USE OF BIONANOTECHNOLOGY TO DECIPHER THE PATTERNS OF ASSEMBLAGE AND INTERACTIONS OF MULTI- PROTEIN COMPLEXES

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A Thesis

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ABSTRACT

Carol A. Heckman, Advisor

PAKs (p-21 activated kinases) are serine/threonine kinases that interact with the adaptor protein Nck-α through its Src homology3 (SH3) domains. Nck is an adaptor, i.e. its only function is to assemble two or more unlike proteins in a precise 3D orientation. Interaction of PAK and Nck-α has been reported to be mediated by the autophosphorylated state of the kinase. The amino-terminal portion of PAK has a sequence that binds Nck. An assay was designed using molecular genetics techniques to express Nck-α in *Escherichia coli (E.coli)* and capture Nck on PAK. Here, the bacterial lysate is used to place a gold-label on the Nck. Nck thus attached to 2 nm gold particles was retrieved by capturing it on PAK bound to 10 nm gold particles. Capture of gold-adsorbed Nck-α on gold-adsorbed PAK displayed patterns in the transmission electron microscope image indicating the interaction of PAK1 molecules with the Nck-α molecules.

Several proteins from the bacterial lysate were captured along with Nck on PAK. Treatment of the bacterial lysate with anti-phosphotyrosine antibodies in order to block the interaction between the phosphotyrosine residues of the bacterial proteins and the Src homology (SH2) domain of Nck-α demonstrated that this type of interaction did not exist between these proteins. The results, however, suggested that Nck-α was captured on PAK-GST irrespective of the activity state of PAK. I conclude that it is possible to synthesize arrays consisting of one to many adsorbed gold particles around a single large particle, such as a quantum dot.

I dedicate this work to my advisor and Dr. Donald Deters.

ACKNOWLEDGEMENTS

This thesis is an end to a wonderful journey that I embarked on two and a half years back at Bowling Green State University. I would not have been able to complete this journey without the invaluable input of many people.

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INTRODUCTION

PAK-regulated structures

Focal contacts (FCs) are structures that connect the actin to the extracellular matrix (Schoenwaeldera and Burridge, 1999). Several terms are used in the literature to refer to this type of structure. Here, the term FC is used as a general term to indicate all integrin-mediated close contacts between the plasma membrane and extracellular proteins. Some other investigators refer to FCs interchangeably with focal adhesions, although the latter term is frequently used to indicate larger, stable, and elongated adhesive specializations that are tightly attached to actin bundles. At the other extreme, there are FCs that turn over rapidly at the cell periphery, which may be termed focal complexes or point contacts in the literature. Cells migrate with the help of dynamic assembly and disassembly of FCs and protrusion formation. Paxillin is a key regulator in the assembly and disassembly of FCs (Brown and Turner, 2004). Paxillin belongs to a class of proteins known as adaptors. Adaptors do not exhibit any catalytic activity and function by creating a platform or scaffold for the assembly of other proteins. Adaptors play a vital role in signal transduction pathways. Paxillin interacts with G-protein coupled receptor kinase interacting proteins, GIT1 and GIT2, through its LD4 domain. This interaction occurs through the phosphorylation of the Ser 273 site that resides in the LD4 domain. This phosphorylation was shown to be mediated by cell division cycle 42/ Ras-related C3 botulinum toxin substrate 1 (Cdc42/Rac) effectors PAKs. Paxillin-GIT binding has been identified as a regulator of adhesion turnover and protrusion formation (Nayal et al., 2006). Manser and coworkers demonstrated that GIT1 binds to PAK-interacting exchange factor (PIX) through its Spa 2 homology domain (Manser et al., 1998). PIX in turn binds to PAK. Phoshophorylation of paxillin by PAK targets

the GIT1-PIX-PAK module to the small dynamic FCs near the cell edge, resulting in rapid turnover (Nayal et al., 2006). Thus coupling of FCs and protrusion formation is regulated by the localization of the paxillin-GIT1-PIX-PAK complex (Manser et al., 1998).

PAKs

PAKs are serine/threonine kinases whose activity is stimulated by the binding of an enzyme that binds GTP specifically, Rac or Cdc42 GTPase. Two groups of PAKs have been identified: Group 1 and Group 2. Group 1 (PAK1, PAK2, PAK3) is involved in cell motility, gene transcription, cell transformation, apoptosis (Chernoff, 2001) and actin organization (Sells et al., 1997), whereas Group 2 (PAK4, PAK5, PAK6) is involved in these processes in addition to hormone signaling (Chernoff, 2001). Both groups of PAKs have well-conserved C-terminal catalytic domains and differ in the N-terminal regulatory domains. Group 1 proteins have a more complex arrangement of domains in the N-terminal than do Group 2 proteins (Fig.1).

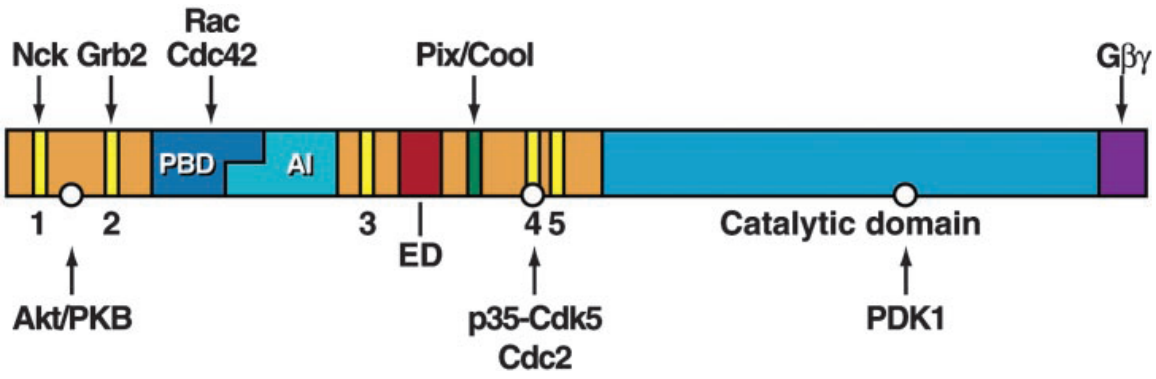

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Fig. 1. Structure of PAK1. N-terminal region of PAK1 is depicted in orange color while the blue color portrays the well conserved C-terminal domain (amino acids 255-529). The five canonical Src homology (SH3)-binding motifs are shown in yellow color while the noncanonical SH3-binding site is shown in green color that interacts with PIX/Cool (Cloned-out of library).

PAKs 1-3 have an extensive N-terminal regulatory domain. PAK1 has five proline-rich PXXP SH3 binding motifs whereas PAK2 and PAK3 have two and four PXXP SH3 binding motifs respectively (Bokoch, 2003). All of them have one non-classical SH3 binding motif that interacts with the PIX family of proteins (Manser et al., 1998). The first PXXP SH3 binding motif in PAK1 isoform interacts with SH3-containing protein Nck (Bokoch et al., 1996; Galisteo et al., 1996), and the second interacts with the adaptor protein Grb2 (Puto et al., 2003). The Cdc42/Rac-interactive binding (CRIB) domain is located within the p21-binding domain (PBD). Overlapping the PBD is a kinase inhibitory domain (KID) that regulates the kinase activity of PAKs 1-3 (Lei et al., 2000). PAK1 is thought to be a homodimer wherein the kinase domain of one molecule is in contact with the KID of another molecule, which keeps the kinase inactive (Lei et al., 2000). Fig. 2 shows the effect of these allosteric rearrangements.

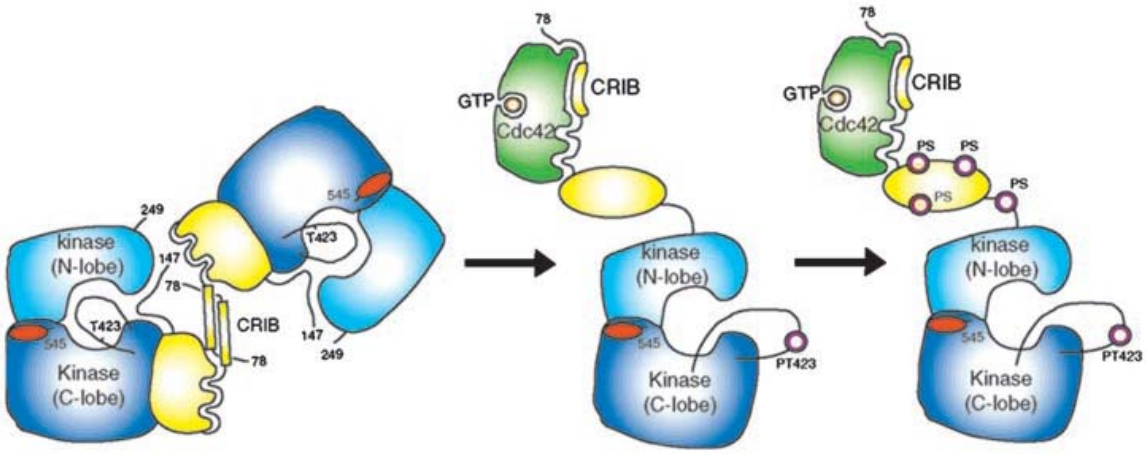

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Fig. 2. Activation of p21 activated kinases by binding at the CRIB PAK (aa 75-90). The KID is shown in yellow and the kinase domains are shown in blue. The interaction of the KID with the kinase domain promotes dimerization and renders it in an inactive state (shown in left). When the GTP-loaded Cdc42 binds the CRIB sequence within the KID, the dimer dissociates and unblocks the kinase domain.

