PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link. http://hdl.handle.net/2066/20928

Please be advised that this information was generated on 2018-07-07 and may be subject to change.

Molecular and Cellular Biochemistry 143: 59-65, 1995. © 1995 Kluwer Academic Publishers. Printed in the Netherlands.

Production of native creatine kinase B in insect cells using a baculovirus expression vector

Yvette J.M. de Kok¹, Monique P.A. Geurds¹, Erik A. Sistermans¹,

Magda Usmany², Just M. Vlak² and Bé Wieringa¹

¹Department of Cell Biology and Histology, Medical Faculty University Nijmegen, P.O. Box 9101, 6500 HB Nijmegen; ²Department of Virology, Agricultural University, Wageningen, The Netherlands

Received 25 July 1994; accepted 21 September 1994

Abstract

73,

ilę

1y,

m zic

0;

A full-length human creatine kinase B (B-CK) cDNA was used to produce a recombinant baculovirus (AcDZ1-BCK). Sf9 cells infected with this recombinant expressed a homodimeric protein composed of 43 kDa subunits which, under optimal conditions, formed up to 30% of the total soluble cellular protein. Upon analysis by PAGE, zymogram assay and gel filtration chromatography the recombinant protein behaved like authentic dimeric human BB-CK protein. Studies with a newly produced monoclonal antibody (CK-BYK/21E10) directed against an epitope in the N-terminus of the protein confirmed the identity of the product. The recombinant BB-CK protein was purified to over 99% homogeneity from the total protein extract of AcDZ1-CKB infected cells in one single step involving anion exchange column chromatography on MonoQ in FPLC. Dialysed protein had a specific activity of 239 U/mg protein. (Mol Cell Biochem 143: 59–65, 1995)

Key words: creatine kinase B, baculovirus, SF9 cells, monoclonal antibody

Introduction

Different isoforms of creatine kinase (CKs¹; EC 2.7.3.2) play an important role in the energy metabolism of vertebrate cells by catalyzing the reversible exchange of high-energetic phosphate between ATP and creatine phosphate (CrP) [1]. CKs, the homo- or heterodimeric cytosolic BB, BM and MM enzymes [2] or the dimeric and octameric mitochondrial proteins [3], are indeed most prominently expressed in cells and tissues with a high and fluctuating energy demand such as cells in the central nervous system (CNS), cardiac and skeletal muscle, kidney, retina and spermatozoa [2 and refs. therein]. Consequently, expression of the different enzymatic subforms is subject to complex tissue and cell-type specific regulation. The B-CK subunit forms an especially interesting study object in this respect. Not only are transcriptional and translational principles involved in its expression, also

the modulation of enzymatic activity by protein modification [phosphorylation; 4] and non-covalent protein and membrane associations [5, 6] do play a role. B-CK studies may therefore form a paradigm for the study of other CKisoforms or enzymes with a similar role in the ATP regulatory networks. The BB-CK isoenzyme has also a direct link to clinical applications. The determination of BB-CK in circulation is of importance in the investigation of neuromuscular (genetic) disorders, the monitoring of different events in the process of cardiac infarction, strokes, CNS and muscular traumas and the development of malignancies [7-11]. Development of reliable standardized tests for B-CK activity in human serum thus far has been hampered by the limited availability of pure enzyme as a control standard for enzyme activity determinations and by the lack of antibodies specific against B-CK.

The baculovirus expression vector system developed by

Address for offprints: B. Wieringa, Department of Cell Biology and Histology, Medical Faculty University Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Smith et al. (1983) has been used for high level expression of eukaryotic proteins, including multimeric proteins and proteins which undergo post-translational modifications [13, 14]. Here we present the use of the baculovirus expression vector system for the production of large quantities of authentic, enzymatically active, human BB-CK dimeric protein. Furthermore, we report on the production of a monoclonal antibody (CK-BYK/21E10) highly specific against the N-terminal portion of B-CK which was used to establish the identity of the in vitro produced protein. The availability of pure subunits and a highly specific monoclonal antibody opens up future possibilities for the study of the pleiotropy of associations and modifications involved in the subcellular compartmentalization of B-CK. Moreover, it forms a valuable asset in the development of standardized immuno-assay kits for clinical chemical monitoring.

