ELSEVIER

FEBS Letters 342 (1994) 255–260

FEBS 13878

FEBS LETTERS

Expression of the catalytic domain of cyclic GMP-dependent protein kinase in a baculovirus system

Nancy J. Boerth, Thomas M. Lincoln*

Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Received 8 February 1994; revised version received 7 March 1994

Abstract

The Type I cGMP-dependent protein kinase catalytic domain (residues 336–671 from the Iα isoform) has been expressed as a cGMP independent kinase in a baculovirus system. Using peptide substrates, the protein retains similar substrate specificity as the native holoenzyme. The recombinant catalytic domain catalyzes the phosphorylation of histone, but does not display the inhibition using non-substrate histones which has been described for the holoenzyme. The catalytic domain is an active kinase in mammalian cells also since vascular smooth muscle cells transfected with the cDNA encoding the catalytic domain display altered morphology. The catalytic domain of G-kinase may be a useful tool for delineating the role of cGMP-mediated protein phosphorylation in cell systems.

Key words: Cyclic GMP; Protein kinase; Phosphorylation; Catalytic domain; Baculovirus

1. Introduction

Cyclic GMP-dependent protein kinase (G-kinase) mediates many of the actions of cGMP in vascular smooth muscle, platelets, and neutrophils (for reviews, see [1–3]). The enzyme was first identified in arthropod tissues [4] and was subsequently purified from bovine lung [5,6]. There are two classes of G-kinase, a Type I which exists as a dimer of identical subunits, and a Type II which exists as a monomer. The Type I enzyme is expressed as two isoforms, an α and a β isoform which differ from each other only in the amino terminus where dimerization is believed to occur [7–9]. The cDNAs encoding the Type Iα and Iβ have been isolated from bovine [10] and human sources [11], and the Type II from *Drosophila* [12] and mouse [13]. The predicted sequences of the Type I enzyme from bovine and human tissues are nearly identical, indicating a high degree of conservation. Furthermore, the G-kinase cDNAs isolated from *Drosophila* are highly homologous with the mouse Type II enzyme as well as bovine and human Type I enzymes in regions consisting of cGMP binding and catalytic function.

The domain structure of G-kinase indicates that the enzyme consists of a cGMP binding domain homologous with that of the regulatory subunit of cAMP-dependent protein kinase (A-kinase), and a catalytic domain homologous with that of the catalytic subunit of A-kinase as well as catalytic domains from other protein kinases. This suggests that the catalytic domain of G-kinase might be functional as a protein separated from the regulatory domain. Although the cDNAs encoding G-kinase have been expressed in transfected cells [13,14], expression of an active enzyme in bacteria has not been successful. This has hampered further characterization of the enzyme, especially those designed to understand the catalytic mechanism of action.

It has been know that proteolytic treatment of the enzyme results in a catalytically active kinase that still retains cGMP binding activity as well as other functional domains [15,16]. No studies to date have addressed the existence of an isolated catalytic domain of G-kinase, however. In this report, we describe a PCR-mediated cloning procedure for the isolation of cDNAs encoding the cGMP-dependent protein kinase catalytic domain. This cDNA has been successfully expressed in the baculovirus-Sf9 insect cell system. Some properties of the expressed protein are described.

2. Materials and methods

2.1. Construction of cDNAs for G-kinase Iα using PCR

Fresh bovine lung mRNA was isolated using a modified guanidinium isothiocyante method [17], and purified on oligodT cellulose. The mRNA was converted to first strand cDNA using Abelson Maloney virus reverse transcriptase, and the cDNA was used as the template for the construction of individual segments using PCR. For constructing the catalytic domain of G-kinase Iα, (bp 999–2016), sense oligonucleotides (CCC CTC GAG TTA AGC ATA TGA AGA TGC AGA AGC

*Corresponding author. Fax: (1) (205) 934 1775.

Abbreviations: G-kinase, cGMP-dependent protein kinase; A-kinase, cAMP-dependent protein kinase; PEM, 20 mM potassium phosphate, 2 mM EDTA, 15 mM β-mercaptoethanol buffer.