PAK1 in its dimeric form is in an off state with both the monomers arranged in an inverse orientation namely, KID motif of one PAK1 molecule binds to the catalytic domain of the other molecule. When GTP-loaded GTPase (Rac or Cdc42) comes in contact with the CRIB domain, the dimer unfolds leaving the kinase domains exposed. Thus, binding of Rac or Cdc42 leads to conformational changes resulting in the dissociation of the PAK dimer and rearrangement of the proteins into a catalytically competent state. Since the GTPase may need to be loaded onto the PAK molecule by a guanine nucleotide exchange factor (GEF), merely supplying GTP-loaded GTPase in the cytoplasm of the cell may be insufficient to activate PAK. Furthermore, it is not clear whether both the monomers are activated by the GTPase although both may be freed from the autoinhibition site binding. Further autophosphorylation of the activation loop fully activates the enzyme (Bokoch, 2003).

All the PAKs 1 through 6 can interact with the GTPases but only PAKs 1-3 are activated by the interaction with the GTPases (Manser et al., 1994). PAKs 4-6 cannot be activated by the interaction with the GTPases (Abo et al., 1998; Chernoff, 2001). There are various other mechanisms that have been identified that point to GTPase-independent activation of the PAKs. First, PAK1 can be activated by lipids such as sphingosine and other sphingolipids (Bokoch et al., 1998). Regulation of the kinase activity of the PAKs by phosphatidylinositol 3-kinase (PI3-K)-dependent kinase 1 (PDK1) occurs in the presence of sphingosine (King et al., 2000). Kinase activity of the PAKs can also be stimulated by a few protein substrates of PAKs such as histone 2B and histone 4 (Gatti et al., 1999). Activity of PAK1 and PAK2 is also affected by any disruption in Ca^{++}/CaM signaling (Lian et al., 2001). Other phosphate-handling enzymes, for example serine/threonine phosphatase 2A (PP2A) and p70 S6 kinase, have also been supposed to play a role in regulating the kinase activity of PAK1 and PAK3. Kinase activity stimulated by

the GTP-bound GTPases can be impeded by PAK1 interacting proteins such as hIP1 (Xia et al., 2001). A transforming retrovirus, product (AKT)/protein kinase B**,** a growth factor-regulated protein kinase, is also involved in the stimulation of PAK1 activity (Zhou et al., 2003). Several growth factors, such as epidermal growth factors, heregulin, platelet derived growth factor and hepatocyte growth factor, are also involved in the stimulation of PAK1 activity mediated by the activation of the GTPases.

Kinase activity of PAK2 was disrupted by the phosphorylation of its tyrosine residues by non-receptor tyrosine kinases such as Abelson (Abl) kinase (Roig et al., 2000). PAK1 is phosphorylated by other non-receptor tyrosine kinases such as epithelial and endothelial tyrosine kinase (Etk/Bmx) (Bagheri-Yarmand et al., 2001). Binding of αPix, Rac and Cdc42-specific nucleotide exchange factors to PAK1 stimulates its activity independent or dependent on the nucleotide exchange activity of αPix (Daniels et al., 1999). On the contrary, kinase activity of PAK bound to a GTP-GTPase is inhibited in vitro by β-Pix. Kinase activity of PAK1 was found to be inhibited by the binding of $G_{\beta\gamma}$. Interaction of Nck, an adaptor protein, with the first proline-rich domain of PAK results in the relocalization of PAKs which further results in its activation. Activation of PAK1 and PAK2 is also stimulated by the heterotrimeric G proteins (Bokoch, 2003).

Nck

The adaptor, Nck, has three SH3 domains near its N-terminus and one SH2 domain in its C-terminal sequence. The middle SH3 domain, SH3[2], was identified to be a site where most of the target molecules bound themselves, although some also relied on sites in SH3[1]. Nck links cell surface receptors via their phosphotyrosine residues to the downstream effectors through

Nck's SH3 domain**.** Nck links the cell surface receptors to the actin cytoskeleton, which is vital for processes such as axon pathfinding, directional migration, chemotaxis and endocytosis. Nck also regulates cell proliferation, gene expression and transformation in mammalian cells (Li et al., 2001)**.** Nck is capable of activating PAK by recruiting it to the tyrosine kinase receptor at the plasma membrane (Lu et al., 1997)**.** AKT\PKB mediates phosphorylation of PAK at serine 21, and prevents the binding of Nck to PAK (Zhou et al., 2003). Nck is capable of binding to a number of different target proteins that are subject to phosphorylation on specific motifs (e.g. proline rich motifs (Zhao et al., 2000). The formation of complexes with Nck depends on the phosphorylation states of these target proteins. The role of Nck in FC assembly, if any, is not clear (Fig. 3).

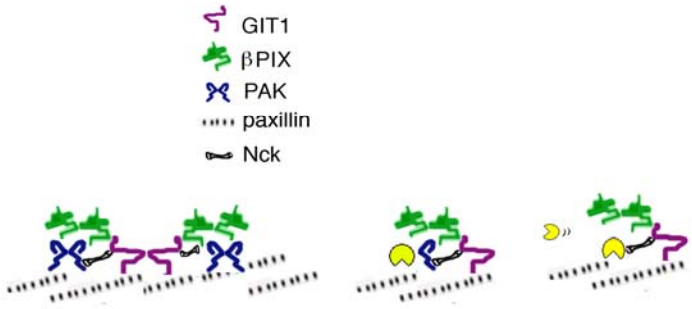

Fig. 3. Schematic diagram showing the hypothesized role of Nck in FC assembly. This illustration is a crude imagination of how the entire multi-protein assemblage might work in nature.

Proteins have great potential for use in devices, because a variety of binding surfaces have been developed through evolution. The concept of using protein assemblages to form nanofibers and nanotubes has been discussed by Reches (Reches et al., 2006). To date, it has not been proved possible to manipulate biomaterials so as to create junctions, create branches at a predictable angle, and to pre-program complex patterns and then execute them in a material.

Although nucleic acids undergo self-assembly and can be programmed for device formation, nature has designed a much greater variety of switches, hinged structures and junctions in proteins. Thus, more patterns can be implemented in protein designs. PAK is an ideal choice for the design of complex structures, because investigators can simultaneously gain insight into the biological functions of the protein and work out principles of nanostructure design. Structures formed in nature by PAK and its binding partners have a level of complexity far exceeding that of such fibers. PAK and PIX form a complex with GIT, and GIT attaches it to paxillin (Fig. 4).

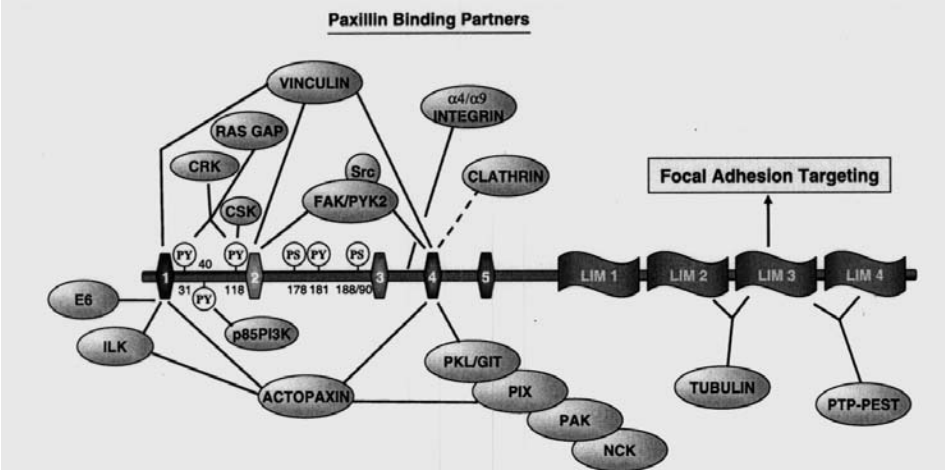

Fig. 4. Diagram showing the proposed arrangement of components on the focal contact scaffold. (Brown and Turner, 2004)

As the understanding of PAK interactions with its binding partners increases, it may be feasible to design modules based on its attachment to the scaffold protein, paxillin. This will allow one to create "U"-shaped junctions. By attaching colloidal gold particles to each of the proteins through an electrostatic interaction, the upright stroke of the "U" could be constructed from the linear array of proteins as shown in Fig. 5. Attachment of a different-sized gold particle to each protein would enable us to determine whether the nature of the assembled complex is that

shown in Fig. 4.

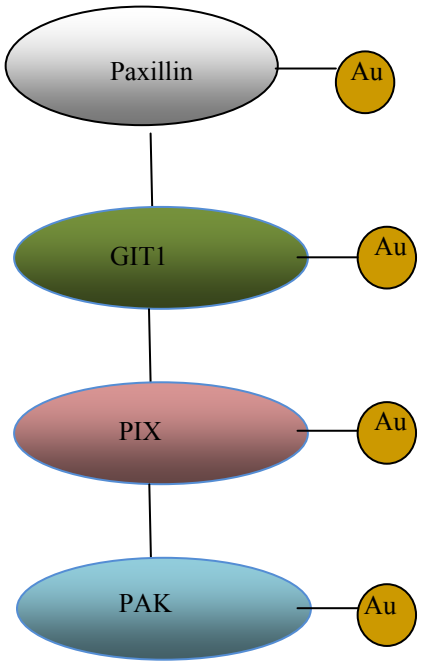

Fig. 5. U-shaped junction constructed from the linear array of proteins.

