Expression of a 19kD fragment of B3 and its crossreactivity with Pyruvate Kinase

Chapter III
3.1 INTRODUCTION:

Recently, several proteins have been identified as having more than one function. A few glycolytic enzymes are known to belong to this group of moonlighting or multifunctional proteins (Jeffery C, 1999).

One of the best characterized molecules belonging to this category is Phosphoglucose Isomerase (PGI). In the cytoplasm it catalyzes the interconversion of D-glucose-6-phosphate to D-Fructose-6-phosphate, an essential step of glycolysis and gluconeogenesis. The functionally active form of this enzyme is a dimer with a subunit molecular mass of 55 kD. A lymphokine product of lectin stimulated T cells, was identified to function as a neutrotrophic factor (Neurleukin) for spinal and sensory neurons, as well as an autocrine motility factor. Further studies indicated that it was identical to PGI. (Chaput M et al, 1988; Faik P et al, 1988).

Glyceraldehyde-3-phosphate dehydrogenase is another glycolytic enzyme with more than one function (Modun B et al, 2000.). In both prokaryotes and eukaryotes, its major role is in the glycolytic pathway. It catalyzes the phosphorylation of Glyceraldehyde-3-phosphate to give 1,3-diphosphoglycerate, in the presence of organic phosphate. The process generates NADH from NAD.

In eukaryotes, it functions as a fibronectin and laminin binding protein, has phosphotransferase-phosphokinase activity, it is also known to play a role in DNA repair and replication, nuclear RNA export and translational regulation, and in endocytosis (as a microtubule binding protein).

Prokaryotic GADPH also has multiple functions, it is known to bind to mammalian proteins such as lysozyme, laminin, fibronectin, plasmin, actin and myosin. It is also known to play a role in ADP-ribosylation and prokaryotic-eukaryotic cell to cell communication. The surface expressed Staphylococcal Tpn (Tranferrin binding protein) was found to be identical to GADPH. It maintains the enzyme activity in the functional tetrameric form, 170 kD in size. The transferrin binding capacity is independent of the native confirmation. The surface expressed protein is 42 kD in size.

There is currently no data to indicate how these proteins are targeted to different compartments in the cell. It has been seen that cytoplasmic and surface expressed protein show no difference in antibody reactivity, enzyme activity and amino acid composition.
The functionally active form of Pyruvate Kinase exists as a tetramer, with a subunit size of 55 kD. The monomer is known to have additional functions as a cytosolic thyroid hormone binding protein (Kato et al 1989; Parkison SC et al, 1991).

The experiments in our lab demonstrated that B3 costimulates CD4+ T cells in conjugation with TCR ligation. Further characterization of B3 revealed that B3 has homology with Pyruvate Kinase. Therefore, there exists the possibility that, either Pyruvate Kinase or a part of it might have costimulatory activity. Alternatively, B3 could be a Pyruvate Kinase-like protein. In order to resolve this issue, the following strategy was adopted.

The fragment obtained (450bp) after PCR using B3 specific primer was cloned in an expression vector. The required fragment was amplified using B3 specific primer, containing a Hind III site and a PK L primer containing a Nde I site. With these modifications the product was in-frame in the expression vectors used.

Cloning was done in two vector systems. Initially the fragment was inserted at the Ndel- Hind III site of the pT7Nd vector (Fig. 3.1). The protein expressed (19 kD) from this system was used for developing a polyclonal antibody in hamster. This antibody was used to functionally characterize B3 in relation to PK.

To obtain a pure population of antibody, a His-tagged version of the same protein was prepared and utilized in an affinity column. In order to do this, the product amplified by the same primers, was ligated at the Hind III – Nde I site in the pET 15b vector (Fig 3.2). This had a short His-tag stretch attached at the N-terminal of the protein. The His-tagged protein was linked to a Ni-NTA column and the polyclonal sera was passed through the column. The affinity purified antibody was obtained on elution.
Fig 3.1: Cloning of 450 bp product in pT7Nd
Fig 3.2: Cloning of 450 bp product in pET 15b vector
3.2 MATERIALS:

3.2.1 Bacterial strains:
JM 109 (DE3)
JM 109

3.2.2 Vectors:
pT7Nd (modified pGEMEX Promega)
pET 15b Novagen

3.2.3 Primers, Gemini Biotech:
B3 Sense /Nde I site: 5’ GAA TTC ACT AGT CAT ATG GCT GCC
PK Lower/ Hind III site: 5’ ATA TCT GGA AAG CTT TTA AGG TAI
CAC TAC

3.2.4 Restriction enzymes from New England Biolabs:
T4 DNA Ligase
Taq polymerase
Nde I, Hind III

3.2.5 Media and Antibiotics:
RPMI 1640 Gibco-BRL
FCS Gibco-BRL
Penicillin Sigma, USA
Streptomycin Sigma, USA

3.2.6 Media and Antibiotics for Bacterial cultures:
LB Media
Ampicillin

3.2.7 Antigen:
Pyruvate Kinase M2, from Rabbit Muscle, Sigma, USA

3.2.8 Protease Inhibitors:
Antipain hydrochloride Boehringer Mannheim
Aprotinin Boehringer Mannheim
Leupeptin Boehringer Mannheim
3.2.9 Adjuvants:
Complete Freund's Adjuvant  Sigma, USA
Incomplete Freund's Adjuvant  Sigma, USA

3.2.10 Reagents for SDS-PAGE:
Acrylamide and Bis acrylamide  Sigma, USA
SDS  Sigma, USA
Coomassie Brilliant Blue R-250  Sigma, USA
Glycine  Qualigens, India

3.2.11 Protein Estimation & Purification:
Extracti-D-Gel  Pierce, USA
Micro BCA Reagent  Pierce, USA
Bradford Reagent  BioRad

3.2.12 Reagents for Western Blot:
CAPS Buffer  Sigma, USA
Nitrocellulose paper, 0.22 μ, type SCN,  Advanced Microdevices, Ambala, India.
Ponceau S  Sigma, USA
Bovine Serum Albumin, Fraction V  Sigma, USA
Lumiglo Reagent  New England Biolabs.
X-Ray films  Konika
Developer  Kodak
Fixer  Kodak
3.3 METHODS:

3.3.1 Subcloning of 450 bp fragment in expression vector:
The 450bp fragment cloned in TEasy vector, was subcloned in an expression vector. For this purpose the 450bp was reamplified with the B3 and PK L primers, these had been modified such that the B3 primer contained a Nde I site and the PK L primer contained the Hind III restriction site. Amplification was carried out as described earlier. The PCR product was purified from the gel and was used for cloning. On amplification, the fragment obtained was cloned inframe in the vector, and the protein was expressed on induction with IPTG.

Vector Arm: The pT7Nd vector was digested with the enzymes Nde I and Hind III. The required fragment was purified from the gel and utilized to set up the ligation.

Ligation reaction:
Ligation was set up as before (Section 2.3.9). Competent cells and transformation protocols were as described before (Sections 2.3.10 and 2.3.11).

Insert Release:
The 10.0-ml enmass cultures were pelleted and miniprep carried out as before, to obtain the plasmid. Double digestion using the enzymes Nde I and Hind III confirmed the presence of the 450bp insert.

3.3.2 Expression:
The expressed protein was expected to be approximately 19 kD in size. Briefly, single colonies obtained from JM109 DE3 were inoculated in 10.0 ml LB media containing ampicillin. Cultures were grown at 37 °C with shaking till the OD reached 0.15. A few aliquots of 1.0 ml each were removed prior to induction.

Induction was carried out with 5.0 µl of 0.5 M IPTG. Cells were maintained at 37 °C with shaking for 2 hours. Aliquots of 1.0 ml were removed to analyze protein expression. 1.0 ml aliquots were centrifuged and the cell pellet obtained was lysed in 40 µl of 1X LSB and boiled for 10 min. Samples were run on a 13% SDS- PAGE gel and visualized by Commassie staining.