SSDI 0014-5793(94)00263-U

TAA GGC) and antisense oligonucleotides (CCC CTC GAG TAA GAA GTC TAT GTC CCA TCC TGA GTT GTC) were used, where the underlined sequences represent *Xho*I restriction sites for subcloning. PCR was performed using 10 ng of cDNA constructed from 3 μg mRNA, or in some cases 10 ng of lamda gt11 DNA from a bovine lung cDNA library. Ten μM of each of the primer oligonucleotides were used to amplify the various cDNAs from template. Forty cycles of thermal denaturation and renaturation were performed with steps at the following temperatures and times: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. An initial 94°C denaturation step for 3 min prior to thermal cycling was included. The amplified fragments were isolated on 1% agarose gels and sub-cloned into the *Xho*I restriction sites in pSK+ and sequenced using the dideoxynucleotide chain termination method [18].

2.2. Ligation of the cDNA encoding the G-kinase catalytic domain

The fragment consisting of the entire coding region for the catalytic domain was ligated in pSK+ at the *Xho*I site. Confirmation of the orientation was obtained by restriction enzyme analysis and eventually by sequencing the cDNA encoding G-kinase. The aim of the PCR strategy was to design specific cDNA sequences encoding the individual domains of G-kinase, especially that encoding the catalytic domain. Thus, we prepared the catalytic domain for expression using linkers encoding the translational initiation codon. The vector was digested with *Nde*I and the overhanging ends were blunt-ended using Klenow polymerase. Linkers containing the *Nco*I restriction site having the ATG in frame with the remaining coding sequence was ligated to the blunt *Nde*I site. The 10-mer linker, AGC CAT GGC T, was ligated to the blunt *Nde*I-cleaved fragment to yield the following coding sequence for the catalytic domain:

```
... A GCC ATG GCT TAT GAA ...
          M  A  Y-336 E
```

where the underlined sequence indicates the correct reading frame for the catalytic domain.

2.3. Expression of the catalytic domain of G-kinase in insect cells

The coding sequence for G-kinase catalytic domain was removed from pSK+ using *Xho*I digestion and cloned into the *Nhe*I site in BlueBac2 (pBB) transfer vector after blunt-ending with the Klenow polymerase to create pBB/GK. The recombinant vector was grown in *E. coli* and purified by Magic Maxi prep (Promega). Sf9 cells were co-transfected with 10 μg of pBB/GK and 1 μg linear wild type baculovirus DNA, AcMNPV (Invitrogen), using liposome-mediated transfection. Cells were grown for 5 days in Grace's medium supplemented with 10% fetal bovine serum (FBS), after which the medium containing virus particles was harvested. Recombinant virus was isolated from wild-type virus by plaque purification using β-galactosidase as a marker. After six rounds of plaque purification, the recombinant virus was judged near homogeneous since a 10^6-fold dilution of virus yielded only blue colonies of Sf9 cells in soft-agar plates, and the plaques were occlusion body-negative. The protein kinase inhibitor, H-8 (50 μM) was added to Grace's medium in order to prevent detachment of cells during expression of the active catalytic domain.

For expression of G-kinase catalytic domain, Sf9 cells were grown in 60 mm^2 tissue culture plates and infected with 10 pfu/cell or MOI = 10. After 1 h, the medium was changed and cells were grown for up to 5 days at 27°C. Cells were harvested at various points and assessment of expression was determined by Western blot analysis and activity assays.

2.4. Expression of the catalytic domain in cultured vascular smooth muscle cells

The coding sequence for the catalytic domain was removed from pSK+ using *Xho*I digestion and cloned in to the *Xho*I site of pMEP4 eukaryotic expression vector to create pMEP/GK. The recombinant vector was grown in *E. coli* and purified. Passage 3 rat aortic smooth muscle cells (RASMC) deficient in endogenous G-kinase were transfected with 5 μg of pMEP/GK or 5 μg pMEP4 as a control using 10 μg of Transfectam reagent for 15 min at room temperature to precipitate the DNA/liposome complex. The precipitate was added to the cell monolayer and incubated for 6 h at 37°C and 10% CO_2. The transfection was terminated by adding DMEM with 20% FBS. Stably-transfected cells were selected using 40 μg/ml hygromycin b, and the established cell lines were maintained in culture in 25 μg/ml hygromycin b. For expression of the catalytic domain, the transfected RASMC were plated at 30% confluence in 100 mm^2 tissue culture plates and were allowed to attach overnight at 37°C and 10% CO^2. The cells were then serum deprived for 48 h in DMEM and 1 mg/ml bovine serum albumin followed by induction by the addition of DMEM plus 10% FBS with 100 μM $ZnSO_4$.