The three isoforms of PAK (see below) are capable of binding to a number of other proteins and may do so at a predictable angle. PAK is capable of binding to other partners to form large assemblages, e.g. Rac, PIX and GIT1 or Nck, Rac, PIX and GIT1 simultaneously. In principle, if one were able to attach nanoparticles of gold (Au) to each component of this module and then get the organic component dissolved by heat treatment, what would be left behind is the nanoconstruct of gold. Such structures would have great utility, because their propagation would terminate when the last protein was added. My project involves the application of nanoparticles of gold to PAK and an adaptor protein Nck and observing this assembly using transmission electron microscopy techniques. The objective of my project is to determine whether conditions can be set that would allow more extensive constructs to be made.

Nanoparticle-protein conjugates

Application of nanoparticles to proteins have not as yet been used to get a better understanding of protein folding and assembly. The use of nanoparticle-protein complexes, however, would have important implications for technological challenges in nanosensors, drug delivery and imaging. It should be possible to fuse any noble metal particle with a protein. One of the many problems in the nanoparticle-protein conjugation is that this union can result in the unfolding of the protein and loss in function owing to the complex nature of the nanoparticles. Since it is not possible to control the points in the proteins where the Au nanoparticles attach, the project mainly relies on attaching enough proteins to each particle to ensure that one or more moieties are oriented in the right direction, i.e., facing outwards.

There are several ways in which nanoparticles can be linked to the proteins, which are summarized in Fig. 6.

1. Electrostatic adsorption: This involves the establishment of conditions such as ionic strength and pH to enhance the interaction of charges present on the nanoparticle surface with the amino acid chains of the proteins. Electrostatic adsorption is not a very specific interaction, because the protein can interact with the nanoparticle in a number of different orientations.
2. Linking to the nanoparticle ligand: This method involves covalent binding of the protein with the nanoparticle.
3. Linking using specific affinity of protein for a cofactor: The nanoparticles can also be labeled with a cofactor such as biotin which can then be specifically targeted to streptavidin.

4. Direct reaction with nanoparticle surface atoms: This method involves the direct application of proteins to the nanoparticles without any linking molecules. The Au nanoparticles form covalent bonds with thiol groups, and hence proteins with cysteine residues will bind to the nanoparticles.

It is possible that the proteins used in this project have cysteine residues. For the most part, however, the binding between the protein and Au is thought to be the structurally nonspecific electrostatic binding. This binding has the advantage that at a minimum only one part of the protein is adsorbed to the surface of a gold particle (Aubin-Tam and Hamad-Schifferli, 2008).

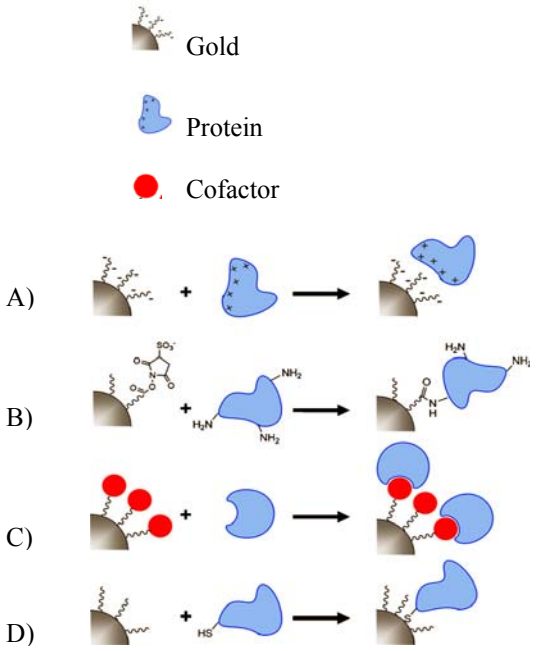

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Fig. 6. Different methods in which gold nanoparticles can be linked to proteins. A) electrostatic adsorption, B) linking to the nanoparticle ligand, C) linking using specific affinity of protein for a cofactor, D) thiol-dependent reaction with nanoparticle surface atoms (Aubin-Tam and Hamad-Schifferli, 2008).

PAKs in cytoskeletal reorganization

The eukaryotic cytoskeleton is composed of actin filaments which are the thinnest filaments in the cell, intermediate filaments, and microtubules. Actin cytoskeletal reorganization

is an essential process underlying physiological phenomena such as cell polarity, shape and motility. Actin cytoskeletal dynamics are regulated by the interaction between actin and myosins. This interaction is primarily regulated by the myosin light chain kinase (MLCK) which phosphorylates the myosin light chain (MLC). PAKs are involved in the phosphorylation of MLCK which discontinues the phosphorylation of the MLC (Sanders et al., 1999). Alternatively, PAK2 has been found to directly phosphorylate MLC which helps in cell retraction (Chew et al., 1998; Zeng et al., 2000). PAK1 stimulates the activity of LIM kinase 1 which in turn phophorylates cofilin, an actin depolymerization factor which is then inactivated. Inactivation of cofilin results in the reduction of actin depolymerization and also promotes stability of the actin filaments (Edwards et al., 1999). A role for PAK has also been identified in the formation of lamellipodia and focal adhesion points. Phosphorylation of desmin, type III intermediate filaments by PAK1 inhibits its ability to bind to the intermediate filaments (Ohtakarab et al., 2000). PAK1-mediated phosphorylation of vimentin, a protein involved in the stabilization of cytoskeletal interactions, maintenance of cell shape and cell integrity, by PAK1 affects the assembly of vimentin filaments (Goto et al., 2002). Stathmin, a protein involved in the destabilization of microtubules is also phosphorylated by PAK1,which in turn promotes the stabilization of microtubules (Daub et al., 2001). PAK1-mediated phosphorylation of tubulin cofactor B (TCoB), a cofactor in the assembly of the α/β tubulin heterodimers, is required for its normal functions, formation of new microtubules (Vadlamudi et al., 2005).

MATERIALS AND METHODS

Plasmids and bacterial transformation

The gene constructs of interest, KID fused in frame with GST and Nck-α were obtained in eukaryotic expression vectors, pXJ40 and pGEX, respectively. The pGEX plasmid containing Nck-α was a gift from Sohail Ahmed (National University of Singapore). The pXJ40 vector (Xiao et al., 1991) has sequences ordered as shown in Fig.7.

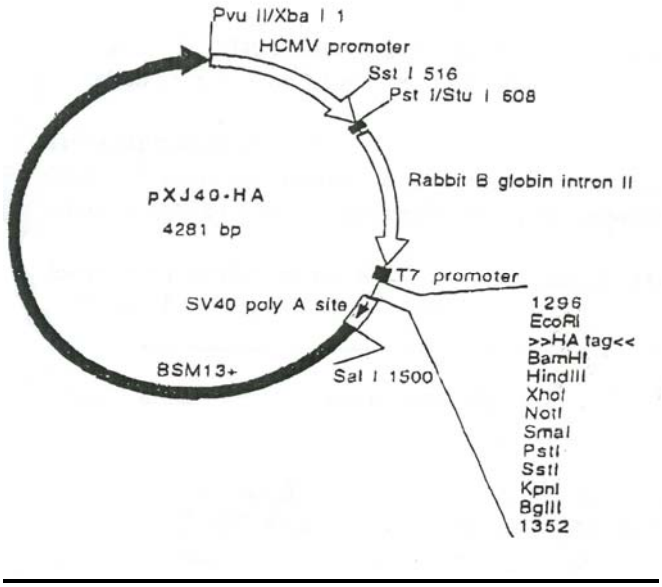

Fig. 7. Illustration of vector sequences in pXJ40 (personal communication from Zhou-Shen Zhao). The vector has the following structural components: a human cytomegalovirus (hCMV) promoter that facilitates the increased expression of the inserted gene, rabbit β-globin intron II that facilitates the splicing event, T7 bacteriophage promoter that allows transcription of the inserted gene, a multiple cloning site (MCS) that facilitates the introduction of many gene sequences, a SV40 polyadenylation sequence that brings about the increased levels of protein expression, BSM13+ sequence that subsumes an origin of replication, the ampicillin resistance gene and lastly a hemagglutinin (HA)/FLAG tag at the N-terminal which assists in the expression and purification of the expressed proteins.

KID was expanded and expressed in BL21 (DE3) cells obtained from Invitrogen. The DE3 indicates the presence of λDE3 lysogenic phage, which has the gene for T7 polymerase under the influence of *lac*UV5 promoter. The expression of T7 polymerase can be induced by

the addition of isopropyl β-D-1-thiogalactopyranoside. These BL21 (DE3) cells lack the *lon* and the OmpT proteases. The *lon* protease is involved in the degradation of some regulatory and abnormal proteins. OmpT protease is present in the outer membrane of Gram-negative bacteria such as *E. coli* and it is involved in the cleavage of several endogenous and exogenous proteins. BL21(DE3) cells are appropriate for the expression of non-toxic proteins. BL21(DE3) strain of cells was not working for expression of Nck-α, and therefore the BL21-AI strain (Invitrogen), which is appropriate for the expression of toxic proteins, was used for Nck-α expansion and expression. BL21-AI *E. coli* strain has a chromosomal copy of the T7 RNA polymerase gene inserted under the influence of the arabinose-inducible *araBAD* promoter. This promoter holds the expression of T7 RNA polymerase gene under tight regulation to control the expression of the plasmid gene. Upon induction with arabinose, this expression system produced a high yield of Nck-α protein.