3.3.3 Preparation of 19 kD antigen for immunisation:
A single colony of JM109 DE3 containing the required plasmid (either pT7Nd or pET15b) was inoculated in 25.0 ml of LB containing ampicillin. Cultures were maintained at 37 °C at shaking. When OD was 0.15, the cells were centrifuged. The pellet was transferred to two 500.0ml cultures of LB containing Ampicillin.
Cultures were maintained at 37 °C until the OD reached 0.2. Induction was done by addition of 125 μl of 1.0 M IPTG / 500 ml culture and cultures were maintained until an OD value of 0.4 was reached. Cells were centrifuged at 2050g and stored at −20 °C till further processing. The cell pellet (from 500 ml) was resuspended in 4.0 ml of buffer containing Tris, EDTA and NaCl. The cell suspension was sonicated on ice for 6 to 8 min with 20 cycles and then spun at 500g for 10 min. The pellet was resuspended in 1.0 ml of 1X LSB and boiled for 7 min. The presence of the protein was analyzed by checking the fraction on 13% SDS-PAGE. A strip of the gel was stained and aligned with the remaining unstained gel, and the corresponding region containing the protein of interest was cut out. The protein was eluted in buffer containing Tris, SDS overnight at 37 °C. The protein was estimated using Bradfords’ reagent.

3.3.4 Generation of antibody against 19 kD protein:
Polyclonal antibodies were raised in Syrian Golden hamster (6 to 8 weeks old). The first immunization was done with 100 μg of the protein. Adjuvants were not used as the protein contained SDS, which has adjuvant like properties. The next boost was given three weeks later, with 50 μg of the protein. The animal was bled 4 days after the boost to estimate the antibody titer. Further immunizations were given at intervals of 2 weeks and blood collected 4 days after the immunization (Antibodies ; A Laboratory Manual, 1988). The titer was determined by Western blot. Briefly, 50 μl of the expressed protein was run on a 13% gel. Protein was transblotted to nitrocellulose using Tris 50mM, Glycine 380 mM, 0.5% SDS and 20 % Methanol. Transfer was carried out at 60 mA, constant current, for 45 min at 4 °C. Blocking was done with 5 μg / ml BSA in TBST for 2 hours at room temperature. All antibody dilutions were prepared in 1.0 mg / ml BSA. Antibodies were tested at dilutions of 1:1000, 1:2000 and 1:5000, pre immune sera was utilised as negative control. Incubation was done for 1.5 hours followed by washing with TBST containing 1.0 mg / ml BSA, four times for 15 min each. The secondary antibody used was anti-hamster -HRP conjugate at a dilution of 1:4000. The washing step was repeated as before and the blots developed using the ECL kit (NEB).
to obtain large amounts of this protein. The TIB 208 (A20) cell line was used for this purpose.

A20 cells were cultured in bulk for membrane preparation. Cells were grown in RPMI 1640 with 10% FCS. Each culture was of 250.0 ml, since the cells grow in suspension bottles were maintained at 37°C on a roller unit. Cells were pelleted at 290 g for 10 min. They were washed thrice with 1X PBS, 10 min each. The cell pellet was now stored at –70 °C till further processing.

3.3.7 Membrane preparation:
The cell pellet was resuspended in 2.5 ml of homogenization buffer containing a cocktail of protease inhibitors (10 μg/ml of Aprotinin, antipain, leupeptin and pepstatin, 1mM PMSF and 10 mM of lodoacetamide). In order to achieve complete cell lysis, glass beads were also added to the suspension. Homogenization was done for 50 strokes in a glass homogenizer. Cell suspension was centrifuged at 300g for 15 min and the supernatant collected. The remaining pellet was resuspended in fresh homogenization buffer (2.5 ml) and the entire procedure repeated. Supernatants were pooled and centrifuged in a Hitachi ultracentrifuge at 100,000g for 2 hrs at 4°C.

The pellet consisted of the membrane fraction while the supernatant consisted of the cytoplasmic material. Pellet was resuspended in 500 μl of solublisation buffer. Solublisation was done overnight, at 4°C, with continuous stirring, using a flea magnet. Once the pellet was completely solubilised ultracentrifugation was carried out at 100,000g for 1 hour at 4 °C (Dass S Vinay et al, 1995).

The supernatant containing the membrane fraction was utilized for further work.

Analysis on SDS- PAGE:
The membrane preparation (50 μl) was mixed with an equal volume 2X Lammeli’s Sample Buffer (LSB) without β ME and incubated at 37 °C for 30 min. The membrane preparations were examined on a 10% polyacrylamide gel and aliquots of 5.0 μl, 10.0μl and 20.0μl were loaded per well to check the membrane sample. A suitable concentration was determined and all further gels utilized this concentration for purification of the B3 molecule.

