.1. *Cells and virus*

Standard methods of gene expression using the baculovirus/insect cell system were used as described by O'Reilly et al. [12]. *Spodoptera frugiperda* (Sf9) cells were propagated as a monolayer culture at 27°C in TC 100 insect medium (Biochrom) supplemented with 10% fetal calf serum (Biochrom), 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Suspension cultures were grown in the same medium additionally supplemented with 1 × lipid concentrate (Gibco/BRL) and 0.1% pluronic F-68 (Gibco/BRL) at 27°C in 2 liter Erlenmeyer flasks on a rotary shaker at 100 rounds per minute. Total cell density was measured with a hemocytometer and viability determined by Trypan blue dye exclusion. Recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) was propagated using monolayer cultures of Sf9 cells and titered by end-point dilution. Viral infections were generally carried out at a multiplicity of infection (MOI) of 1.

2.2. *Construction and purification of recombinant virus*

Molecular biology techniques were performed according to Sambrook et al. [17]. A 2.1 kb *Eco*RI-*Eco*RI cDNA fragment, coding for bovine cGMP kinase Iα, was excised from plasmid p91023Iα [8] and ligated into the *Eco*RI restriction site of baculovirus transfer vector pVL1393 [12]. The cGMP kinase coding sequence was properly orientated for transcription from the polyhedrin promotor. The translation initiation context was ccaaaaacATGAGCGAG starting at the last residue of the *Eco*RI restriction site. The cGMP kinase encoding sequence is indicated by capital letters and a stop codon is underlined, which was in frame with the mutated upstream polyhedrin start codon. The ligation mixture was electroporated into *Escherichia coli* DH5α and correct transformants were identified by PCR colony screening, restriction analysis of the plasmid DNA and dideoxy sequencing of the manipulated regions. The recombinant transfer vector pVL1393/acgk contained nucleotides (nt) −6 to 2119 of the bovine cGMP kinase Iα cDNA [5] including the entire coding sequence (nt 1–2013).

Recombinant baculovirus was prepared by co-transfection of BaculoGold baculovirus DNA (Pharmingen) (50 ng) and anion-exchange-purified transfer vector pVL1393/acgk (500 ng) into Sf9 cells using the liposome-mediated method [12]. Recombinant virus was isolated by plaque purification and was subsequently used to infect Sf9 monolayer cultures. Two days post-infection (p.i.) cells and supernatants were harvested and analyzed for expression of cGMP kinase Iα by immunoblotting, by binding of cGMP and by the phosphotransferase reaction. High titer stocks of recombinant virus (10^7 to 10^8 plaque-forming units/ml) were obtained after three amplification steps using Sf9 monolayer cultures and were stored at 4°C.

2.3. *Expression of cGMP kinase Iα in Sf9 cells*

A 200 ml suspension culture of Sf9 cells was seeded with 3×10^5 cells/ml and grown to a cell density of 2×10^6 cells/ml. Subsequently the cells were collected by centrifugation at $300 \times g$ at 27°C and resuspended in fresh medium to a final volume of 10 ml. 5 ml of this cell suspension were infected with recombinant baculovirus at a MOI of 1 for one hour at room temperature and 5 ml left uninfected as a control. Then the infected and the uninfected cells were each added to fresh medium to obtain two 100 ml suspension cultures.

The following procedures were carried out at 4°C. At various time points p.i., 5 ml aliquots of the cultures were harvested by centrifugation at $300 \times g$. Culture supernatants were stored at −30°C until use. Cells were washed twice in phosphate-buffered saline, resuspended in 0.25 ml of buffer A (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM dithiothreit, 5 mM benzamidine and 2 mM aminoethylbenzenesulfonylfluoride) lysed by freeze/thawing and homogenized with a teflon-coated glass homogenizer. Centrifugation at $18{,}000 \times g$ yielded the soluble cell extract and the insoluble cell debris. Cell extracts were made up 40% (w/v) in glycerol in a final volume of 0.5 ml. The insoluble material was washed twice with buffer A and was then solubilized in 0.5 ml of buffer A + 1.25% SDS. Both preparations were stored at −80°C until use.