Eppendorf tubes were taken and labeled as A, B and C. The contents were respectively: A, 1µl of Puc19 (10 µg DNA in 28.8 µl water) and 20 µl of competent BL21 cells, B, 20 µl of the competent BL21-AI cells, and C, 5 µl of Nck-α in pGEX and 30 µl of competent BL21-AI cells. Tube A serves as the positive control for the transformation procedure. DNA enters the bacterial cell membrane through the holes introduced by incubating the tubes on ice for 30 minutes followed by heat shocking for 1½ min at 42º C in a water bath and subsequently incubating on ice for 2 minutes. To tubes A and C, 200 µl of super optimal broth with catabolite repression (SOC) medium was added, and to tube B 100 µl of SOC medium was added. The tubes were incubated in a water bath at 37º C for 40 minutes, which allows the cells to grow and also facilitates the expression of the ampicillin resistance protein beta-lactamase that facilitates

survival of these cells on Luria-Bertani ampicillin (LB Amp) media (Lengeler et al., 1990). The contents of tubes A-C were distributed on sterile agar plates as shown in Table 1.

Table 1. Contents of agar plates seeded with cells from tubes A-C[a]

Number of the plate	**Contents of the plate**
plate 1	50 µl Tube C
plate 2	200 µl Tube C
plate 3	100 µl Tube A
plate 4	No cells
plate 5 (no Amp plate)	20 µl Tube B

[a]Control procedures are used to determine whether the cells were viable and the transformation was successful. Transformed cells are plated at two concentrations (plates 1 and 2). The purpose of plate 3 is to check if the cells are competent or not. The purpose of plate 4 is to check if the media are sterile. The purpose of plate 5 is to check if the *E. coli* cells are viable or not.

DNA purification

For plates transformed with the Nck-α plasmid, the plates were left overnight at 37º C. The next day, the plates were checked for transformants. For the next phase of experimentation, 2 colonies were picked up and used to inoculate 5 ml of LB media containing 50 µl of 20 mg/ml ampicillin. These tubes were left overnight at 37°C, and the plasmid DNA was extracted the following day. This same procedure was used to expand the KID-GST plasmid in pXJ40. DNA purification was carried out using the Qiagen Plasmid Mini Kit 25 (Valencia, CA). The procedure followed the manufacturer's protocol.

A. 5 ml of the cells were harvested by centrifuging them down at 14,000 rpm for 30-40 seconds in an Eppendorf 5415 C microfuge.

B. 0.3 ml of P1 buffer supplied with the mini-prep kit was added to resuspend the cells. The cell suspension was vortexed gently.

C. 0.3 ml of P2 buffer as added, vortexed gently and incubated the tube at room temperature (15-25º C) for 5 minutes.

D. Following the incubation period, 0.3 ml of P3 buffer was added and the contents of the tubes were mixed by inverting the tubes several times. The tubes were incubated on ice for 10 minutes.

E. The tubes were centrifuged at 10,000 rpm for 5 minutes.

F. The supernatant from Step E was applied to the two pre-equilibrated Qiagen columns and the columns allowed to empty due to surface tension.

G. The columns were washed 4 times, each time with 1 ml of QC buffer supplied with the mini-prep kit.

H. Following the washing step, 0.8 ml of QF elution buffer was added to the columns and the solution running down the column was captured in Eppendorf tubes. This solution contains the DNA.

I. DNA was precipitated by the addition of 0.56 ml (0.7 volumes) of isopropanol. The tubes were incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 15 minutes.

J. The supernatant was removed and DNA pellet washed with 0.6 ml of 80% ice-cold ethanol. The tubes were once again centrifuged at 14,000 rpm for 10 minutes.

K. The supernatant was thrown away and the DNA pellet was allowed to air-dry for 10 minutes.

L. The pellet was resuspended in 60 µl of TE buffer (10mM Tris-HCl, pH 7.6 in 0.1mM ethylenediaminetetraacetic acid [EDTA]). Tris is Tris[hydroxymethyl]aminomethane.

Restriction enzyme digestion and agarose gel electrophoresis

As the last step in the purification procedure, the presence of the DNAs was confirmed by restriction enzyme digestion and agarose gel electrophoresis. In an Eppendorf tube, I combined 3 µl of the purified DNA, 7 µl of sterile deonized water, 1 µl of New England buffer 2 (NEB 2) and 1 µl of *Eco*R 1 restriction enzyme. This tube was then incubated at 37°C for 2 hours. Then, 4 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added. The tubes were vortexed and 8 µl of this sample was loaded into a well of a 0.8% agarose gel (0.28 g of agarose in 35 ml of TBE buffer) along with the marker *λBst* II.

Nck-α complex formation with PAK1

In order to form the Nck-PAK complex, the protein Nck-α was expressed in a fresh culture of cells as follows.

A. Bacteria transformed with the Nck plasmid were scraped off with a loop and 2 ml of LB media was inoculated with the cells. 100 µg/ml of ampicillin was added. This was then grown overnight at 37° C.
B. An aliquot of 30 µl of this culture was taken and added to 5 ml of LB media, and then 120 µg/ml ampicillin was added. This was left overnight at 37° C with constant shaking.
C. This culture was added to 50 ml LB media containing ampicillin. The culture was grown for 4 hours at 30° C to allow the cells to reach an exponential rate of growth.
D. Following this incubation, the bacterial cells were induced with 20% arabinose to allow the expression of Nck. After 1 1/2 hours, the cells were spun down at 6,000 rpm for 10 minutes at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor). The pellet was resuspended

in 10 ml of 1X phosphate buffered saline (PBS) (8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4).

E. An equal volume of lysis buffer, consisting of 1% Triton X-100, 100 mM phenyl methane sulfonyl fluoride (PMSF), 0.1% sodium dodecyl sulfate (SDS) and one protease inhibitor tablet (Roche) in every 10 ml, was added to the resuspended cells. The solution was rapidly pipetted several times to ensure proper suspension.

F. To lyse the cells, they were sonicated 5 times for 20 seconds each time.

G. The cells were then spun down at 15,000 rpm for 15 minutes at 4°C (JA-20 rotor). The supernatant containing the expressed protein was retained.

H. Steps A through G were carried out to get the expression of the protein KID-GST. KID-GST protein was purified by incubating the supernatant obtained from step G with 1 ml of prewashed (to get rid of the ethanol) glutathione-Sepharose beads for 2 hours. The tube was then centrifuged briefly at 6,000 rpm to collect the protein-bead complex. The pellet was washed with immunoprecipition buffer (equal volume of the PBS buffer and the above prepared lysis buffer) 4 times and recovered by centrifugation each time. The pellet was finally suspended in 1 ml of the same buffer.

I. KID-GST was eluted from glutathione-Sepharose beads

   1. 1 ml of glutathione elution buffer (0.154 g glutathione in 50 ml of 50 mM Tris-HCl) per ml of bed volume was added.
   2. The pellet obtained above was mixed gently to resuspend and incubated at room temperature for 10 minutes.
   3. Then, it was centrifuged at 247 rpm for 5 minutes to pellet the beads. The supernatant was collected.

J. The supernatant was exhaustively dialyzed to get rid of the glutathione. Then, tubes were loaded with Nck-α captured on PAK-GST in the presence or absence of KID (Table 2).

Table 2. Capture of Nck-α on PAK-GST in the absence and presence of KID-GST

Tube 1	500 µl of 2X immunobuffer[a] + 170 µl of cell lysate (Nck) + 400 µl of water + KID-GST + 0.1 µg of PAK-GST
Tube 2	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 400 µl of water+ 0.1 µg of PAK-GST
Tube 3	500 µl of 2X immunobuffer + 400 µl of water

[a]2X immunobuffer: 2% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40

K. Tubes 1-3 were then incubated in the refrigerator with rotation for 2 hours.

L. 75 µl of glutathione-Sepharose beads was added. The sample tubes were vortexed and turned on a rotor placed in the refrigerator for 1 1/2 hrs. This allows the capture of Nck--PAK-GST complex on the glutathione-Sepharose beads via the interaction between glutathione and GST as illustrated in Fig. 8. The procedure for recovering the complex is shown graphically in Fig. 9.

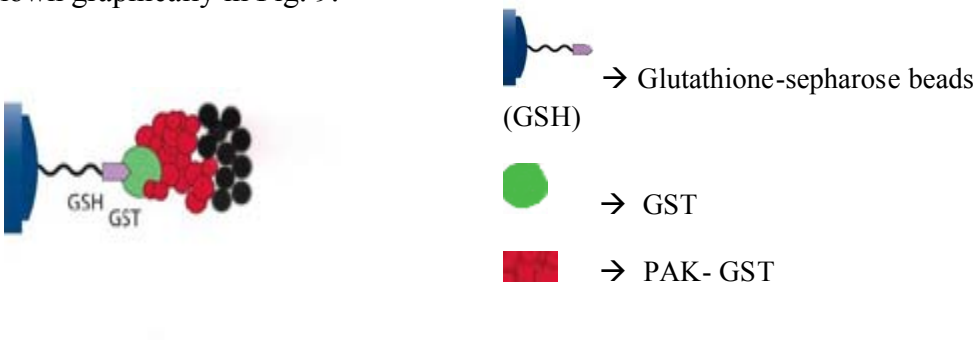

Fig. 8. Illustration of the capture of Nck---PAK-GST complex on glutathione-Sepharose beads.

M. The tubes were centrifuged at 2,000 rpm for 4 minutes.

N. The supernatant was replaced with 1X immunobuffer.

O. The tubes were once again centrifuged at 2,000 rpm for 4 minutes.

P. The supernatant was replaced with 100 µl of SDS disruption mixture (10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.01 mg/ml bromophenol blue, 5% β-mercaptoethanol).