Preparative gels (10%) were employed and a small strip was cut vertically down the length of the gel. This was stained with Coomassie R250 and destained. It was then aligned with the remaining unstained gel. The required B3 fragment was now cut from the unstained region.
The gel fragments were crushed and soaked in elution buffer (50 mM Tris, 0.1% SDS, 100 mM NH₄HCO₃, 0.1 mM EDTA and 150 mM NaCl, pH 8.8) overnight at 37°C. Supernatant was collected after a low speed spin and the volume minimized to 100 µl using Amicon concentrator with 10 kD cut off. Since the protein was needed in it’s functional form for cell assays, it was essential that the SDS be removed. This was done using the Extracti-D-Gel column (Pierce), sample was processed according to the manufacturer’s instructions and once again concentrated to the required volume before use. Protein estimation was carried out using the Micro BCA kit from Pierce Chemicals. A standard curve was prepared using BSA (1.0 mg/ml) in the range of 2.0 µg to 10.0 µg. The final reaction volume was 110 µl, OD was measured at 570 nm in an ELISA reader.

3.3.8 Polyclonal sera against B3 molecule
Syrian Golden Hamsters, age 6 to 8 weeks were used for this immunization. Initially 100 µg of the B3 protein was immunized subcutaneously as an emulsion with Complete Freunds adjuvant. After a period of 20 days, 50 µg was used per boost, with incomplete Freunds adjuvant. Test bleeds were carried out 4 days after the immunization. The serum was tested for positivity by Dot blot or ELISA. The second boost was given after a period of another 10 days and the animal test bled as before.

3.3.9 Polyclonal sera against Pyruvate Kinase M2:
Antibodies were raised in Syrian Golden Hamsters, all immunizations were subcutaneous. Commercially available Rabbit Muscle Pyruvate Kinase M2 from Sigma was used as a source of antigen. Initially, 100 µg was used for immunization with complete Freunds adjuvant, after a period of 20 days the first boost was given. All boosts were given with Incomplete Freunds adjuvant. The amount of antigen used was 50 µg, the animal was test bled after 4 days. The serum was tested using Western blot. Another booster was given 10 days later and the sera collected after the antibody titer was confirmed (Antibodies; A Laboratory Manual, 1988).
3.3.10 Determination of antibody titer:
The required antigen (Pyruvate Kinase or purified B3) was loaded on a 10% polyacrylamide gel at a concentration of 5.0 μg per lane. Gels were run till the dye front reached the base of the gel, and were then transblotted to nitrocellulose. Transfer buffer contained Tris, SDS, Glycine and Methanol, and was carried out at constant current. Pyruvate Kinase and B3 were transferred at 100mA for 45min. Blocking was done in TBST containing 5% BSA for 2hrs at room temperature. TBST containing 1% BSA was used to prepare all dilutions of sera and in the washing steps. The blots were then incubated with the test sera or suitable controls of normal sera for a period of 11/2 hrs at RT. Dilution of test sera tested were in the range of 1:500 to 1:5000. This was followed by three washes of 15 min each. Conjugate used was a 1:2500 dilution of Prt A-HRP, incubation was done at RT for an hour, again followed by three washes as before. Blots were developed using the Lumiglo Reagent (New England Biolabs).

3.3.11 Crossreactivity of Antibodies:
It was necessary to determine whether Pyruvate Kinase had any crossreactivity with B3. Pyruvate Kinase was obtained from Sigma Chemicals, immunization was done in Syrian Golden hamsters. B3 was purified from the membrane fraction of A20 cells and used for immunization. All three antibodies (α-19 kD, α-PK, α-B3) were used in Western blot system to determine whether, there are common regions between the enzyme, B3 and the 19kD expressed protein. Pure proteins were individually transblotted as described before and screened with all three antibodies. In addition Western blot was carried out with A20 membrane followed by screening with α-19kD antibody. This was done to indicate the number of membrane proteins with homology to B3 or PK.
3.4 RESULTS AND DISCUSSION:
3.4.1 Cloning of 450 bp fragment:
The 450 bp fragment was obtained by amplification with B3 sense and PK Lower primers, containing Nde I and Hind III sites respectively (Fig 3.3, Panel A, lanes 1&2). This fragment was cloned at the Nde I-Hind III site in pT7Nd vector. It was also cloned in the pET 15b vector at the same site. This was confirmed by restriction digestion with the above mentioned enzymes. A 450bp fragment corresponding to the PCR product was obtained (Fig 3.3 Panel B and C).