2.4. *Enzyme assays*

Equilibrium binding of [^{3}H]cGMP was performed according to [18] at 4°C. The binding to the high affinity site was determined 1 min after the addition of 1 mM unlabeled cGMP. The cGMP binding capacity of cell extracts was determined in the presence of 0.7 μM [^{3}H]cGMP and 0.1 μM cAMP. Phosphotransferase activity was measured as described in [8,18] at 30°C in the presence of 2 μM protein kinase inhibitor peptide (amino acids 5–24), 40 μM substrate peptide (GRTGRRNSI) and 10 μM cGMP.

The amount of functional cGMP kinase Iα in Sf9 cell extracts was calculated by using the binding capacity and specific phosphotransferase activity of purified bovine lung cGMP kinase determined always in the same assay. Almost the same expression levels of the recombinant enzyme were estimated by cGMP binding and phosphotransferase assays. Mean values are indicated in the text.

2.5. *Protein analysis methods*

Protein concentrations of samples were measured by the method of Bradford [19] with bovine serum albumin as a standard. SDS-PAGE was carried out according to Laemmli [20] using 9% acrylamide. Gels were stained with Coomassie blue R-250 or transferred onto an Immobilon-P membrane (Millipore) and probed with an anti-cGMP kinase antibody, raised against the carboxy-terminus of the enzyme [9]. Densitometric evaluation of Coomassie-stained gels was performed using a LKB Ultroscan XL laser densitometer.

3. RESULTS

3.1. *Construction and purification of the recombinant baculovirus*

To express cGMP kinase Iα in insect cells using a recombinant baculovirus, initially a cDNA fragment encoding bovine cGMP kinase Iα was inserted into the baculovirus transfer vector pVL1393. The recombinant transfer vector pVL1393/acgk contained the 2013 nt coding sequence properly orientated to allow transcription from the strong polyhedrin promotor. Two factors which may be important for an effective translation of a non-fused cloned gene [12] are: (a) a short upstream leader region which provides a suitable translation initiation context; and (b) an out-of-frame placement of the ATG start codon of the cloned gene with the mutated polyhedrin initiation codon (ATT), where some translation may still initiate. Both these criteria were met by the construct. Furthermore, unwanted translation into the cGMP kinase coding sequence was hindered by an early stop codon in the polyhedrin reading frame.

The recombinant transfer plasmid and modified baculovirus DNA containing a lethal deletion, which could only be complemented by plasmid encoded sequences, were cotransfected into Sf9 cells. Three virus plaques were isolated and used to infect Sf9 cells. As analyzed by immunoblotting, cGMP binding and phosphotransferase assays, each independently purified virus efficiently directed the expression of functional cGMP kinase Iα in Sf9 cells, but to a varying extent of 2–10% of the total cell extract protein. Culture supernatants contained only a minor fraction of the total enzymatic activity, possibly due to cell lysis (not shown). Endogenous cGMP kinase activity could not be detected in cell extracts of uninfected Sf9 cells using the same low protein concentration (but see below). The determination of the expressed cGMP kinase was not affected by the presence of an endogenous cAMP kinase [21]. The binding assay contained 0.1 μM cAMP to block binding of cGMP to the cAMP kinase. The cAMP kinase activity was blocked by the inhibitor peptide from the heat-stable inhibitor protein. For further experiments the recombinant virus isolate, which showed the highest level of cGMP Iα expression, was selected.

