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Technical Approach to Generate Polyclonal Antibodies Against Bacterially Expressed GST-PYK-C: Evaluation of Cross-Reaction and Recognition in Some Mammalian and Yeast Cell Lysates

Abstract: Pyk-2 is a protein tyrosine kinase, highly expressed in human hippocampus, dentate gyrus and olfactory bulb. In this study, polyclonal antibodies against the C-terminal of pyk-2, starting from Pro-759, were generated by injecting rabbits with the bacterially expressed GST fusion construct, generated by cloning the C-terminal domain of pyk-2 into pGEX-3X vector and affinity purification to increase the specificity of recognition. Anti-pyk-C was shown to recognize a 123 kD band in Western Blots of 293 cells transfected with pyk-2, as well as PC-12, COS and 3T3 cells and not to cross-react with FAK transfected Sf-21 lysates, in

spite of high sequence homology with FAK. This is an early study to develop quantitative immunochemical diagnostic assays, to determine the importance of pyk-2 in human physiology through clinical laboratory investigations. The use of bacterial expression systems in preference to other choices for antigen generation against native mammalian proteins are discussed, in context of the description of 'functional epitope'.

Key Words: pyk-2, Central Nervous System, Molecular Cloning, Gene Fusion, Bacterial Proteins, Antibody Formation, Antibody Specificity, Immunochemistry, Immunoblotting.

Introduction

Pyk-2 (protein tyrosine kinase-2) is a protein tyrosine kinase expressed in human brain, embryonic lung, liver, bone marrow cells and megacaryocytes (1-3). The tissue distribution by Northern blot studies of human brain sections and in situ hybridization analysis in rat brain showed that the highest levels of pyk-2 mRNA are seen in the hippocampus, dentate gyrus and olfactory bulb (1). The so-named RAFTK (related adhesion focal tyrosine kinase) (2) and CAK- β (cell adhesion kinase β) (3), named after their identification by separate groups are identical with pyk-2 in their amino acid sequences. Thus pyk-2 is a 1009 amino acid protein with a calculated molecular mass of 123 kD. The homology comparison of the amino acid sequence of the catalytic domain of pyk-2 with the four most related protein tyrosine kinases FAK (focal adhesion kinase), Fer, Her and Abl demonstrated 61, 43, 40 and 41 % sequence identity (1). The molecular physiologic studies done on cell cultures indicate that pyk-2 is phosphorylated on stimulation by nicotinic acetylcholine receptors, in response to calcium influx, by protein kinase

C dependent and independent mechanisms, as well as on stimulation of G-protein coupled receptors such as bradykinin through elevation of cytosolic calcium, mediated by inositol 1, 4, 5 P activation. In addition, pyk-2 phosphorylates and activates Kv1.2 potassium channels and also recruits shc/grb-2/sos complexes activating MAP kinase functions.

The knowledge acquired is through in vitro experiments and there is no information about the possible importance of pyk-2 as a diagnostic and therapeutic target for human health.

The use of immunochemical assays for description of clinical states is widely available. However, it is well known that the epitope selection at the antibody generation step as well as the use of antigens generated in procaryotic expression systems may lead to variations in the antigen antibody recognition reaction. The purpose of this study was to generate antibodies to detect pyk-2, to be used in mammalian immunochemical assays to evaluate the clinical usefulness of the molecule for future clinical laboratory studies.