Q. The samples were then boiled in water at 100° C for 5 minutes.

R. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). 12% polyacrylamide was used and the proteins were separated at 150 V for 1½ hours alongside the standard Precision Plus Protein™ Kaleidoscope™.

S. Following SDS-PAGE, the gel was washed with deionized water twice for 15 minutes and left for staining in Coomassie Blue R-250 solution (0.125g Coomassie blue, 37.5 ml glacial acetic acid, 95% ethanol 250 ml ddH_2O: make volume up to 500ml) overnight.

T. The stained gel was then washed repeatedly with the destaining solution (7% glacial acetic acid and 12% methanol in ddH_2O).

The effect of kinase activity on the capture of Nck-α on PAK-GST

Since autophosphorylation of PAK reportedly reversed the binding of Nck to PAK, an attempt was made to study the difference between the ability of Nck to interact with active PAK and inactive PAK. Steps A through G repeated in order to get the bacterial cell lysate with the overexpression of the Nck-α. Tubes were loaded with the Cdc42 activator, GTP, as shown in Table 3. Then, steps K through T from the capture of Nck-α on PAK-GST experiment were repeated.

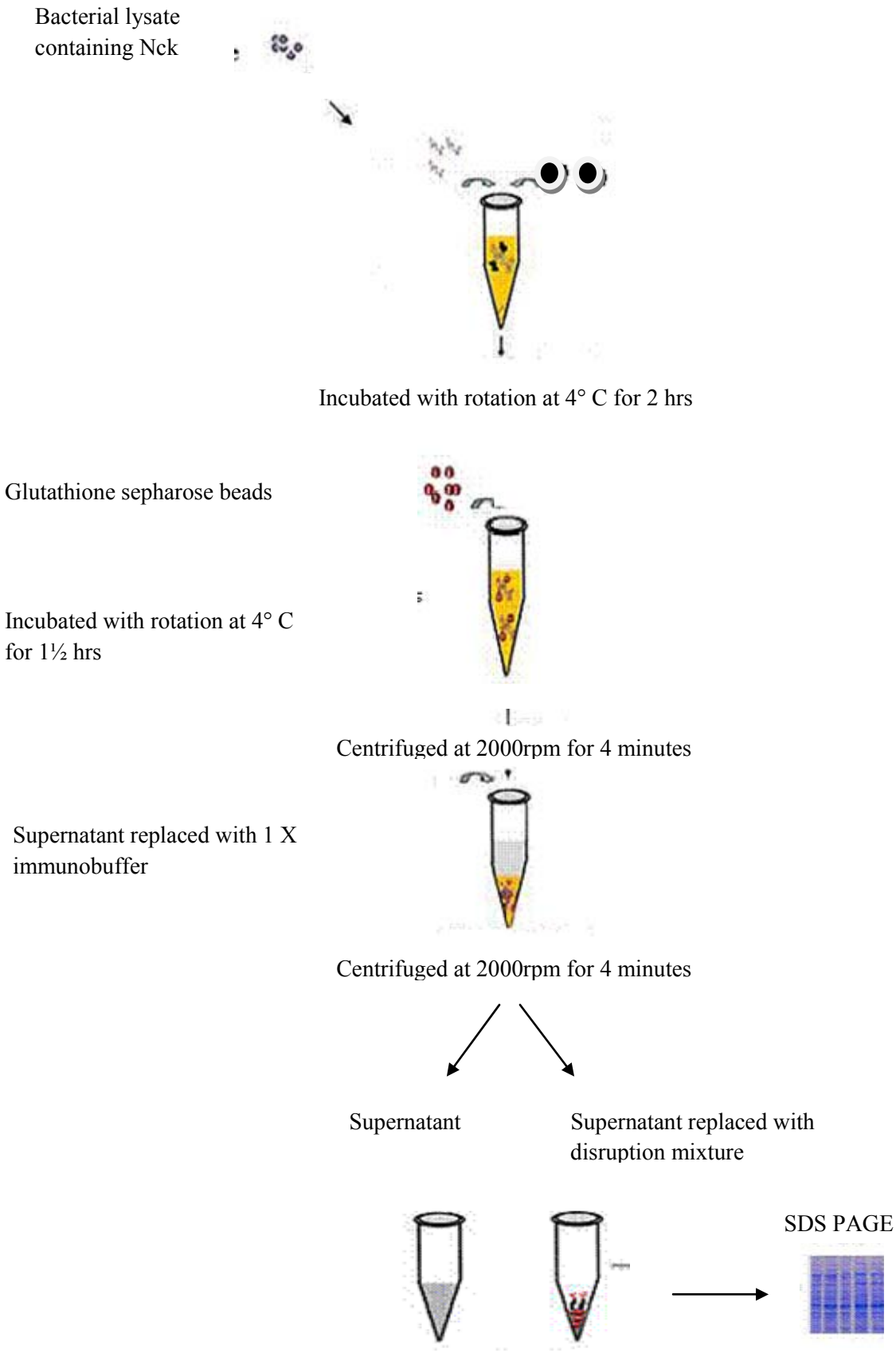

Fig 9. Illustration of Nck---PAK-GST complex formation

Table 3. Capture of Nck-α on PAK-GST while activating the kinase

Tube No.	Contents of the tube
Tube 1 (Experimental sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 400 µl of water + 0.1 µg PAK-GST/Cdc42 active + 3 µl of GTP (conc of GTP ~10mM-80 mM GTP)
Tube 2 (Experimental sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 400 µl of water + 0.1 µg PAK-GST/Cdc42 active + 3 µl of GTP (the fusion protein and the GTP was added into an Eppendorf tube and activated for an hour or so before exposing it to the bacterial cell lysate)
Tube 3 (Control sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 400 µl of water + 0.1 µg PAK-GST/Cdc42 active

Construction method and geometry of the nanodevice

The procedure was adapted to again capture Nck on PAK, but this time the particles were recovered after adsorption of the proteins separately on gold particles as opposed to glutathione-Sepharose beads. PAK was marked with 10 nm particles, and proteins of the bacterial lysate with 2 nm particles. The 2 nm particles were too small to be sedimented in the microfuge, so that only 2 nm gold in a complex could be captured in the procedure. A control sample was made by using BL21-AI cells untransformed with the Nck plasmid by following steps A through G. The supernatant obtained from Step G was passed through a 0.22 µm Millipore filter, and then tubes were loaded to perform the experiment as shown in Table 4.

Table 4. Capture of gold-adsorbed Nck on gold-adsorbed PAK-GST

Tube No.	Contents of the tube
Tube1 (Experimental sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 400 µl of water + 130 µl of 2 nm gold + 0.1 µg PAK-GST (already treated with 130 µl of 10 nm gold)
Tube 2 (Control sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (No Nck) + 400 µl of water + 130 µl of 2 nm gold + 0.1 µg PAK-GST (already treated with 130 µl of 10 nm gold)

The recombinant protein, PAK1-GST, was purchased from SignalChem**.** The protein was produced in insect cells and supplied at a concentration of 1 µg/10 µl in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25 mM dithiothreitol, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM PMSF, and 25% glycerol.

It was important to know the number of PAK1 molecules on the colloidal gold, because this would set an upper limit on the number of Nck molecules that could be recruited to the particle. The appearance of the pattern in the electron microscope must be interpreted in accordance with these estimates. To determine how many PAK1-GST molecules and gold particles were in the replicate tubes designated above, I did the following calculations. (UN stands for unknown.)

<u>How many molecules of PAK1-GST were in the tube?</u>

Constants:

Molecular weight of the protein PAK1-GST = 9.6×10^4 Daltons

Number of molecules/mole (Avagadro's number) = 6.023×10^{23}

Mass of protein added to tube = 1×10^{-7} g

thus, 1×10^{-7} g/UN1 moles = 9.6×10^{4} g/mole

1.04×10^{-12} moles = UN1

and UN2 = (1.04×10^{-12} moles) **x** (6.023×10^{23} molecules/mole)

UN2 = 6.27×10^{11} molecules

How many particles of gold were in the tube?

Molecular weight of gold = 196.97 g/mole

Diameter of gold spheres = 10 nm

Density of gold = 19.3 g/cc

Concentration of gold = 0.01% or 0.01 g/100 ml

for the 130 µl added to the tube this was 13 µg.

Mass = density x volume

thus, the mass of gold = 19.3 g/cc **x** volume of each sphere

Volume of the sphere = $4/3\pi R^3$ (where R is the radius of the sphere and $\pi = 3.14$)

$= 4/3\pi (5)^3 = 523.5$ nm^3 [1 nm^3= 10^{-21} cm^3]

$= 523 \times 10^{-21}$ cm^3

$= 523 \times 10^{-21}$ ml

Mass of each 10nm particle = density x volume

= 19.3 g/ml **x** 523×10^{-21} ml

= 1×10^{-17} g or 1×10^{-11} µg

thus, number of gold particles in 13 µg of gold = 1.3×10^{1} µg/1×10^{-11} µg

$= 1.3 \times 10^{12}$ particles

Here, only approximations could be derived for the experimental conditions, as the concentrations of the starting ingredients were not confirmed in our laboratory. The activity of the moieties could easily vary by two-fold or more. Although it appears that the gold beads slightly exceeded the PAK-GST molecules in number, the actual final concentrations in the tube could be the reverse. The tubes set up as shown in Table 4 were then processed as follows:

H. The tubes were incubated in the refrigerator for 2 hours with rotation.

I. The tubes were centrifuged at 14,000 rpm for 12 minutes.

J. The supernatant was replaced with 1X immunobuffer.

K. The tubes were once again centrifuged at 14,000 rpm for 12 minutes.

L. The supernatant was discarded and the pellet was resuspended in a drop of 0.02 M ammonium bicarbonate buffer, pH 7 (a volatile buffer is used to prevent the formation of crystals).