2.5. Assay for G-kinase and catalytic domain activity

Cells (approximately 1×10^8) were harvested by rinsing the monolayers twice with phosphate-buffered saline, followed by the addition of 0.5 ml of 20 mM potassium phosphate, pH 6.8, 2 mM EDTA, 15 mM β-mercaptoethanol (PEM buffer). The cells were scraped from the plates and sonicated, and the particulate fraction removed by centrifugation at 10,000 × *g* for 10 min. Ten μl of the extract was assayed for G-kinase in 100 μl of assay buffer consisting of 50 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (200 cpm/pmol), and either histone F2b (0.1 mg/ml) or 100 μM of peptide substrate selective for G-kinase, RKISASEFDRPL, initially described by Colbran et al. [19]. All assays for G-kinase were performed in the presence of 0.9 μM protein kinase inhibitor peptide (5–24). Assays were conducted at 30°C for 5 min and terminated by aliquoting histone or the peptide to Whatman 3MM filter paper or P81 phosphocellulose paper, respectively. A-kinase activity was determined using either histone or kemptide as the substrate using the same assay conditions.

2.6. Materials

The bovine lung cDNA library from which the catalytic domain was cloned was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Restriction endonucleases were from BRL (Gaithersburg, MD) or Stratagene (La Jolla, CA). Deoxyribonucleotides were purchased from Pharmacia, Inc. (Piscataway, NJ). Reagents for baculovirus expression, transfer vectors, and wild type virus and Abelson Maloney Virus reverse transcriptase were obtained from Invitrogen (LaJolla, CA). Transfectam Reagent was purchased from Promega, Inc (Madison, WI). Radioactive ATP was from New England Nuclear (Wilmington, DE). Peptides were synthesized and purchased from Research Genetics, Inc. (Huntsville, AL). Histone Type VIII-S was from Sigma, Co. (St. Louis, MO) while F2b was from Worthington (Freehold, NJ).

3. Results

3.1. Cloning and expression of the G-kinase catalytic domain

The sequence homology between the catalytic subunit of the A-kinase and the G-kinase Type Iα catalytic domain is shown below:

```
C-subunit:
     ...EFLAKAKEDFLKKWENPAQNTAHLDQFERIKTLGTGSFG...
G-kinase:
          MAYEDAEAKAKYEAEAAFFANLKLSDFNIIDTLGVGGFG...
```

The glycine loop is underlined for residues 50–55 in the catalytic subunit and residues 34–39 of the catalytic domain. Little homology is observed between the two proteins before the glycine loop. It will also be noted that the G-kinase catalytic domain is truncated by 16 residues relative to the catalytic subunit of A-kinase.

Expression of immunoreactive G-kinase catalytic domain in Sf9 insect cells following infection with baculovirus is shown in Fig. 1. Polyclonal antibodies to the holoenzyme recognized a band of 38 kDa which was maximally expressed at 48 and 72 h post-infection (Fig. 1A). This corresponded to the predicted size of the