This study was conducted on a graduate fellowship grant from the Turkish Higher Educational Council, at Cornell University Biochemistry, Molecular and Cell Biology Department, Ithaca, NY, USA

Materials and Methods

Construction of vectors carrying GST-pyk-C fusion protein genes

The sequence at the C-terminal of pyk-2 starting from Pro-759 was selected for antibody production with respect to abundance of surface residues, higher hydrophilicity, high antigenicity and least homology with FAK as well as the existence of convenient restriction sites for in frame cloning into pGEX-3X (Pharmacia, NJ). Amino acid sequence analysis of pyk-2 was done by a sequence analysis software (CGC Scientific, Inc, MO) (Figure 1). DNA was excised from pBS-pyk2 cloning vector (Stratagene, CA) by Hpa I and Eco RI digestions and inserted into the SmaI-EcoRI digested PGEX-3X in frame. The resulting plasmid named pGEX-3X-pyk C was used to generate the fusion protein GST-pyk-C (Figure 2). Transformation of *E. coli* DH5 α by pGEX-3X-pyk-C was done as described previously (6) and selection for ampicillin resistance was carried out.

Expression and Purification of GST-pyk-C fusion protein

GST-pyk-C fusion proteins were expressed in *E. coli* and purified according to the directions supplied by the manufacturer (Pharmacia, NJ). Briefly, bacteria with recombinant plasmids were grown to A600 of 0.6-0.8 with vigorous agitation at 37°C in LB medium with 50 μ g/ml ampicillin. After the assessment of the expected cellular density, fusion protein expression was induced by adding 10 μ l/ml of 100 mM IPTG. After incubation for 3 more hours at 37°C equal volumes of cells were collected by centrifugation, pellets were resuspended in 300 μ l ice cold phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (aprotinin, 2 μ g/ml; leupeptin, 20 μ g/ml; antipain 25 μ g/ml) and lysed by sonication 3x30 seconds. Bacterial cell lysis was followed by increased color formation on dye-binding protein assays (Bio-Rad laboratories, CA). Soluble supernatant was obtained by centrifugation at 16,000g for 15 minutes and GST-pykC fusion proteins were purified from the soluble fraction by chromatography on Gluthathione-Sepharose 4B (Pharmacia, NJ). 20 μ l of 50% Gluthathione Sepharose 4B was added to each supernatant, mixed gently for 5 minutes, centrifuged and washed 4 times with 50 μ l PBS. The GST beads with bound fusion protein were boiled with SDS loading dye, run at 70 Volts constant voltage at 10% SDS-PAGE and stained with Commassia Blue for detection of clones expressing GST-pyk-C.

The selected colony named *E. coli* DH5 α pGEX 3X pyk-C was regrown in abundance and GST-pyk-C protein was purified by and eluted from the Glutathione-

Sepharose 4B chromatography resin by 10 mM reduced glutathione in 50 mM Tris. HCl (pH 8.0)

Animal Immunizations

The immunization schedules and procedures were performed in the Animal Handling Facility of the Veterinary Medical School at Cornell University, Ithaca, NY as described elsewhere (5) in compliance with ethical regulations. Injections were given to two New Zealand white rabbits once every four weeks with a concentration of 200mg in 0.5 ml PBS. The rabbits received Freund's complete adjuvant antigen for the primary vaccination and Freund's incomplete adjuvant antigen for boosting. Preimmune sera was collected from both rabbits as well as immune sera one week following every immunization. Generation of immune response was followed by Western blots at each step of immunization. A total of 3 immunizations and one booster injection were given before sacrificing the rabbits.

Western Blotting for test of recognition

The antisera obtained from the test bleeds were tested for pyk-2 recognition by Western Blotting, the lysates obtained from Sf-21(ATCC, MD) transfected with pyk-2 constructs as well as 293 (ATCC, MD) cell extracts transfected with pCMV-LIC-pyk2 (Invitrogen, CA). Western Blotting was performed at 1:3000 dilution of the serum using the Amersham electrochemiluminescence system as previously described (6). The anti-serum was stored at -70°C in 0.2 % NaN₃. On proof of recognition, the rabbits were sacrificed.