BamHI cleaved pAcDZ1 vector standard cloning methods were used [18]. The orientation and integrity of the reading frame was checked by supercoil DNA sequencing [16] using B-CK specific primers.

Generation of recombinant AcMNPV

One μ g of purified pAcDZ1-BCK transfer vector DNA was cotransfected with 2 μ g pure wt AcMNPV DNA into Sf9 cells (80–85% confluency) using lipofectin transformation (GIBCO-BRL) according to the manufacturers protocol [see also Peng *et al.*, 20]. Transfected cells were assayed for the production of recombinant AcMNPV-BCK virus using routine β -galactosidase staining procedures. Recombinant virus was further isolated from culture supernatant through four rounds of plaque purification. A soft agar overlay was added to assist in picking of pure clones. Recombinant plaques were scored as lacking polyhedra and positive for β -galactosidase [21]. One purified plaque was chosen and expanded to a large seeding stock of recombinant virus (stored at 4°C).

Material and methods

Viruses and cells

Sf9 cells, derived from the armyworm Spodoptera frugiperda, were grown at 27°C in flasks using Grace's Insect medium (Gibco) [14] supplemented with 3.3 g/l yeastolate, 3.3 g/l lactalbumin hydrolysate (Gibco), 5.6 g/l BSA and 10% fetal bovine serum. The wild-type strain E2 of Autographa californica multiple-nucleocapsid nuclear polyhedrosis virus (AcMNPV) was used. The wild-type and recombinant viruses were propagated and titrated (plaque assay, p.f.u. determination) on loosely adherent monolayers of Sf9 cells as described [15].

Production and purification of B-CK protein from baculovirus infected Sf9 cells

For optimization of production of B-CK protein, Sf9 cells were infected at different multiplicities of infection (moi's ranging from 1-10). To this end, Sf9 cells were seeded in 25 cm²-flask in 4 ml medium at a density of 1.10⁶ cells/ml and were infected with recombinant virus at the appropriate dilution for 1 h. For harvest, the cells were collected by brief centrifugation at the appropriate time interval after infection (48-72 h), washed in cold phosphate-buffered saline (PBS, 7.3 mM NaH, PO_4 ; 55 mM KCl, 47 mM NaCl, 6.8 mM CaCl, pH6.2), resuspended in 8 ml of 0.05 M Tris-HCl (pH = 7.5), 5 mM 2-mercaptoethanol in the presence of 50 µg/ml PMSF and lysed by 3 cycles of freeze-thawing at --80°C. The lysate was centrifuged at 4,000 ×g for 10 min and either used directly or stored frozen until use. Recombinant B-CK protein production was verified by SDS-PAGE [22] and standard CK-activity assays. The total protein concentration in the lysate or in purified fractions was measured using the method of Bradford et al. [23]. For preparative purification, cell lysates were adjusted to 10 mM MgCl, and subjected to a 1-h treatment with 10 μ g/ ml DNAsel followed by an additional centrifugation at 12,000 rpm to remove protein aggregates, cell debris and unbroken cell organelles. Approximately 3.5 ml of the lysate (7.6 mg of protein) was then applied on a MonoQ FPLCcolumn and protein was eluted using a 0-0.8 M NaCl gradient in the above Tris/HCl-buffer. B-CK activity in in-

Construction of the recombinant expression vector

One forward primer (5'-CTTGGATCCATGCCCTTCTCC-AACAGCCAC-3') and one reverse primer (5'-CTTGGA-TCCGGCT<u>TCA</u>TTTCTGGGCAGGCA-3') were designed for PCR amplification of the 1148 bp DNA fragment spanning the complete B-CK open reading frame (start and stop codon underlined). GGATCC sequences (bold print) were included in the primers to bracket the segment between flanking BamHI restriction sites. For PCR amplification, alkaline denatured [16] plasmid pCKB-131 DNA [17] served as template and standard conditions were used for generation and isolation of the DNA product [18]. Next, the 1158 bp BamHI fragment (including primer sequences) was cloned downstream of the polyhedrin promoter in the baculovirus transfer vector pAcDZ1 which contains an E. coli LacZ marker for facilitating recombinant-virus identification [19]. To introduce the B-CK DNA insert into the dividual fractions was determined and individual fractions were resolved by SDS-PAGE. The fractions highest in 43 kDa B-CK protein were pooled, dialysed against phosphate-buffered saline (PBS), and protein concentrations determined.