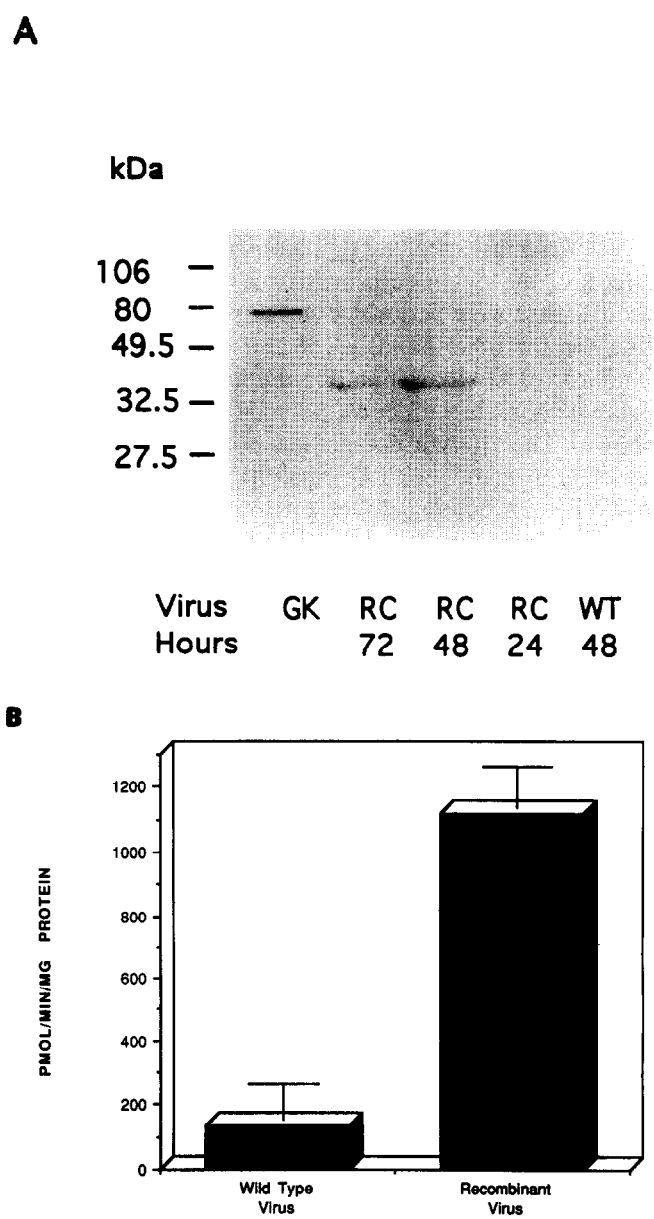

Fig. 1. (A) Immunoreactivity of G-kinase catalytic domain in extracts of Sf9 cells. Sf9 cells were grown as monolayers in T-75 flasks in Grace's medium supplemented with 10% FBS, and harvested at the times indicated. Cells were scraped from the plates, centrifuged, and homogenized. Extract proteins were then resolved by 10% SDS-PAGE. After transfer to nitrocellulose, the blots were probed with rabbit anti-bovine G-kinase. (B) G-kinase activity in Sf9 cell extracts. Extracts were prepared as described in section 2. G-kinase activity was assayed in the absence of cGMP and in the presence of the protein kinase inhibitor peptide 5–24 using 100 μM BPDEtide as the substrate.

catalytic domain. For comparison, 10 ng of purified G-kinase holoenzyme, M_r = 79 kDa, is shown (GK). Extracts from wild-type (WT) virus-infected cells displayed no immunoreactive G-kinase at 79 kDa or catalytic domain. Activity of the catalytic domain was assessed using a selective G-kinase substrate, the BPDEtide [19], assayed in the absence of cGMP and the presence of the protein kinase inhibitor peptide 5–24. As shown in Fig 1B, cells infected with recombinant virus demonstrated a 10-fold increase in peptide phosphorylation relative to cells infected with wild-type virus. Very little kinase activity was present in the wild-type virus extracts either in the presence or absence of 2 μM cGMP. These results demonstrate that the catalytic domain is fully active in Sf9 cells and is independent of cGMP for activity.

3.2. *Substrate specificity of the catalytic domain*

Previous studies from several laboratories have shown that while G-kinase demonstrates overlapping substrate specificity with several other protein kinases, especially the A-kinase, there appear to be specificity determinants for the G-kinase as well [20,21]. Nevertheless, the molecular basis for specificity is not well-defined and may relate to the recognition of specific sequence motifs in substrates by the catalytic domain, by the targeting of the holoenzyme to subcellular structures containing substrates for protein kinases, or both. Thus, we examined the phosphorylation of different peptide substrates for the G-kinase catalytic domain and holoenzyme. As shown in Table 1, G-kinase and the G-kinase catalytic domain phosphorylated the BPDEtide substrate slightly better than the Kemptide substrate which was preferentially phosphorylated by the catalytic subunit of A-kinase. In fact, the catalytic subunit of A-kinase catalyzed the phosphorylation of the BPDEtide only 3% as well as kemptide; both G-kinase preparations however catalyzed the phosphorylation of the BPDEtide more than 5 times the rate of that by the A-kinase catalytic subunit, thus confirming previous observations by