Affinity Purification of the Specific IgG recognizing GST-pyk-C

300 ml bacterial culture containing pGEX-3X-pyk-C was induced with 0.1 M IPTG, centrifuged, and lysed by sonication in 50 μ l/ml PBS+Triton X-100. 1.8 ml of lysate was loaded on a 10% SDS gel and run with 70 V. A thin slice of gel was stained with Commassia Blue to identify the position of the fusion protein as well as for quantitation. The remainder was transferred to nitrocellulose paper and incubated with 5% milk to block the paper followed by TBST wash (7). Meanwhile rabbit anti-GST pyk-C serum was incubated with 250 μ l/ml 50% slurry of GST beads equilibrated with PBS for 1 hour to eliminate anti-GST antibodies. After centrifugation the supernatant was used to incubate the blocked blot for 6 hours at 4°C. The specific antibodies bound were eluted by washing the paper with 200 μ l of 100 mM glycine (pH 2.8) followed by immediate neutralization (1M Tris.Cl pH 8.0). For quantitation SDS-Page loaded and Commassia stained eluate were compared with albumin standards.

Detection of Specificity

The improvement in specificity was detected by Western Blotting of the total cell lysates obtained from COS, 3T3, PC-12 cells and 293 cells transfected with pyk-2, Sf-21, Sf21 transfected with FAK and Sf-21 transfected with FAK. N. COS, 3T3 and PC-12 cell lysates were prepared in 1% Nonidet P-40 lysis buffer (20mM Tris pH8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃ VO₄, 1mM phenylmethylsulfonylfloride, 0.2 trypsin inhibitory unit/ml aprotinin and 20 mg/ ml leupeptin) as previously described (6). Sf21 lysates were a kindly donated by Jun Lin Guan.

Results

Figure 1 demonstrates the strategy to decide on the peptide sequence to be used for highest antigenicity. The sequence between the amino acids 750-880 shows an array of highly hydrophilic sequences with considerably high surface probability and antigenicity. In addition to these, the existence of an Hpa I restriction site prior to Pro-759 makes in frame cloning into the Sma I blunt end convenient. Assuming the average molecular weight (MW) of an amino acid was approximately 110 Daltons, the calculated MW for GST-pyk-C would be 53.5 kD of which 26 kD is acquired from GST. Figure 3 shows several bacterial expression patterns. Lane 1 is *E. coli*

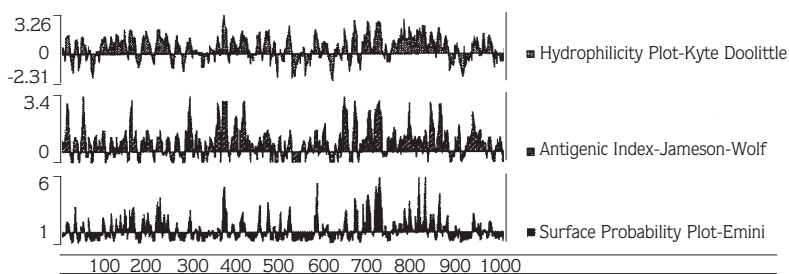


Figure 1. Analysis of pyk-2 protein sequence for selection of the peptide of best antigenicity.

Hydrophilicity, high surface probability and antigenicity were the features leading to selection of the peptide sequence starting from Pro-759 to generate the GST-fusion protein.