M. This was then transferred onto carbon-coated silicon monoxide grids.

N. The grids were observed under the TEM at magnifications of 100,000x or greater.

Control procedure for the origin of "extra" bands

An experiment was done to block the phosphotyrosine moiety of bacterial proteins. This was done as a control procedure to determine whether any bacterial proteins containing a phosphotyrosine moiety were binding to Nck via the Nck SH2 group, thus accounting for bands in the gel that were at other molecular mass ranges and might not be PAK1-GST, Cdc42, or Nck-α. The SH2 domain of the Nck-α could not bind proteins with high efficiency if they were adsorbed to the anti-phosphotyrosine antibody. Steps A through G were repeated in order to get the bacterial cell lysate with overexpression of Nck. A control sample was also made using

BL21-AI cells untransformed with the Nck plasmid. The supernatants obtained from Step G were passed through a 0.22 µm filter and 3 sample tubes were made and loaded as shown in Table 5. To visualize the proteins recovered by the procedure, steps K through T from Nck---PAK-GST complex formation experiment were repeated.

Table 5. Interfering with Nck-α recognition of bacterial proteins via the SH2 domain

Tube No.	Contents of the tube
Tube 1 (Experimental sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 400 µl of water + 2 µl of phosphotyrosine antibody (incubated for 2 hours) + 0.1 µg PAK-GST
Tube 2 (Control sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (Nck) + 402 µl of water + 0.1 µg PAK-GST
Tube 3 (Control sample)	500 µl of 2X immunobuffer + 170 µl of cell lysate (No Nck) + 400 µl of water + 2 µl of phosphotyrosine antibody (incubated for 2 hours) + 0.1 µg PAK-GST

RESULTS

Bacterial transformation, plasmid expansion and DNA purification of KID-GST and Nck-α plasmids.

When the BL21 (DE3) cells were transformed with the KID-GST and Nck-α plasmid, plated on LB ampicillin media and grown overnight at 37°C, there were numerous colonies on the KID-GST plates but no colonies on Nck-α plates. When the transformation process was tried again with another strain of cells, BL 21-AI cells, numerous colonies grew. These colonies were well rounded which is an indication of healthy transformants. Isolated colonies were picked up from these plates and inoculated into liquid LB media. The tubes showed good turbidity which is an indication of good growth of the transformed cells. Figs. 10 and 11 show the presence of the KID and the Nck-α DNAs, and they confirm that the size of the inserted gene matches the size expected.

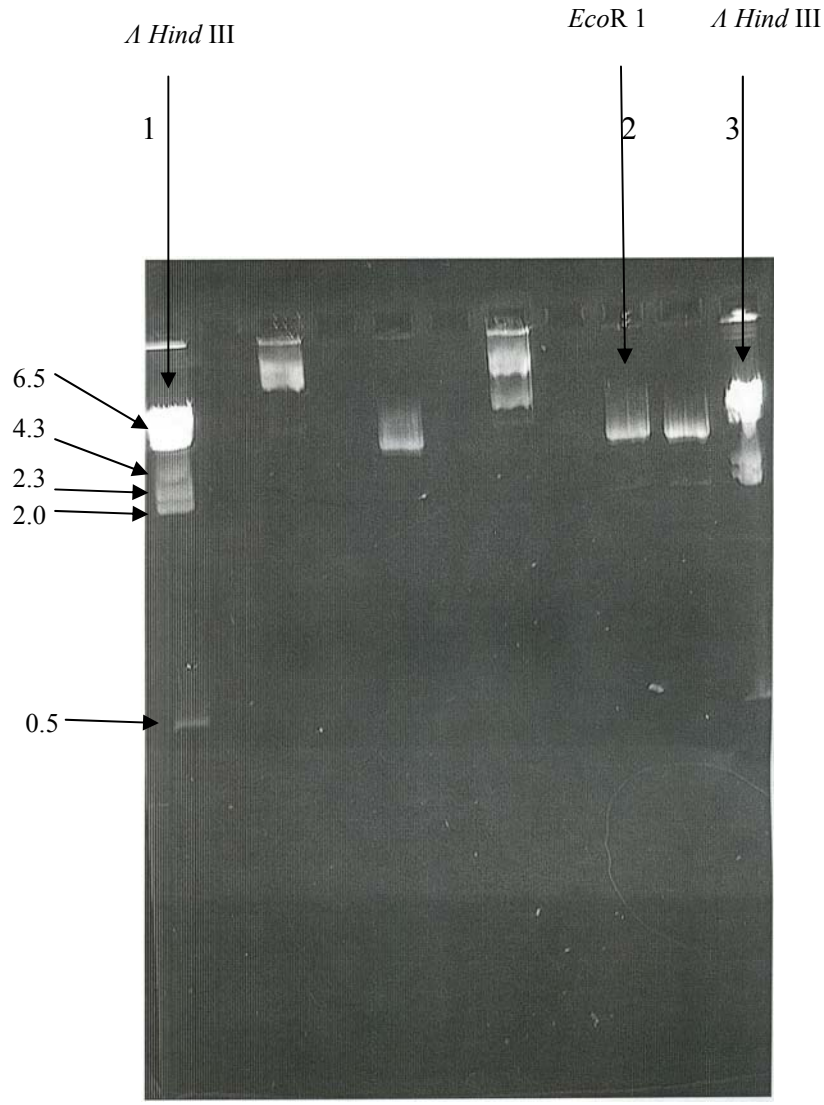

Fig. 10. Restriction digest of KID-GST plasmid by restriction enzyme *Eco*R 1, prepared by gel electrophoresis. Standard marker *λHind* III was loaded in lanes 1 and 3. Lane 2 shows the digest of KID-GST. The extra lanes show plasmids for other genetic constructs, which were restricted but were not of interest for this project.

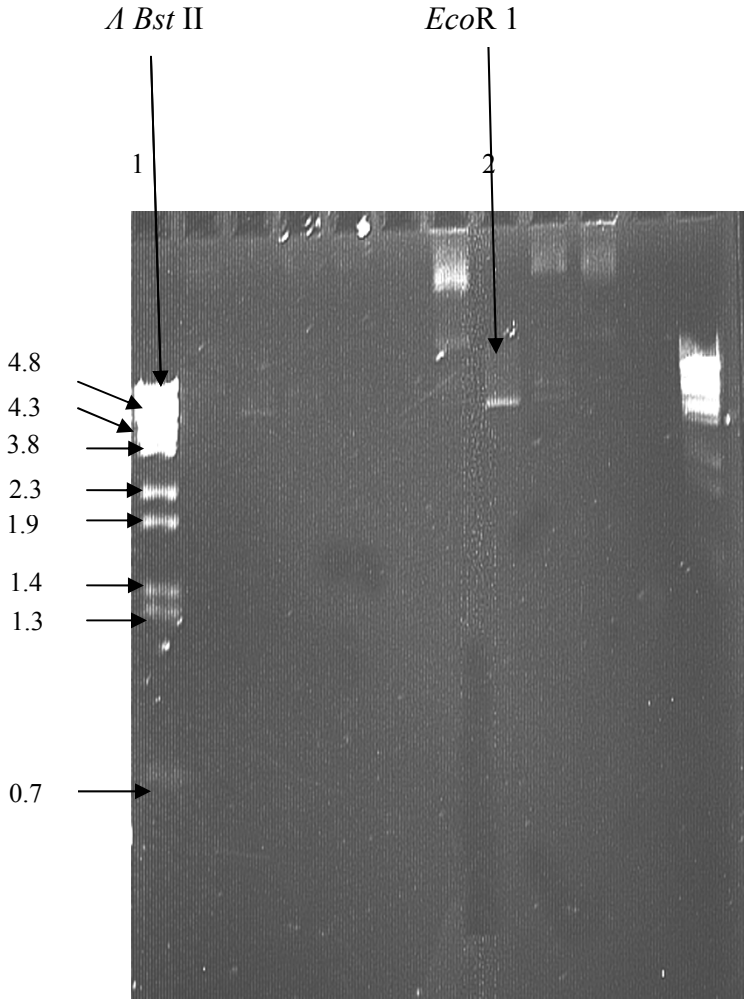

Fig. 11. Restriction digest of Nck-α plasmid by restriction enzyme *Eco*R 1, prepared by gel electrophoresis. Standard marker *λ Bst* II was loaded in lane 1. Lane 2 shows the restriction digest. The extra lanes show plasmids for other genetic constructs, which were restricted but were not of interest for this project.

Nck-α complex formation with PAK

The complex formed by Nck-α with PAK1 was recovered by a pull-down procedure similar to the immunoprecipitation procedure, as described in the Materials and Methods section. Bacterial lysate containing the overexpressed Nck-α was added to the PAK-GST preparation, and this complex was attached to glutathione-Sepharose beads via the interaction between GST and glutathione. When SDS-PAGE was run to confirm the presence of Nck, a band

corresponding to Nck-α was observed at 47 kD, and a band corresponding to PAK-GST was observed at 92 kD. Additional bands were observed at various molecular weights. There was a band of around 137 kD and a band of around 184 kD (Fig. 12). Although it can be speculated that these bands represent PAK-Nck complex and a PAK dimer respectively, the retention of any protein-protein was highly unlikely due to the use of a high concentration of SDS in the gel protocol. These proteins remain unknown. KID-GST failed to alter the bands observed. The band corresponding to KID-GST, which should be at ~33 kD, was not obvious on the gel (not shown), and so it was possible there was too little KID present to exert the desired effect.