3.4.2 Protein Expression:
The expressed protein was found to be associated with the inclusion bodies (Fig 3.4). The lane containing bacterial lysate alone showed no expressed protein. Several colonies of pT7Nd containing 450 bp insert were picked and individually tested for expression of the protein. Alternate lanes were loaded with uninduced (Fig. 3.5, lanes 1, 3, 5) and induced lysate (Fig. 3.5, lanes 2, 4, 6), a 19kd protein was visible in the cultures after induction with IPTG. Similarly, with the pET15b system, several colonies were screened, the 19 kD protein was expressed on induction with IPTG (Fig. 3.6, lanes 3,5,7) as compared to uninduced cultures (Fig 3.6, lanes 2,4,6). This expressed protein contains an N-terminal His Tag.

3.4.3 Immunization of 19kD protein:
The 19 kd protein from pT7Nd was immunized in hamster and a polyclonal antibody was raised. Normal Hamster sera showed no reactivity against the pure protein. Test sera was checked at three dilutions 1:500, 1:1000 and 1:2500 (Fig.3.7, Panel A, lanes 2,3 & 4 respectively). The titer was determined to be 1:1000 on Western blot and this dilution was used for all further studies.

3.4.4 Affinity purified antibody:
Using the His-tagged version of the same protein an affinity column was prepared using Ni-NTA matrix. The polyclonal was purified by this system. A highly pure antibody was obtained. Three dilutions, 1:1000,1:2500 and 1:5000 were tested by western blot(Fig 3.7, Panel B, Lane 2, 3, 4). The titer was determined to be 1:5000 on western blot, this dilution was utilized for all further blots. Control Hamster sera failed to react with the transblotted protein at lowest dilution (Fig 3.7, Panel B, lane 1).
Fig 3.3: Cloning and Screening of 450 bp proc.
Expression vectors. cDNA from A20 was amplified by PCR and PK lower primers. The product obtained was cloned in pT7Nd and expression vectors. Positive clones were identified by insert release from and samples were analyzed on a 1% agarose gel. Panel A, lanes 1 & 2.
Fig 3.4: Expression of protein as Inclu
JM109 DE3 strain of E coli was transformed with pET 15 I
the 450 bp Insert. A single colony was inoculated in LB me
processed, as explained in methods, to identify whet
protein was in the soluble fraction or in the form of inclu
were run on 13% SDS-PAGE.Lanes, 1. 2.5 μl inclusion
5.0μl inclusion body fraction, 3. 2.5 μl supernate, 4. 5.1
Markers (kD)
Fig 3.5: Expression of 19kD protein in pT7l
Plasmids containing the 450 bp insert were transformed in JM101 E.coli. Individual colonies were inoculated in LB media and induced. The pellet was resuspended in 1X LSB and analyzed on 13 % SDS-PAGE gels. Lanes: 1. Bacterial Lysate 2. Clone1/uninduced 3. Clone1/Induced uninduced 5. Clone2/Induced 6. Clone2/uninduced 7. Clone3/Induced 8. Clone3/uninduced (kD)
Fig 3.6: Expression of His Tagged-19 kD protein 15b vector. Individual colonies were inoculated and induced with IPT was lysed with LSB and samples analyzed on 10% SDS-PAGE Lanes uninduced 2. Clone 1/ Induced 3. Clone 2/ uninduced 4. Clone 2/ Induce uninduced 6. Clone 3/ Induced 7. Markers (kD)
Fig 3.7: Confirmation of antibody titer

protein. The 19 kd protein expressed in bacterial expression to raise an antibody. The protein expressed with His-Tag (pHis) was purified to prepare an affinity purified antibody. Western blot was used.