Table 1
Peptide phosphorylation by G-kinase, the G-kinase catalytic domain, and the catalytic subunit of cAMP-dependent protein kinase

Substrate	Kinase	Percent maximum phosphorylation
LRRASLG (kemptide)	G-kinase	16.2
	Catalytic domain	15.0
	C-subunit	100.0
RKISASEFDRPL (BPDE-tide)	G-kinase	17.6
	Catalytic domain	14.4
	C-subunit	3.2
GRRESLTSFG (IP_3Rtide)	G-kinase	100.0
	Catalytic domain	100.0
	C-subunit	83.4

Percent maximal phosphorylation is the pmoles/min/mg protein of each peptide divided by the best peptide substrate for each kinase. Peptides were used at 100 μM. The G-kinase (10 nM) was the Iα isoform purified to homogeneity from bovine lung; the C-subunit (20 nM) was purified to homogeneity from bovine heart; the Catalytic domain of G-kinase was partially purified from Sf9 cell extracts by Econo Q chromatography. The specific activity of the G-kinase was 2.4 μmol/min/mg using the IP_3Rtide, of the C-subunit was 4.3 μmol/min/mg using the kemptide, and of the catalytic domain 86.6 nmol/min/mg using the IP_3Rtide.

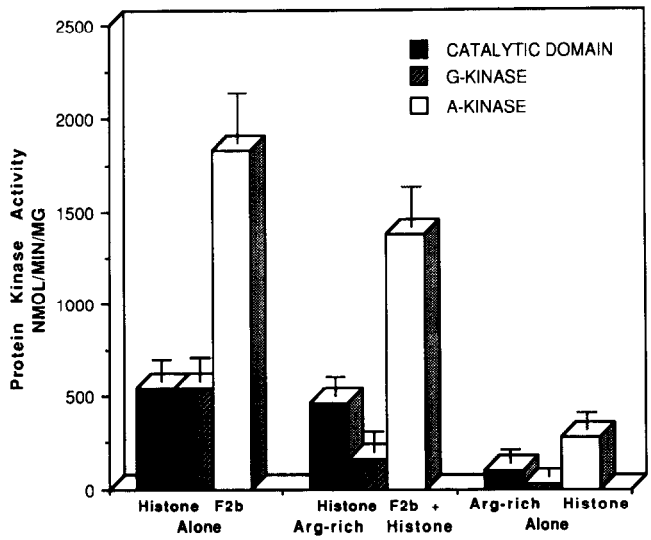

Fig. 2. Effects of arginine-rich histone (histone VIII-S) on F2b histone phosphorylation by the holoenzyme of G-kinase (10 nM), the catalytic domain of G-kinase, and the catalytic subunit of A-kinase (20 nM). Proteins were diluted buffer containing 1 mg/ml bovine serum albumin to yield similar specific activities before assay. Histone concentrations were 0.5 mg/ml in each instance.

Colbran, et al [19]. Our laboratory has observed that the purified inositol 1,4,5-trisphosphate (IP_3) receptor is an excellent substrate for G-kinase in vitro. As shown in Table 1, all three kinase preparations phosphorylated the IP_3 receptor peptide substrate with similar potency. Collectively, these results demonstrate that the catalytic domain of G-kinase and the holoenzyme recognize similar substrates in vitro and indicate that the G-kinase does indeed recognize specific substrates at the peptide binding domain.