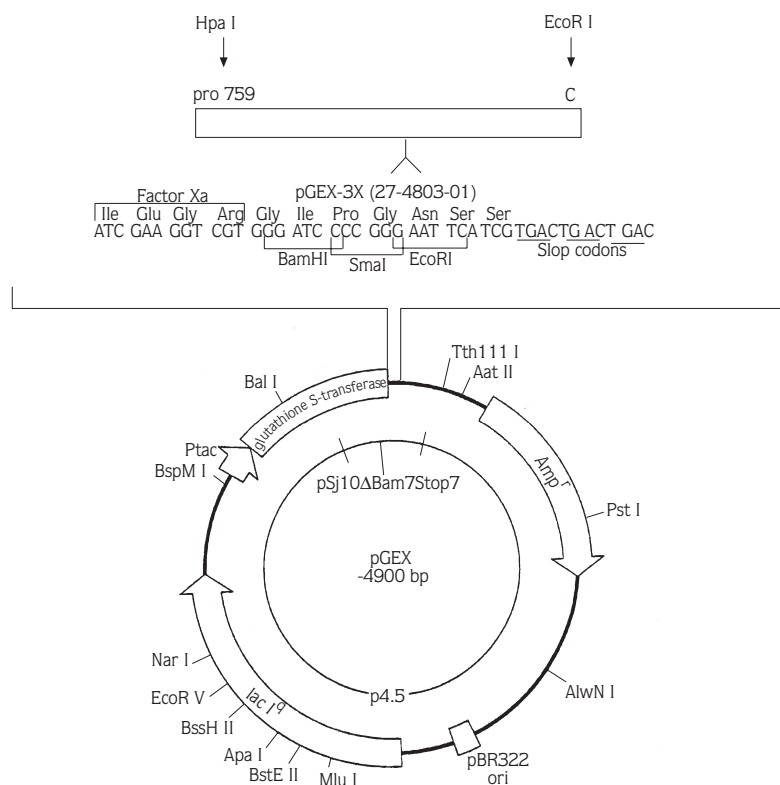


Figure 2. Strategy for construction of pGEX-pyk-C.

Pyk-2 was digested with the restriction enzymes Hpa1 and EcoR1 and obtained C-terminal peptide was inserted inframe into Sma1-EcoR1 cloning site on pGEX-3X in frame.

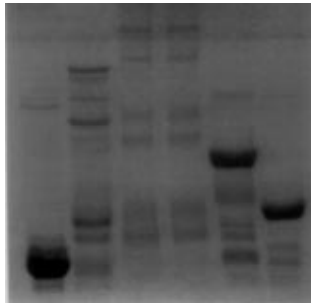


Figure 3. Commassia stained SDS-PAGE demonstrating expression of GST-pyk-C.

Colonies from *E.coli* transformants were grown and induced with IPTG for expression of bacterial GST fusion proteins. Cells were lysed by sonication and resuspended in PBS. The supernatants were incubated with 50% glutathione sepharose 4B except lane 2 and loaded on SDS-PAGE after boiling in SDS-PAGE loading dye for 3 minutes.

Lane 1. pGEX-3X transformant Lane 2. Untransfected but induced *E.coli* DH5a.

Lane 3 and 4. Molecular weight markers. Lane 5. pGEX-3X-pyk-C transformant

Lane 6. PGEX-FAK-C transformant.

transfected with pGEX-3X, induced with IPTG and finally adsorbed on GST-beads. The prominent band corresponds to the 26kD GST whereas lane 6 is pGEX-FAK-C induced and purified in the same fashion. These experiments were conducted to observe the efficiency of induction. Lane 2 is native *E. coli* DH5a induced with IPTG. Lane 3 and 4 are the molecular weight markers. In lane 5, it is observed that cloning and expression has led to successful synthesis of the GST-pyk-C fusion protein. However, it is interesting to observe the variety of bands indicating the changes in the metabolism of bacteria implied by changes in protein expression patterns due to the expression of the exogenous transfected proteins (15). After elution of the GST-pyk-C specific immunoglobulins by affinity purification, the eluate was evaluated by Commassia stained SDS-PAGE in comparison with known quantities of albumin. Figure 4 shows evidence of IgG eluted on affinity purification. By visual estimation, it is calculated that the concentration of purified antibody is 0.04mg/ml. IgG is a 150 kD protein with 2 heavy and 2 light chains. The upper band corresponds to the double overlapping heavy chains and the faint lower band in lane 4 corresponds to the light chains. For detection of specificity and possible cross-reaction with FAK, the experiment in Figure 5 was conducted. A band of approximately 123 kD is observed