Production of a peptide immunogen and of a monoclonal antibody against human B-CK

The peptide immunogen BW-17 (Ser-Asn-Ser-His-Asn-Ala-Leu-Lys-Leu-Arg-Phe-Pro-Ala-Glu-Asp-Glu-Phe) correTris-buffered saline/Tween 20 (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20; TBST) followed by incubation with MoAb antibody CK-BYK/21E10 at the appropriate dilution (1:50.000 for ascites MoAb). Filters were washed extensively in TBST and then incubated with a secondary antibody consisting of alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Biotec). The color was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate using the Protoblot AP system according to the specifications of the manufacturer (Promega).

sponding to a unique stretch of amino acids (residues 4–20) from the N-terminal end of the human B-CK was produced by standard solid phase synthesis [24]. The purity of the peptide was assessed by determination of its amino acid composition and by routine HPLC analysis. Next, activated BW-17 peptide was coupled to BSA using MHS as described (25), yielding a ratio of 16 moles of peptide per mole of BSA.

For immunisation of Balb/C mice, 80 µg of the BW-17-BSA conjugate in 300 μ l PBS was mixed in with 300 μ l Freunds adjuvant and injected intraperitoneally at day one. Preimmune serum was taken prior to immunisation. Booster injections of 80 µg immunogen in incomplete Freunds adjuvant were given at days 14 and 28. Fusion experiments for hybrydoma production were started at around day 33. The standard methodology for fusion of immunized Balb/ C spleen cells with SP2/0AG14 myeloma cells was used [26]. Hybridomas were screened by standard ELISA on plates coated with (i) recombinant B-CK/ β -galactosidase fusion protein produced in E. coli, (ii) synthetic BW-17 peptide conjugated to casein or thyroglobulin, (iii) Sf-9 lysate from wt or recombinant AcMNPV-BCK or (iv) commercially available M-CK protein (Boehringer) as a negative control. Promising clones were recloned twice by limiting dilution and their specificity was further verified on Western blots as described below. Ultimately, monoclonal (MoAb) CK-BYK/21E10 was chosen for its high specificity and stable growth characteristics. Nude Balb/C mice were used to produce ascites fluid. MoAb CK-BYK/21E10 is an IgG2b.

Protein sizing by gel filtration

Protein in the dialyzed pure pooled peak fractions of the Mono-Q column (950 μ g/ml; 2.5 ml total) was brought on a FPLC-Superdex 200 column (gelfiltration column of Pharmacia/LKB FPLC system) to see whether B-CK was present in its monomeric or dimeric form. Retention in the elution was calibrated with a series of known proteins such as aldolase, bovine serum albumin and ovalbumin. The various fractions of the Superdex 200 elution protein were tested in standard CK-activity assays for the specific activity of the protein (in standard CK Units per mg protein/l). Finally, the immunogenicity of B-CK in the pooled fraction of the MonoQ column was retested by Western blotting.

Determination of CK-activity

Typing of purified recombinant B-CK protein: SDS-PAGE analysis

Protein was resolved by SDS-PAGE in 10% (w/v) slab gels and then, for Western blotting, electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schull) Accurate determination of CK-activity was performed using the CK-NAC activated method (CBR-program Boehringer Mannheim; CBR-CK NAc-activated kit no. 475742) adapted for a Cobas Myra automatic analyser (Hoffman La Roche, Basel, Switzerland).