3.3. *Phosphorylation of histones by the G-kinase catalytic domain*

It has been known for a number of years that G-kinase interacts with histone substrates and other polycationic proteins at undetermined sites [22,23]. This interaction results in a decrease in the activity of the enzyme and has hampered assays and characterization of the enzyme from tissue extracts. In order to aid in the identification of the putative interaction site(s) on G-kinase for polycationic proteins, we compared the phosphorylation of histone F2b (a good histone substrate for G-kinase) by the holoenzyme and the catalytic domain in the presence and absence of arginine-rich histone (a poor substrates for G-kinase and an inhibitory polycationic protein). As shown in Fig. 2, arginine-rich histone inhibited the capacity of the holoenzyme to phosphorylate F2b histone by approximately 80%; such inhibition was not observed for the catalytic domain of G-kinase. For comparison, the effects of arginine-rich histone on the capacity of the catalytic subunit of A-kinase to phosphorylate F2b is also shown. Thus, the inhibition of the holoenzyme of G-kinase can be attributed to interaction(s) of polycationic proteins at sites distinct from the catalytic domain. In this sense, the catalytic domain may function more like the A-kinase catalytic subunit in catalyzing the phosphorylation of protein substrates.

3.4. *Other properties*

The G-kinase catalytic domain was insensitive to the effects of the A-kinase protein kinase inhibitor peptide as all assays were performed in the presence of the peptide. On the other hand, KT5823 a reportedly selective inhibitor of G-kinase, inhibits the holoenzyme and the catalytic domain with equal potency (< 1 μM) (data not shown). The catalytic domain has been partially purified by elution from Econo Q columns with 400–500 mM NaCl at pH 8.0, suggesting a strongly anionic protein (Fig. 3). The dilute enzyme is rather unstable, and in its purified form the half-life was approximately 2.5 h at 4°C, similar to the A-kinase C subunit.

3.5. *Effects of the G-kinase catalytic domain on cell morphology*

Cultured RASMC stably transfected with the cDNA encoding the catalytic domain of G-kinase demonstrated conspicuous morphological changes. Specifically, the cells were transformed from a phenotype characterized by flattened phase-lucent morphology (Fig. 4A) to cells that were characterized by an elongated, 'hill and valley' structure (Fig. 4B). Cells transfected with the catalytic domain also tend to grow in parallel arrays rather than randomly. These phenotypic alterations resemble super-

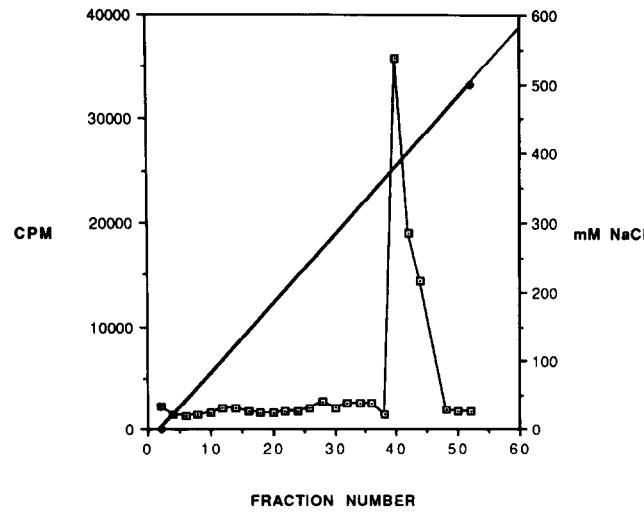

Fig. 3. Econo Q chromatography of the G-kinase catalytic domain expressed in Sf9 cells. Approximately 3×10^6 cells/ml were harvested from a 500 ml spinner culture and homogenized in 1 ml of PEM buffer using a ultraturax homogenizer. The homogenate was centrifuged at $10{,}000 \times g$ for 10 min, and the extract was applied to a 1 ml Econo Q column equilibrated with PEM buffer. The column was eluted with a 75 ml NaCl gradient (0 to 600 mM) in PEM buffer using a Bio-Rad Econosystem pump. Fractions were collected and assayed for G-kinase as described in section 2.