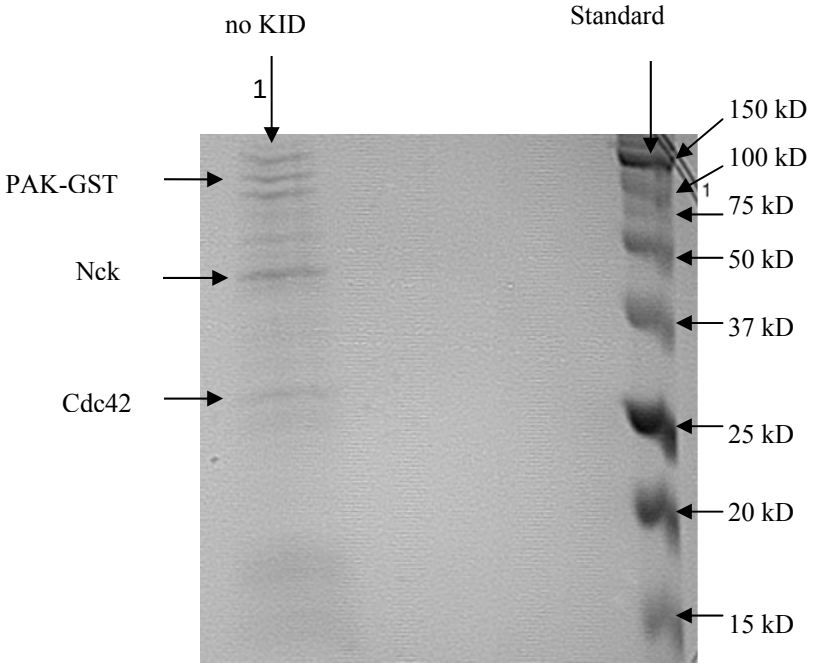

Fig. 12. SDS-PAGE demonstrating the presence of the overexpressed Nck-α (47kD) precipitated with PAK-GST. Lane on left shows PAK-GST at 92 kD and Nck-α at 47kD, as inferred from their molecular weights. In addition, a number of unidentified proteins may have been bacterial in origin. The protein around 25 kD is Cdc42. The lane on right shows protein standard markers.

Effect of kinase activity on the capture of Nck-α on PAK-GST

It is thought that, when GTP-loaded Cdc42 binds to the CRIB sequence, PAK1 converts from its autoinhibited, dimeric form into a dissociated, active form. To explore whether the

PAK1 in the commercial preparation was active, I made three samples. The first sample served as a control. It contained the overexpressed Nck-α pulled down on PAK-GST but without any GTP which should be expected to activate the Cdc42 GTPase (lane 1). The second sample contained Nck-α on PAK-GST which had been activated with GTP for an hour before it was combined with the bacterial lysate, and the third sample contained Nck-α pulled down on PAK-GST with GTP added only a few minutes before it was combined with bacterial lysate. These attempts to activate the kinase had little effect on Nck---PAK-GST complex (Fig. 13). Since the manufacturer stated that there was some active kinase in the preparation, even without the addition of GTP prior to use, it can be assumed that the PAK-GST already had some active molecules. These were bound to active Cdc42 and were not further activated by GTP addition. One possible interpretation of this observation was that the active and inactive PAK molecules are both capable of capturing Nck in the pull down procedure.

Control procedure for the origin of extra bands

In an attempt to establish some identity to the extra proteins found repeatedly associated with the Nck----PAK-GST capture gel, 3 samples were made: one with Nck expression and anti-phosphotyrosine antibodies loaded in lane 1, the other with Nck expression but without the anti-phosphotyrosine antibodies loaded in lane 2 and the last one with no Nck expression which was loaded in lane 3. There was no significant difference between the first two samples as shown in Fig. 14, indicating the lack of interaction between the SH2 domain of Nck-α and the bacterial proteins. Insufficient amount of PAK-GST may have been added to the sample loaded in lane 3 which explains the absence of any bands. However, this clearly indicates that the bacterial proteins were getting pulled down on either Nck-α or PAK-GST.

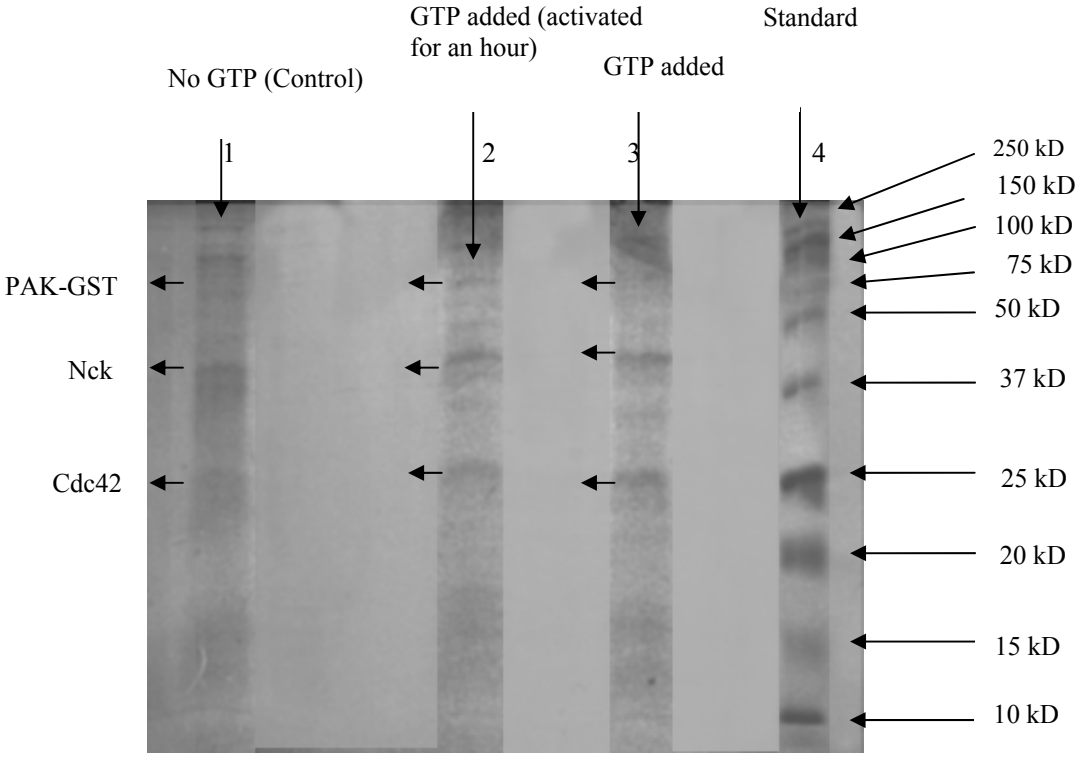

Fig. 13. SDS-PAGE demonstrating the effect of kinase activity on the capture of Nck-α on PAK-GST. The 1st lane shows the presence of the overexpressed Nck-α and PAK-GST in the absence of GTP. This served as a control for the experiment. Lanes 2 and 3 show the capture of Nck-α by PAK-GST in the presence of GTP. PAK-GST present in lane 2 is activated for an hour with GTP before bacterial lysate containing the Nck-α is added. Nck-α in lane 3 is added to the PAK-GST immediately after the addition of GTP. Bands corresponding to Nck-α (47kD) can be found in all the three samples.

Proposed construction of nanodevices

The above results suggested that, if the PAK-GST were attached to a sizeable gold particle, and the Nck-α to a smaller particle, the two sizes of particles would be sedimented in tandem because of Nck-PAK binding. Results were obtained by TEM in order to study the assembly of 2 nm gold particles attached to Nck-α molecules on 10 nm gold particles attached to PAK-GST. These images were taken at magnifications greater than 125,000x. Study of these images showed that frequently there were two to three 2 nm gold particles surrounding a single 10 nm particle. Occasionally, it was also observed that a single 10 nm particle was surrounded

by a halo of 2 nm gold particles. These results are demonstrated in the images below (Figs. 15-16).

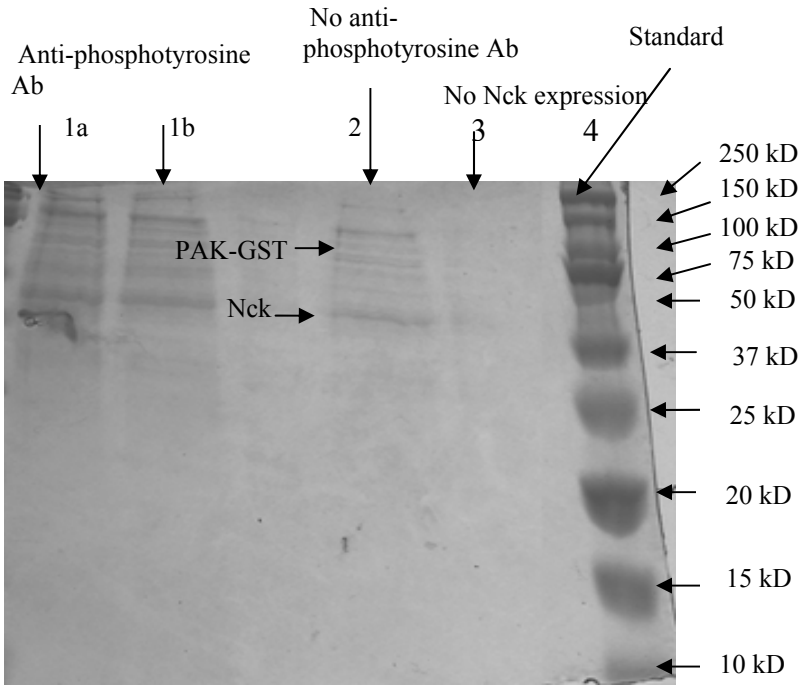

Fig. 14. SDS-PAGE demonstrating the effect of blocking the ligand to which Nck-α SH2 domain would bind, by treatment with anti-phosphotyrosine antibody (Ab). Lanes 1a and 1b wells are duplicates showing the presence of the overexpressed Nck-α and PAK-GST in the presence of the anti-phosphotyrosine antibody. Lane 2 shows the capture of Nck-α by PAK-GST in the absence of anti-phosphotyrosine antibody. The sample loaded in lane 3 failed to pick up Nck-α because insufficient PAK-GST was added to the sample while performing the pull down experiment. Kaleidoscope^{TM} standard marker proteins are shown in well 4.