Zymogram typing of CK isoenzymes

Typing of cytosolic CK isoenzymes in cell lysates was done by applying the lysate to cellulose acetate membrane strips (Sartorius) prior to electrophoretic separation at 200 V for 1 h in Tris-barbital buffer pH 8.6 as described by Harm *et al.* [28]. The positions of CK activity were vizualized by activity staining according to Kanemitsu *et al.* [29].

(1 h; 250 mA) in a buffer containing 25 mM Tris-HCl (pH = 8.3), 192 mM glycine, and 20% (v/v) methanol. Alternatively, for routine analysis the gels were directly stained with Coomassie Brilliant Blue or silver stain [27] to verify the purity of the B-CK preparation. For immunoblot analyses, nitrocellulose sheets were first blocked with 1% gelatin in

Results and discussion

Recombinant baculovirus expressing the B-CK protein was isolated as described under 'Materials and methods'. To determine whether insect Sf9 cells infected with this plaque-









Fig. 1. A) SDS-PAGE of protein extracts from Sf9 cells. Lane 1, lysate (4 μ g) of mock infected SF9 cells; lane 2, lysate (4 μ g) of recombinant baculovirus AcMNPV-BCK infected Sf9 cells; lane 3, molecular weight markers: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14.4 kDa). Proteins were resolved on a 10% (w/v) SDS/PAA gel and stained with Coomassie brilliant blue. The position of the B-CK subunit protein (43 kDa) is indicaed by an arrow.



Fig. 1. C) Immunoblot analysis of native and recombinant B-CK containing protein extracts with the monoclonal antibody CK-BYK/21E10. SDS-PAGE was performed as given in Materials and methods, the samples were transferred onto a nitrocellulose membrane, and immunoreactive proteins detected with the monoclonal antibody CK-BYK/21E10. The position of native and recombinant B-CK is noted. Lane 1, lysate of CKB-recombinant virus infected Sf9 cells (0.5 μ g of protein total); lane 2, lysate of uninfected Sf9 cells 2 μ g of protein); lane 3, lysate (2 μ g) of ovarium carcinoma cell line OVCAR-3 which produces significant amounts of authentic human B-CK; lane 4, human heart extract (2 μ g); lane 5, human brain extract (1.5 μ g); lane 6 human skeletal muscle extract (2 μ g).

show CK activity (not shown).

.t

Optimization of production of B-CK protein revealed that infection with a MOI of I gave exactly the same result as a MOI of 10. We could only achieve a maximum p.f.u. of 3.107/ml. Normally, in baculovirus preparations easily 10-20 fold higher titers can be obtained. Though we cannot provide direct evidence it is conceivable that excessive overproduction of B-CK interferes with normal cellular ATP levels needed to sustain transcription and translation processes required for completion of the viral replication cycle. Estimates of the maximal amount of recombinant protein based on the intensity of signals on stained gels suggest that the 43 kDa band of B-CK subunits accounted for approximately 30% of the soluble protein in the lysate under optimal conditions. These results affirm maxima reported for other eukaryotic cytoplasmic proteins expressed from the polyhedrin promoter in this system [30, 31]. To provide independent evidence for the authenticity of the recombinant protein we used a monoclonal antibody, MoAbCK-BYK/21E10, which is specific against the human B-CK protein. The generation of this monoclonal is briefly described in 'Materials and methods'. Relevant characteristics of the antibody showing its specificity on Western blots of human cell and tissue extracts are given in Fig. 1C. Further description of the antibody and its use in immunohistochemical analysis will be detailed elsewhere (Sistermanset al. Cell and Tissue Res, in press). Immunoblotting (Fig. 1C) demonstrated that the recombinant protein was specifically recognized by the anti-B-CK peptide monoclonal an-

Fig. 1. B) Time course of B-CK expression in Sf9 cells after infection with recombinant virus. Sf9 cells were infected with AcMNPV-BCK recombinant virus, harvested every 12 h until 96 h after infection, and CK-activity was determined as described in Materials and methods. \blacksquare = total protein concentration; * = CK-activity.