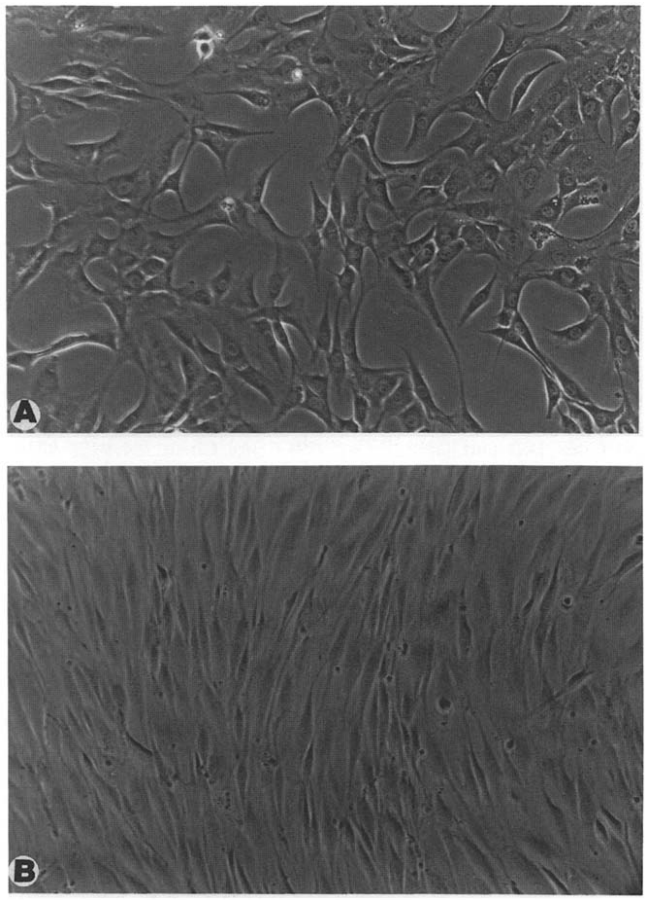

Fig. 4. Morphological properties of RASMC expressing the G-kinase catalytic domain. Cells in passage 3 and deficient in endogenous G-kinase were transfected with pMEP4 vector alone (panel A) or pMEP/GK (panel B) as described in section 2. After serum-deprivation for 48 hrs, expression was induced using DMEM plus 10% FBS containing 100 μM $ZnSO_4$. Cell monolayers were photographed using a phase- contrast Nikon microscope equipped with a camera. Magnification × 800.

ficially at least the differentiated, contractile phenotype associated with primary isolates of VSMC. These results indicate that the catalytic domain of G-kinase can function as an active catalytic moiety in mammalian cultured cells.

4. Discussion

The catalytic domain of G-kinase has been successfully expressed in a baculovirus system. The enzyme is independent of cGMP for activity and displays many properties similar to that of the holoenzyme with respect to substrate recognition and phosphorylation. These results confirm the notion that the catalytic domain of the G-kinase may be independently folded from the regulatory portion of the molecule during synthesis to produce an active catalytic core. The enzyme has been purified approximately 10-fold by mono Q ion-exchange chromatography from Sf9 cell extracts, and appears to be 10% pure at this point. This indicates that the catalytic domain comprises 1% to 0.1% of Sf9 soluble protein. It was observed during expression of the active catalytic domain that cells became detached from the culture dishes, but were still viable as judged by trypan blue exclusion. The mechanism for this effect was not clear, but subsequent plaque purification of recombinant virus was hampered unless the kinase activity was blocked. To do this, we employed H-8, a cyclic nucleotide kinase inhibitor, to allow purification of recombinant virus particles. A similar situation may be encountered by other investigators attempting to express active kinases in the baculovirus system.

For many years, it has been noticed that assay methods using histones as substrates for G-kinase has yielded contradictory results. It is now known that G-kinase activity is suppressed when using high concentrations of histone as the substrate. It had been speculated that histones bind to sites distinct from the catalytic site and inhibit activity [23]. The results reported here demonstrate for the first time that this indeed is the case since the holoenzyme, but not catalytic domain, is inhibited by non-substrate polycationic histones.