3.2. *Expression of recombinant cGMP kinase Iα in Sf9 cells*

The time course of cGMP kinase Iα production in a recombinant culture of Sf9 cells infected with purified recombinant baculovirus is shown in Fig. 1. After infection (MOI = 1) cell growth and cell viability decreased rapidly compared to an uninfected control culture (Fig. 1A). As estimated from the cGMP-binding capacity and cGMP-dependent kinase activity of cell extracts (Fig. 1A), functional cGMP kinase Iα began to accumulate in infected Sf9 cells about 24 h p.i. A sharp optimum was reached at 71 h p.i. yielding 18 mg enzyme per liter culture or per 2.5×10^9 cells. This represented about 10% of the total cell extract protein. Immunoblot analysis of soluble and insoluble cell protein revealed the same time course of expression (Fig. 1B). An immunoreactive 75 kDa species, which co-migrated with

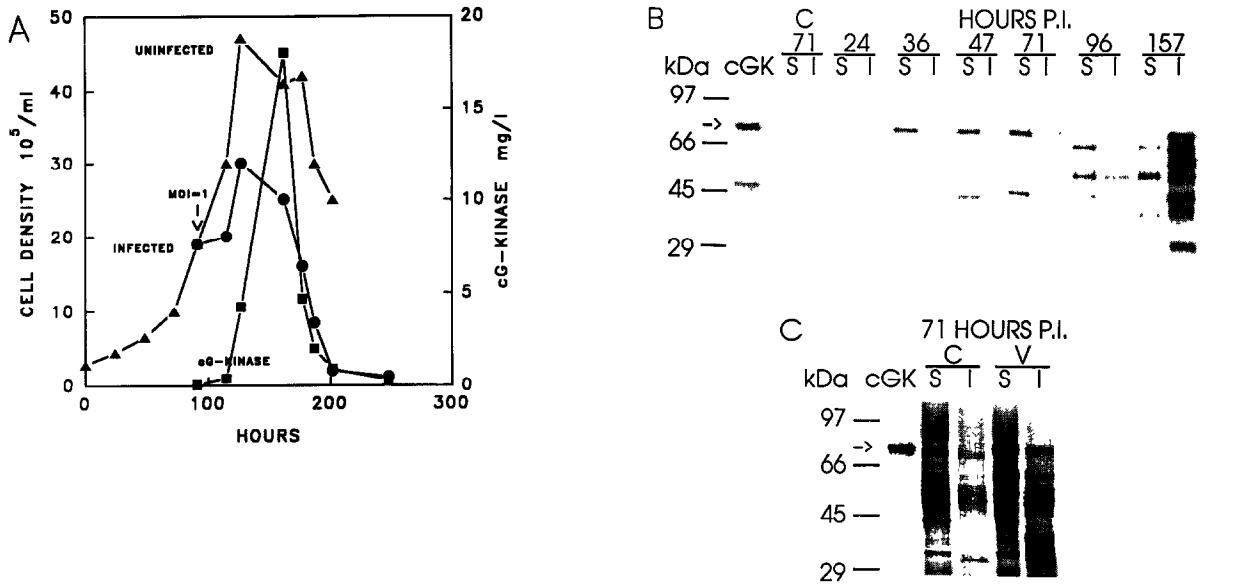

Fig. 1. Time course of cGMP kinase Iα expression in a suspension culture of Sf9 cells. (A) Growth of an uninfected (▲) and an infected (○) culture (viable cells) and production of functional cGMP kinase Iα in infected cells (■). The kinase concentration was calculated from the cGMP binding capacity and phosphotransferase activity using 4 μg and 0.25 μg cell extract protein, respectively. (B) Immunoblot of soluble (S) and insoluble cell protein (I) from the same infected culture as in (A). cGK, bovine lung cGMP kinase; c, soluble and insoluble fraction from uninfected cells. (C) Coomassie stain of soluble (S) and insoluble (I) cell fractions prepared 71 h p.i. from virus infected (v) and uninfected control cells (c) and of the native enzyme (cGK). The amount of protein loaded on gels for immunoblotting and Coomassie-staining was: 1.0 μg and 20 μg of soluble cell extract protein, the same fraction by volume of the corresponding insoluble cell protein and 80 ng and 2.0 μg of the purified bovine lung enzyme. The arrow shows the position of the 75 kDa cGMP kinase subunit.