purified recombinant AcMNPV-BCK indeed expressed protein of the expected size (43kDa), total crude cell lysates were resolved by PAGE. Upon staining a predominant and distinct band of 43 kDa corresponding to the expected molecular weight of creatine kinase B (i.e. 43 kDa; 381 amino acids; [17]) was found (Fig. 1A, lane 2). This band was several fold more intense than a weak 43 kDa band which occurs as an endogenous protein in lysates of mock-infected cells (Fig. 1A, lane 1). The new 43 kDa band appeared first at 24 h after infection, reached an optimal concentration around 48 h after infection and gradually disappeared after 72 h, presumably as a consequence of lysis of the cells. Determination of B-CK activity in cell-lysates revealed that the enzymatic activity of the protein followed exactly the same time course (Fig. 1B). Uninfected Sf-9 cells did not





















Fig. 2. Purification of B-CK from recombinant virus infected Sf9 cells and characterisation of the purified protein. A) Elution profile on the MonoQ-FPLC column. AcMNPV-BCK recombinant virus infected Sf9 cell lysate was prepared and applied on a FPLC-MonoQ anion exchange column of the Pharmacia/LKB FPLC system as described under 'Materials and methods'. The OD280 elution profile after application of a NaCl gradient is shown. The vertical 0-100% NaCl scale ranges from 0-0.8 M NaCl. B) SDS/PAGE and immunoblot analysis of the different peak fractions. Panel to the left, proteins stained with silver stain. Panel to the right, proteins immunoreactive with monoclonal CK-BYK/21E10. Lane 1, pooled peak fraction of the MonoQ column (2 µg); lane 2 B-CK recombinant virus infected Sf9 lysate (2 µg); lane 3, molecular weight markers as described in the legend of Fig. 1. The weak band (right panel, lane 2) at ± 40 kDa is a proteolytic B-CK cleavage product, which was occasionally observed upon prolonged storage of crude lysates. C) Elution profile of the purified protein on a FPLC-Superdex 200 gelfiltration column. Pooled purified protein (2.38 mg) was dialysed against PBS and applied on the Superdex 200 column (Pharmacia/LKB FPLC system). The OD280 elution and activity distribution profiles are shown. B-CK enzymatic activity was determined as described in 'Materials and methods' on an automatic Cobas Myra analyser. Peaks in the elution profiles of reference marker proteins (aldolase (158 kDa) and bovine serum albumin (68 kDa)) are marked with an arrow indicating the native molecular weight in kDa. D) Zymogram assay comparison of recombinant and native BB-CK protein. Migration positions of the MM-, BM- and BB-CK-isoenzymes in the standard marker mix (Boehringer kit) are indicated to the left; also the position of the mitochondrial (ubiquitous) CK isoform which is co-expressed with BB-CK in most cells and tissues is given; lane 1, dialysed pooled pure BB-CK protein (400 ng); lane 2, extract (4 µg protein equivalent) of the human OVCAR-3 cell line which produces authentic human BB-CK protein.

tibody (CK-BYK/21E10). The antibody does not detect any significant quantity of protein in uninfected cells and the recombinant protein has a size that exactly corresponds to that of native B-CK subunits from human brain or heart extracts.

The B-CK expressed in insect cells was purified to homogeneity in one single step by applying the total cell lysate on a monoQ FPLC-column and eluting the column with a 0-0.8 M NaCl gradient. Monitoring of individual fractions showed that virtually pure B-CK eluted as one peak at 0.27 M NaCl (Fig. 2A). The creatine kinase activity in the pooled fractions, after dialysis against phosphate-buffered saline, protein which can be used as standard in serological tests for brain and cardiac infarction [8, 9]. Until now, transient production of CK proteins in cDNA transfected COS cells [34] was the method of choice for production of CKs for potential use as clinical standards. Also the newly generated monoclonal antibody CK-BYK/21E10 can be considered a useful tool in these tests.