An interesting effect on RASMC morphology was observed in cells expressing the G-kinase catalytic domain. The cells expressing the catalytic domain changed to a fusiform, elongated morphology similar to that displayed by primary isolates of RASMC or differentiated SMC found in the medial layer of arteries. Control transfectants, on the other hand, retained the dedifferentiated morphology observed for many types of SMC grown and passaged in vitro. It is tempting to speculate that cGMP-dependent protein phosphorylation might be involved in the phenotypic and growth properties of RASMC. More studies, however, are needed to determine the mechanisms underlying the phenotypic modulation observed in cells expressing G-kinase.

Recently the holoenzyme of G-kinase Iα has been expressed at high levels in baculovirus [24]. this provides an alternate method to the standard lung purification for obtaining G-kinase. On the other hand, the expression of an active catalytic domain of G-kinase may prove useful in delineating the role of cGMP in cellular systems. Because cGMP can interact with multiple receptor proteins to effect cell function [3], the availability of the cGMP-independent G-kinase may help define the roles of specific cGMP-dependent protein phosphorylation in cells. Furthermore, pathways leading to cGMP formation, e.g. nitric oxide (NO), can be by-passed in cells expressing the catalytic domain of G-kinase thus eliminating other potential effects of NO.

Acknowledgements: This work was supported by grants from the National Institutes of Health (HL34636) and the National Science Foundation (DCB9118405). We thank Dr. Will Rading for help and advice with the baculovirus expression system.

References

[1] Walter, U. (1989) Rev. Physiol. Biochem. Pharmacol. 113, 41–88.
[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] Kuo, J.F. and Greengard, P. (1970) J. Biol. Chem. 245, 2493–2498.
[5] Gill, G.N., Holdy, K.E., Walton, G.M. and Kanstein, C.B. (1976) Proc. Nat. Acad. Sci. USA 73, 3918–3922.
[6] Lincoln, T.M., Dills, W.D. and Corbin, J.D. (1977) J. Biol. Chem. 252, 4269–4275.
[7] Lincoln, T.M., Thompson, M. and Cornwell, T.L. (1988) J. Biol. Chem. 263, 17632–17637.
[8] Wolfe, L, Corbin, J.D. and Francis, S.H. (1989) J. Biol. Chem. 264, 7734–7741.
[9] Francis, S.H., Woodford, T.A., Wolfe, L. and Corbin, J.D. (1989) Second Messengers and Phosphoproteins 12, 301–310.
[10] Wernet, W., Flockerzi, V. and Hofmann, F. (1989) FEBS Lett. 251, 191–196.
[11] Sandberg, M., Natarajan, V., Ronander, I., Kalderon, D.,Walter, U., Lohmann, S.M. and Jahnsen, T. (1989) FEBS Lett. 255, 321–329.
[12] Kalderon, D.G. and Rubin, G.M. (1989) J. Biol. Chem. 264, 10738–10748.
[13] Uhler, M.D. (1993) J. Biol. Chem. 268, 13586–13591.
[14] Ruth, P. Landgraf, W., Keilbach, A., May, B., Egleme, C. and Hofmann, F. (1991) Eur. J. Biochem. 202, 1339–1344.
[15] Lincoln, T.M., Flockhart, D.A. and Corbin, J.D. (1978) J. Biol. Chem. 253, 6002–6009.
[16] Heil, W.G., Landgraf, W. and Hofmann, F. (1987) Eur. J. Biochem. 168, 117–121.
[17] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[18] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Nat. Acad Sci. USA 74, 5463–5467.
[19] Colbran, J.L., Francis, S.H., Leach, A.B., Thomas, M.K., Jiang, H., McAllister, L.M. and Corbin, J.D. (1992) J. Biol. Chem. 267, 9589–9594.
[20] Lincoln, T.M. and Corbin, J.D. (1977) Proc. Nat. Acad. Sci. USA 74, 3239–3243.
[21] Glass, D.B. and Krebs, E.G. (1979) J. Biol. Chem. 254, 9728–9738.
[22] Walton, G.M. and Gill, G.N. (1980) J. Biol. Chem. 255, 1603–1609.
[23] Glass, D.B., McFann, L.J., Miller, M.D. and Zeilig, C.E. (1981) Cold Spring Harbor Conf. Cell Prolif., Vol. 8, 267–289.
[24] Feil, R., Muller, S. and Hofmann, F. (1993) FEBS Lett. 336, 163–167.