in PC-12 cells which endogenously express pyk-2 as well as in 293 cells transfected with pyk-2 in mammalian expression vector. There is observable recognition in COS and 3T3 cells as well, though not as prominent as in lane 3 and 4. It is notable that the highest band intensity is observed in the 293 pyk-2 transfected lysates. On the other hand, there is no observable FAK recognition on transfected Sf-21 cells in comparison with native Sf-21 extracts which implies lack of cross-reactivity. Lane 7 shows the Sf-21 lysates transfected with N-terminal FAK where cross-reaction of a low molecular weight band is detectable in Figure 5a. Figure 5b blotted with purified anti-serum shows a decrease in non-specific recognition in comparison with Figure 5a blotted with native anti-serum.

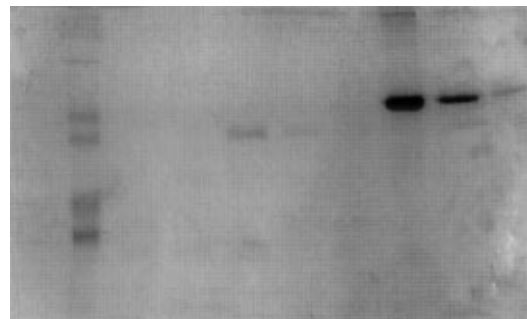


Figure 4. Quantitation of anti-GST-pyk-C antibodies purified by immunoadsorption of rabbit GST-pyk-C antiserum in comparison to albumin.

The pool containing the specific antibodies after affinity purification was run on SDS-PAGE (lane 4: 15 ml, lane 5: 5 ml, lane 6: 1 ml) and compared to albumin (lane 7: 6 mg, lane 8: 2 mg, lane 9: 0.4 mg). Lane 1: molecular weight markers.

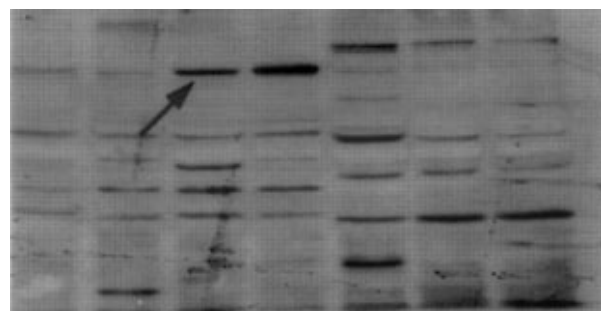


Figure 5a. Western Blot with GST-pyk-C antiserum. Cell lysates were Western Blotted with 1:3000 dilution of rabbit GST-pyk-C antiserum. Arrow indicates the 123 kD band for pyk-2.

Lane 1. COS. Lane 2. 3T3. Lane 3. PC12. Lane 4. 293 transfected with pyk-2.

Lane 5. Sf21. Lane 6. Sf21 transfected with FAK. Lane 7. Sf21 transfected with N FAK.

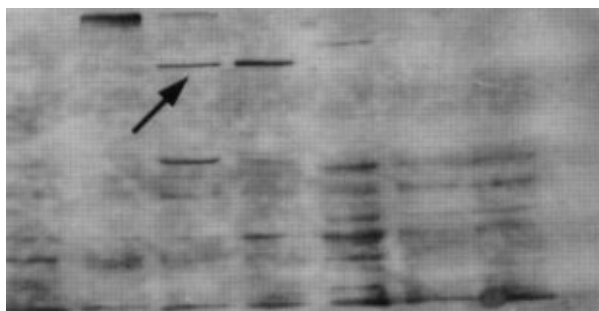


Figure 5b. Western Blot with affinity purified anti-GST-pyk-C antibody.
Western Blot of the same lysates with 1:500 dilution of affinity purified anti-GST-pyk-C antibody.