Acknowledgements

was determined to be 239 U/mg protein, an enrichment of 2.5 fold as compared to the CK activity determined in the total cell lysate before column chromatography (98 U/mg protein). Silver and Coommassie staining showed that B-CK in the fractions with highest activity was essentially pure and comprised over 99% of the total visible protein bands Fig. 2B, left panel). This was confirmed by Western blot analysis with MoAb CK-BYK/21E10 (Fig. 2B, right panel), which also demonstrates that through this purification step the weak \pm 40 kDa proteolytic fragment present in the crude lysate was lost. When the pooled protein eluted from the MonoQ column was brought on a FPLC-Superdex-200 column (gelfiltration column), the protein (and the activity profile) behaved like a 80–90 kDa protein indicating that B-CK was present in its native dimeric form (Fig. 2C). B-CK in brain extracts was used as a control and eluted at exactly the same position (not shown). Zymogram typing (Fig. 2D) showed that the purified recombinant protein migrated ex-

This work was supported by grant 5372/1/6/375/90/06-BCR from the EEC (to B.W.). We thank E. Mariman and G. van Eys for help and critical discussions during the initial phases of this study.

References

- 1. Bessman SP, Carpenter CL: The creatine-creatine phosphate energy shuttle. Ann Rev Biochem 54: 831-862, 1985
- Walliman T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM: Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem J 281: 21-40, 1992
- 3. Wyss M, Smeitink J, Wevers RA, Wallimann T: Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. Biochim Biophys Acta 1102: 119–166, 1992
- 4. Chida K, Tsunenaga M, Kasahara K, Kohno Y, Kuroki T: Regulation of creatine phosphokinase B activity by protein kinase C. Biochem Biophys Res Commun 173: 346–357, 1990

actly as native BB-CK from human cells.

Taken together, these results indicate that most if not all of the recombinant BB-CK produced in insect cells behaves like authentic native, enzymatically functional BB-CK protein. The product is similar to dimeric BB-CK protein as found in human tissues. High levels of BB-CK are for example found in the CNS, the epithelial linings of lung and intestine, photoreceptor cells of the retina, the thick ascending limb of the loop of Henle in kidney, osteoclasts, and uterus during labour [2 and refs. therein]. Several reports emphasize the role of BB-CK in regenerating ATP for sodium transport and its has been suggested that BB-CK is closely associated to Na,K-ATPases in these tissues [32]. The availability of biologically active preparations of purified native CK-B subunits will make possible reconstitution studies to determine the nature of these associations. Fortunately, the expression and correct assembly of functional Na,K-ATPase subunits in baculovirus-infected insect cells has also been reported [33]. Ultimately, these studies may provide us with more insight in B-CK's biological properties and the role of protein-structural flexibility in partitioning and intracellular compartmentalization phenomena. In addition, the baculovirus expression vector system is an attractive venue for producing large amounts of pure native B-CK

- 5. Lim L, Hall C, Leung T, Mahadevan L, Whatley S: Neuron specific enclase and creatine phosphokinase are protein components of rat brain synaptic plasma membranes. J Neurochem 41: 1177–1182, 1983
- 6. Oblinger MM, Brady ST, McQuarrie IG, Lasek RJ: Cytotypic differences in the protein composition of the axonally transported cytoskeleton in mammalian neurons. J Neurol 7: 433-462, 1987
- 7. van der Veen KJ, Willebrands AF: Isoenzymes of creatine phosphokinase in tissue extracts and in normal pathological sera. Clin Chim Acta 13: 312–316, 1966
- Ingwall JS, Kramer MF, Fifer MA, Lorell BH, Shemin R, Grossman W, Allen WD: The creatine kinase system in normal and diseased myocardium. N Engl J Med 313: 1050-1054, 1985
- Silverman LM, Dermer GB, Zweig MH, van Stierteghem AC, Tokes ZA: Creatine kinase BB: a new tumor-associated marker. Clin Chem 25: 1432–1435, 1979
- Gazdar AF, Zweig MH, Carney DN, van Steirteghem AC, Baylin SB, Minna JD: Levels of creatine kinase and its BB isoenzymes in lung cancer specimens and cultures. Cancer Res 41: 2773-2777, 1981
- Kaye AM, Hallowes R, Cox S, Sluyser M: Hormone-responsive creatine kinase in normal and neoplastic mammary glands. Ann NY Acad Sci 464: 218-230, 1986
- Smith GE, Summers MD, Fraser MJ: Production of human beta interferon in insect cells infected with a baculovirus expression vector. Mol Cell Biol 3: 2156-2165, 1983
- 13. Luckow VA, Summers MD: Trend in the development of baculovirus expression vectors. Bio/Technology 6: 47-55, 1988
- 14. Grace TDC: Establishment of four strains of cells from insect tissues