native cGMP kinase Iα, was specifically synthesized in infected Sf9 cells. At the maximal expression level 71 h p.i. the recombinant cGMP kinase Iα was mainly present in the soluble cell extract. At 36 h p.i. proteolytic breakdown products and enzyme associated with the insoluble cell fraction could also be detected. At longer infection times (96 and 157 h p.i.) the soluble 75 kDa enzyme was fully degraded and the amount of insoluble enzyme protein increased. These findings together with massive cell lysis were consistent with the rapid decrease of functional cGMP kinase found in Sf9 cells beyond 71 h p.i. (Fig. 1A).

At the expression optimum the recombinant enzyme represented one major protein of infected Sf9 cells as demonstrated by the Coomassie-stained protein gel in Fig. 1C. A densitometric scan indicated that the recombinant cGMP kinase Iα constituted approximately 10% of the total soluble cell protein. The Coomassie stain may overestimate the expression level of the recombinant cGMP kinase since a protein with a slightly higher mobility was present in the extracts of non-transfected cells. However, an expression level of 10% of the total soluble cell protein was also calculated from the functional assays (see above) suggesting that the correct value for the expression level of the cGMP kinase was not to far off from those values calculated by three different determination methods. About 10% of the expressed enzyme were associated with the particulate cell fraction. Another highly expressed protein with an apparent molecular mass of 58 kDa was not recognized by the anti-cGMP kinase antibody suggesting that it was either an amino-terminal degradation product of the recombinant enzyme or a baculovirus encoded gene product. The latter case was most likely because a protein with a similar apparent molecular mass not related to the cloned gene was already described in baculovirus infected Sf9 cells [21].

Equivalent amounts of protein from uninfected Sf9 cells showed no immunoreactivity (Fig. 1B) and Coomassie-stainable overexpressed proteins (Fig. 1C). However, Sf9 cells possess a very low concentration of endogenous immunoreactive cGMP kinase activity, which could only be detected using large amounts of cell protein. During the whole time course of expression the level of endogenous cGMP kinase in uninfected Sf9 cells was about 0.03% of the total cell extract protein (not shown). In infected Sf9 cells the amount of endogenous cGMP kinase may even be lower since the viral infection results in a shut-off of host gene expression [12]. Thus, at the optimal expression level of the recombinant cGMP kinase Iα (10%) the endogenous enzyme present in the preparation constituted maximally 0.3% of total cGMP kinase and therefore was not expected to interfere with the further characterization of the recombinant cGMP-kinase Iα.

Monolayer cultures of infected Sf9 cells showed essentially the same expression pattern of recombinant cGMP-kinase Iα as described above for suspension cul-