Discussion

It is well known that the most important determinants of antigen-antibody binding specificity are the physical characteristics of the two interacting molecular surfaces, such as their shape, complementarity, electrostatic charge, and polarity (7). The most conspicuous properties common to all humoral antigenic epitopes are their convex shape and the presence of large, polar, and charged amino acid side chains. A typical preparation of immune sera consists of a microheterogeneous mixture of molecules of the same antigenic specificity but different amino acid sequence in the antigen binding parts of the molecule. Through solution binding studies, there is evidence that antibodies are capable of recognizing the smallest structural variations in otherwise cross-reacting ligands (8). Antiprotein antibodies sometimes specifically recognize short peptides; such antibodies can be elicited by synthetic peptide antigens. The majority of antigenic sites in proteins, however, seem to consist of amino acids that are not contiguous in the amino acid sequence (composite or discontinuous epitopes).

A concept of 'functional (energetic)' epitope originally formulated in computational analysis of X-ray crystallographic structures, has found support in extensive alanine-scanning mutagenesis experiments of antigen-antibody complexes (9). Accordingly, only a small part of the total antibody and antigen contact surface (some 30-40% thereof, or three to four amino acid residues) appears to contribute actively to binding, often via hydrogen bonds and buried salt links. This smaller contact area, namely functional, or energetic epitope, belongs to the most protruding parts of the antigenic surface, where side chains such as Arg, Glu, Gln, Lys and Pro are particularly abundant. For the complex formation

to be possible however, there must be a complementarity between the additional surface area of the antibody and the antigen.

The rationale for making antibodies to recognize pyk-2 was to develop immunochemical methods to investigate the function and significance of pyk-2 in human physiology through clinical laboratory investigations. The approach taken for making pyk-2 anti-serum was injecting rabbits with GST-fusion proteins constructed by cloning the C-terminal of pyk-2 into pGEX-3X expression vector in frame. Protein engineering taking advantage of computer aided structural design based on available X-ray crystallographic coordinates has become a widely used tool for antigen engineering by gene cloning technologies (11, 12). It has been postulated that in attempts to generate antibodies against bacterial compounds using synthetic peptides, the response against the protein in its native conformation may be low (13). In order to maximise specific recognition, through increasing the microheterogeneity and preferring to recognize the folded native structure (10) of pyk-2, cloning a longer sequence rather than using synthetic peptides approach was taken in this study. In some bacterial expression vectors, it is possible to engineer secretable proteins. Insolubility of bacterial proteins forming 'inclusion bodies' is another difficulty for bacterial protein expression systems. In this study we were able to obtain pyk-C antigen as a soluble cytoplasmic protein. C-terminal fragment of pyk-2 starting from pro-759 was selected for making recombinant antigen mainly for the convenience of in frame cloning, in addition to hydrophilicity designating solubility. Soluble cytoplasmic protein purified was used for the injections. However, the peptide sequence was evaluated for antigenicity and surface probability by a commercial protein sequence analysis tool, as well. FAK being a protein with high amino acid sequence homology to pyk-2 was tested for cross-reactivity and the antibody was found to be non cross-reactive with FAK. The non-specific recognition bands which were abundant before affinity purification lessened after the purification step.

One concern often voiced in the case of recombinant antigens is the potential structural therefore functional differences between recombinant and native proteins. These differences may include posttranslational modifications occurring in *E. coli* but not in humans that may affect the suitability of an antigen generated against a recombinant bacterial protein as a reagent in immunological assays (14). No size differences were observed in SDS-Page between recombinant -pyk-C and native-pyk-C that cannot be accounted for by the

presence of a GST tag. More importantly, by using several different assays with different cell sources, bands matching in molecular weight with the expected native pyk-2 were observed. Still, it would be nice to compare this antibody with the ones generated against short peptides and recombinant pyk-2 constructs (1, 2) cloned via different strategies for the sake of better specificity and sensitivity of detection in physiological samples possibly using quantitative immunochemical methods.

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