grown in vitro. Nature 195: 788–789, 1962

- Summers MD, Smith GE: A manual of methods for baculovirus vectors and insect cell culture procedures. Tex Agri Exp Sta and Texas A & M University Publishers, College station, Bull No 1555, 1987
- 16. Hattor: M, Sakaki Y: Dideoxy sequencing method using denatured plasmid templates. Anal Biochem 152: 232-238, 1986
- 17. Mariman ECM, Broers CAM, Claesen CAA, Tesser GI, Wieringa B: Structure and expression of the human creatine kinase B gene. Genomics 1: 126-137, 1987
- 18. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2nd ed, 1989
- 19. Zuidema D, Schouten A, Usmany M, Maule AJ, Belsham GJ, Roosien

effect of the three moieties on the immunogenecity of the conjugates. J Immunol Methods 120: 133–143, 1989

- 26. Köhler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497, 1975
- Morrisey JH: Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal Biochem 117: 307-310, 1981
- Harm R, Musolf KM, Sirageldin E, Voigt K: Verteilungsmuster cytosolischer und mitochondrialer kreatinkinase-isoenzyme in menschlicher oberschenkel- und augenmuskulatur. D Arztl lab 33: 259-266, 1987.
- 29. Kanemitsu F, Okigaki T: Creatine kinase isoenzymes. J Chrom 429: 399-417, 1988
- J, Klinge-Roode EC, Van Lent JWM, Vlak JM: Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrinbased baculovirus expression vector. J Gen Virol 71: 2101–2209, 1990
- 20. Peng S, Sommerfelt MA, Berta G, Berry AK, Kirk KL, Hunter E, Sorcher EJ: Rapid purification of recombinant baculovirus using fluorescense-activated cell sorting. Biotechniques 14: 274–277, 1993
- 21. O'Reilly DR, Miller LK, Luckow VA: Baculovirus expression vectors: A laboratory Manual. WH Freeman and Company, NY, 1992
- 22. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970
- 23. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976
- 24. Sarin VK, Kent SBM, Jam JP, Merrifield RB: Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. Anal Biochem 117: 147-157, 1981
- 25. Peeters JM, Hazendonk TG, Beuvery EC, Tesser GI: Comparison of four bifunctional reagents for coupling peptides to proteins and the

- 30. Alvares K, Widrow RJ, Abu-Jawdeh GM, Schmidt JV, Yeldandi AV, Sambasiva Rao M, Reddy JK: Rat urate oxidase produced by recombinant baculovirus expression; formation of peroxisome crystalloid core-like structures. Proc Natl Acad Sci USA 89:P 4908-4912, 1992
- 31. Bhasin R, Van Nostrand WE, Saitoh T, Donets MA, Barnes EA, Quitschke WW, Goldaber D: Expression of active secreted forms of human amyloid β-protein precursor by recombinant baculovirus-infected insect cells. Proc Natl Acad Sci USA 88: 10307–10311, 1991
- 32. Friedman DL, Roberts R: Purification and localization of brain-type creatine kinase in sodium chloride transporting epithelia of the spiny dogfish squalus acanthias. J Biol Chem 267: 4270-4276, 1992
- 33. DeTomaso AW, Xie ZJ, Liu G, Mercer R: Expression, targeting and assembly of functional Na,K-ATPase polypeptide in baculovirus-infected insect cells. J Biol Chem 268: 1470–1478, 1993
- 34. Friedman DL, Kesterson R, Pulco P, Wu AHB, Perryman MB: Recombinant creatine kinase proteins and proposed standards for creatine kinase isoenzyme and subform assays. Clin Chem 39: 1598– 1601, 1993

1