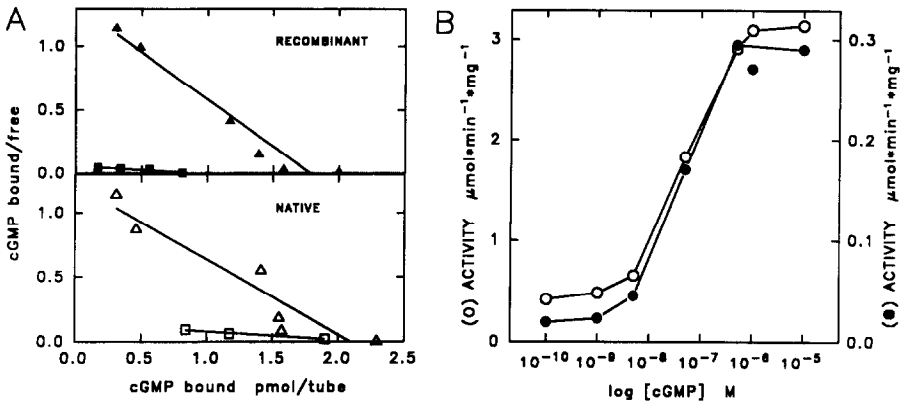

Fig. 2. Enzymological characterization of recombinant cGMP kinase Iα. (A) Equilibrium binding of cGMP to the recombinant (upper panel, 2.5 µg soluble cell extract protein per tube) and the native (lower panel, 0.2 µg protein per tube) cGMP kinase Iα. Scatchard plots for the binding of cGMP to the high affinity (triangles) and low affinity site (squares) are shown. (B) cGMP-dependent phosphotransferase activity of the recombinant (O, 250 ng soluble cell extract protein per tube) and the native (O, 15 ng protein per tube) cGMP kinase Iα. Cell extracts of infected Sf9 cells were prepared 71 h p.i.

tures, with an optimal expression level reaching 10% of the total cell extract protein at 71 h p.i. However, due to the lower cell densities obtained in monolayer cultures the maximal amount of recombinant enzyme reached only 4 mg/l culture or 5×10^8 cells (not shown).

3.3. *Characterization of recombinant cGMP-kinase Iα*

Sf9 cell extracts prepared 71 h p.i. were used to characterize the cGMP binding sites and the phosphotransferase activity of the expressed enzyme. All experiments included samples with the purified native bovine lung enzyme (Fig. 2). The recombinant cGMP-kinase Iα bound cGMP to the low and high affinity sites with apparent K_d values of 73 nM and 7 nM, respectively (Fig. 2A, upper panel). These values are nearly identical to those obtained with the native enzyme, which were 73 nM and 9 nM (Fig. 2A lower panel). The activity of the recombinant enzyme was stimulated 11-fold by cGMP with apparent K_a and V_{max} values of 41 nM and 0.3 µmol/min and mg cell extract protein, respectively (Fig. 2B). In the same experiment, the native enzyme was stimulated 8-fold by cGMP with apparent K_a and V_{max} values of 48 nM and 3.2 µmol/min and mg protein, respectively (Fig. 2B). These data indicate, that the cGMP binding sites and catalytic properties of the recombinant and native cGMP-kinase Iα resemble each other and that the recombinant enzyme is fully biologically active. The kinetic values of the recombinant baculovirus enzyme are not significantly distinct from that of the cGMP kinase Iα expressed in mammalian cell lines [8,9].

4. DISCUSSION

As part of our effort to elucidate the physiological role, biochemistry and structure of cGMP kinase, we report here the high-level expression of the bovine type Iα isozyme using the baculovirus/insect cell system. The identity of the protein expressed in Sf9 cells infected with the recombinant baculovirus with cGMP kinase Iα was established by four criteria: (a) in SDS polyacrylamide gels, the newly synthesized protein comigrated with authentic cGMP-kinase Iα to an apparent molecular mass of 75 kDa; (b) the newly synthesized protein was detected by an antibody specifically directed against the carboxy-terminus of cGMP kinase; (c) the cell extracts of infected cells had a high density of cGMP specific binding sites; and (d) cGMP-dependent kinase activity indicating that the expressed enzyme was also enzymatically active.

Production of the recombinant cGMP kinase Iα in infected Sf9 cells was time-dependent and the time course of enzyme activity correlated well with the appearance of different immunoreactive protein species. The synthesized enzyme was located intracellularly, at the beginning mainly in the soluble cell fraction. After longer infection times proteolysis and a tendency of aggregation or association with particulate cell material became evident. In infected cells soluble and functional cGMP kinase Iα accumulated to constitute a major cell protein. Up to 18 mg/l culture corresponding to 10% of the total soluble Sf9 cell protein could be detected. The same value was obtained when the calculation was based on the protein stain, the binding capacity or kinase activity. This expression level is above average when compared to that of other serine/threonine protein kinases expressed as non-fusion proteins in insect cells [13–15]. The high expression may be attributable in part to the construction of the transfer vector.