Panel A: titer of polyclonal antibody, lanes:

Panel B: titer of affinity purified antibody, lanes:
3.4.5 B3 purification and Immunization:

B3 molecule was purified from the TIB 208 cell line, and purified from the membrane fraction (Fig. 3.8, Panel A). A polyclonal antibody was raised against this molecule. A protein of 40 kD was detected on Western Blot, three dilutions were tested, 1:500, 1:1000 and 1:2000 (Fig. 3.9, Panel A, lanes 2, 3 & 4). The titer was determined to be 1:1000 on Western blot, this dilution was used for all further Western blots. Control Hamster failed to react to the protein (Fig. 3.9, lane 1).

3.4.6 PK M2 Immunization:

Pyruvate Kinase was obtained commercially and immunized in Syrian Golden hamsters. Polyclonal sera was raised against the enzyme and three dilutions were tested, the titer was found to be 1:1500 on Western blot (Fig. 3.9, Panel B), control sera showed no reactivity to the protein. This dilution was used for all further blots.

3.4.7 Crossreactivity of α-19 kD, α-B3 and α-PK:

Purified 19 kD protein, B3 or PK was run on the required percentage of gel and transblotted to Nitrocellulose under previously standardized conditions. Strips of the Nitrocellulose were cut and each was individually tested with the appropriate antibody dilution. Blots were developed using Luminol Reagent.

All three antibodies (Fig. 3.10, Panel A, lanes 2, 3 & 4) recognized the 19 kD protein. Similarly, B3 (39 kD) was recognized by α-19 kD and α-PK polyclonals (Fig. 3.10, Panel B, lanes 2, 3 & 4). Pyruvate Kinase (55 kD) was recognized by both α-B3 as well as α-19kD antibodies (Fig. 3.10, Panel C, lanes 2, 3 & 4). All three antigens therefore show a homology to each other. Control sera failed to react strongly to any of the three proteins (Panel A, Panel B and Panel C, lane 1). It was also observed on Western Blot with A20 membrane, B3 was the major protein recognized, there was no protein corresponding in size to Pyruvate Kinase (Fig. 3.8, Panel B).

In summary, the α-19 kD antibody recognized both PK and B3 indicating that the two proteins share homology. Both proteins contained the common 19 kD sequence as seen by Western blotting experiments. In addition, α-PK and α-B3 crossreact to B3 and PK respectively. The A20 membrane does not express any protein corresponding in size to full length Pyruvate Kinase. Therefore, it is possible that B3 is either an entirely novel protein or a posttranslationally modified
Fig 3.8: A20 membrane preparation blotting. Panel A. Membrane was prepared from explained in methods. The membrane preparation was by 10% SDS-PAGE. The fragment corresponding to B3 protein was purified as described in methods. Lanes: 1. Protein blot membrane; 2. Protein markers (kD).

Panel B. A20 transblotted to a nitrocellulose membrane and western blotted with different dilutions of α 19kD affinity purified anti Ctrl sera (1:5000), 2. α 19kD, 1:2500, 3. α 19kD, 1:500
Fig 3.9: Confirmation of antibody and α PK. Polyclonal antibodies were raised to B3 and PK., as described in methods. Pure B3 or PK were nitrocellulose and the titer determined by Western Blotting. Lanes, 1. Ctrl sera 2. 1:500 α B3 3.1:1000 α B3 4. α PK.
Panel B, Lanes, 1. Ctrl Sera 2. 1:500 α PK 3.1:1000 α PK.
Fig 3.10: Crossreactivity of α19kd, αPurified 19 kD, B3 or PK protein was transblotted to nitrocellulose and the crossreactivity checked, as described in methods. Antibodies α19kD, α PK and α B3. Panel A (13% gel) : Protein was transblotted, lanes, 1. 1:1000 Ctrl Sera 2. 1:1000 α19kD 3. 1:1000 α B3. Panel B (10% gel) : PKM2 was transblotted. Lanes, 1. 1:1000 Ctrl Sera 2. 1:1000 α PK 3. 1:1000 α 19kD 4. 1:1000 α B3
form of Pyruvate Kinase. To confirm whether PK and B3 share any functions needs to be established by invitro assay of costimulatory activity.
3.5 REFERENCES:
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