The apparent molecular mass, immunoreactivity and functional characteristics such as the K_d values for cGMP binding sites or the K_a value for phosphotransferase activity of the recombinant cGMP kinase Iα were indistinguishable from the native enzyme. These find-

ings suggest that Sf9 insect cells are capable of expressing correctly folded and presumably post-translationally modified cGMP kinase Iα, which is equivalent to the protein synthesized in mammalian cells. The endogenous cGMP kinase activity maximally represented 0.3% of the total cGMP-kinase present in baculovirus infected cells at the expression optimum. Thus it should not have interfered with the characterization of the fully active recombinant enzyme.

In conclusion, through the establishment of the baculovirus expression system, large quantities of biologically active cGMP-kinase Iα could be provided, overcoming the problems inherent in mammalian and bacterial expression methods. This expression system should also be an useful tool to produce mutant forms of cGMP-kinase and will facilitate detailed biochemical and structure–function studies on cGMP kinase.

Acknowledgements: We thank Mrs. E. Bosse for constant encouragement, Mrs. I. Schatz for typing part of the manuscript and Mrs. E. Roller for the graphical work. This work was supported by grants from DFG and Fonds der Chemie.

REFERENCES

[1] Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987) Annu. Rev. Biochem. 56, 567–613.
[2] Hofmann, F., Dostmann, W., Keilbach, A., Landgraf, W. and Ruth, P. (1992) Biochim. Biophys. Acta 1135, 51–60.
[3] Lincoln, T.M. and Cornwell, T.L. (1993) FASEB J. 7, 328–338.
[4] Butt, E., Geiger, J., Jarchau, T., Lohmann, S.M. and Walter, U. (1993) Neurochem. Res. 18, 27–42.
[5] Wernet, W., Flockerzi, V. and Hofmann, F. (1989) FEBS Lett. 251, 191–196.
[6] Sandberg, M., Natarajan, V., Ronender, I., Kalderon, D., Walter, U., Lohmann, S.M. and Jahnsen, T. (1989) FEBS Lett. 255, 321–329.
[7] Uhler, M.D. (1993) J. Biol. Chem. 268, 13568–13591.
[8] Ruth, P., Landgraf, W., Keilbach, A., May, B., Egleme, C. and Hofmann, F. (1991) Eur. J. Biochem. 202, 1339–1344.
[9] Keilbach, A., Ruth, P. and Hofmann, F. (1992) Eur. J. Biochem. 208, 467–473.
[10] Feil, R., Bigl, M., Ruth, P. and Hofmann, F. (1993) Mol. Cell. Biochem. (in press).
[11] Luckow, V.A. and Summers, M.D. (1988) Biotechnology 6, 47–55.
[12] O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992) Baculovirus Expression Vectors: a Laboratory Manual, W.H. Freeman and Co. New York.
[13] Brickey, D.A., Colbran, R.J., Fong, Y.L. and Soderling, T.R. (1990) Biochem. Biophys. Res. Commun. 173, 578–584.
[14] Stabel, L., Schaap, D. and Parker, P.J. (1991) Methods Enzymol. 200, 670–673.
[15] Filhol, O., Cochet, C., Wedegaertner, P., Gill, N. and Chambaz, E.M. (1991) Biochemistry 30, 11133–11140.
[16] Barber, G.N., Tomita, J., Garfinkel, M.S., Meurs, E., Hovanessian, A. and Katze, M.G. (1992) Virology 191, 670–679.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[18] Landgraf, W. and Hofmann, F. (1989) Eur. J. Biochem. 181, 643–650.
[19] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Siow, Y.L., Chilcote, T.J., Benfenati, F., Greengard, P. and Thiel, G. (1992) Biochemistry 31, 4268–4275.