After careful analysis of several images, it was observed that edges of the gold nanoparticles adsorbed on proteins were not very sharp as opposed to just the naked gold nanoparticles shown in Fig. 17. This may be attributed to a probable cloud of proteins settling around the gold nanoparticles which leads to the scattering of electrons and subsequently loss of sharpness around the edges.

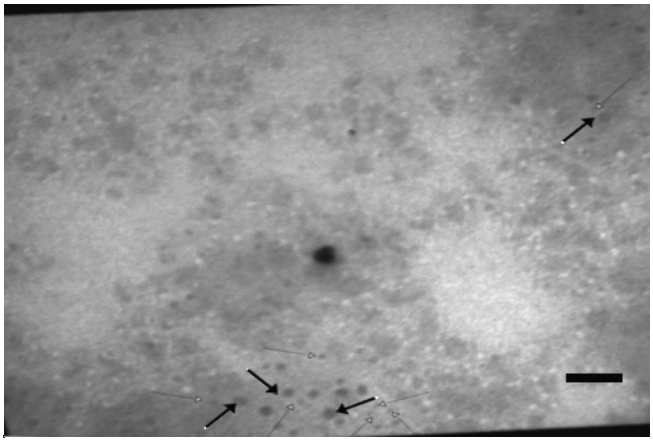

Fig. 15. Electron micrograph demonstrating the arrangement of 2 nm gold particles bound to Nck-α molecules around 10 nm gold particles bound to PAK-GST molecules. In this image, it appears that each 10 nm gold particle (thick arrows) is bound to a single 2 nm gold particle or exists in isolation (thin arrows). The dense black central spot and smaller, uniform spot above it are artifacts from burning in the phosphorescent screen. Bar = 0.04 μ

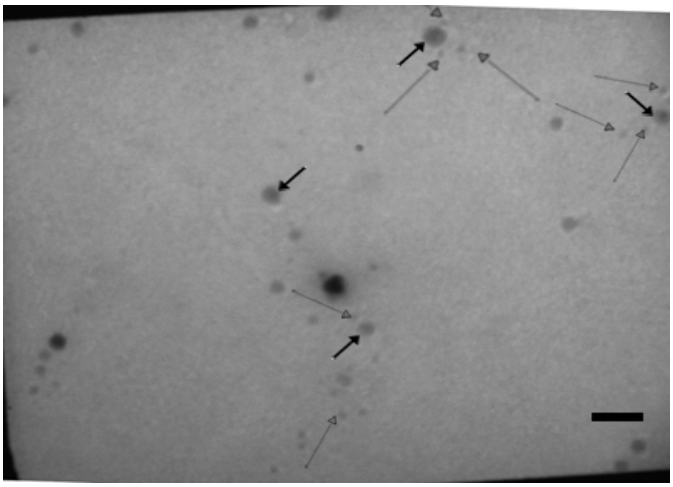

Fig. 16. Electron micrograph demonstrating the arrangement of 2 nm gold particles bound to Nck-α molecules around 10 nm gold particles bound to PAK-GST molecules. In this image, it appears that each 10 nm gold particle (thick arrows) is bound to from one up to four 2 nm gold particles (thin arrows) and there are also a few free 10 nm gold particles. The dense black central spot and smaller, uniform spot above it are artifacts from burning in the phosphorescent screen. Bar = 0.04μ

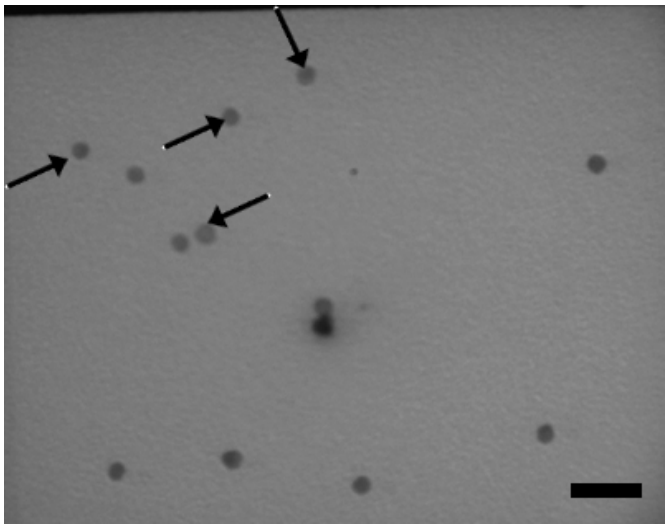

Fig. 17. Electron micrograph of 10 nm gold particles. The dense black central spot and smaller, uniform spot above it are artifacts from burning in the phosphorescent screen. Bar= 0.03μ

DISCUSSION

An effective strategy to study the assembly of proteins present in the FCs is by employing techniques used in bionanotechnology. The chief objective of this project was to demonstrate the feasibility of studying protein-protein assembly on scaffolds by establishing conditions to pull down the Nck-α from the bacterial lysate on PAK1. The second objective was to observe the assembly of Nck-α and PAK1 directly under TEM by applying different sizes of gold to each of these two proteins.

Discussion of Nck-α complex formation with PAK1

It was observed that when Nck-α was overexpressed in bacterial cells and then treated with PAK-GST, it was possible to pull out Nck-α from the bacterial lysate. The introduction of KID-GST to this interaction seemed to have no apparent effect. It has been previously observed by Chernoff et al., (2007) that the expression of KID inhibits the kinase activity of PAK without actually creating any hindrance to the loading of GTP-Cdc42 onto PAK. The interpretation of results obtained from SDS-PAGE (Fig. 10) was equivocal, because the yield of the protein KID-GST was low as observed from the low intensity of the band at 33kD.

The gel also displayed the presence of some extra bands that did not correspond to any of the expected proteins in the preparation. The procedure is analogous to immunoprecipitation whereby the cell lysate is treated with the primary antibody. In this common "pull down" technique, the lysate is treated instead with PAK-GST. The present result is surprising, because only the capture of the protein of interest was anticipated but I observed several bands on the gel. The purification process is apparently not 100% specific. There are around 4500 proteins

produced by *E. coli*. It is perhaps not surprising given the sticky nature of Nck-α, being an adaptor protein, and the number of proteins in the bacterial lysate, if Nck-α were capable of interacting with some of these proteins and they are subsequently pulled down on PAK-GST molecules. Attempts to rule out the possibility is that bacterial proteins can be interacting with PAK1 molecules were thwarted, because of the lack of a reliable source of high-quality PAK-GST. Currently, the nature of the proteins remains a mystery.

Discussion of effect of kinase activity on the capture of Nck-α on PAK-GST

GTP activates Cdc42 and presumably the loading of GTP-Cdc42 activates the kinase. After analysis of the gel (Fig. 13), it was observed that attempt to activate the kinase by supplying GTP did not have any significant effect on the capture of Nck-α by PAK1. This could be consistent with two possibilities. There was no GEF added which could load GTP onto the GTPase. Perhaps, because spontaneous GTP loading on the GTPase was extremely slow, it could be that the GTPase did not get activated and hence there was no difference between the sample containing GTP and the sample without GTP. This might merely indicate that GDP bound Cdc42 is able to remain on the PAK1. This seems unlikely, as a personal communication with the manufacturer indicated that GTP was loaded on the GTPase. However, this possibility cannot be ruled out. On the other hand, another personal communication with the manufacturer indicated that there are always some PAK1 molecules present that are active even in the absence of added GTP. If we imagine, that there are around 100 molecules of PAK1 in the tube, then an arbitrary fraction, e.g. 10 molecules, could be active and so even if the GTP induced further PAK1 activation, the increment in active molecules may not be detectable by TEM. In their studies of Nck and PAK, Zhao et al. (2000), in reported that kinase autophosphorylation blocks

binding by Nck. They show that recruitment of GTP-Cdc42 dissociates the Nck-PAK complex within the cells. On the other hand, Vadlamudi et al. (2002) reported that when the GTPase complexes with PAK, it causes autophosphorylation of the kinase leading to decreased affinity for the GTPase and release of the GTPase. In my results, the addition of GTP in an attempt to activate the PAK did not seem to have any effect on PAK-Nck interaction. Nck still seems to interact with PAK irrespective of the activity state of PAK. A possible mechanism to explain this result would be that the GTP bound Cdc42 activates the PAK and its association with Nck is hindered. But the activated PAK now displays a decreased affinity for the GTPase and releases it. PAK now returns to its inactive state and picks up Nck. Colocalization of PAK and Cdc42 by immunological techniques might be one approach to addressing this problem.

Discussion on the control procedure for the origin of extra bands

In addition to the observation of bands corresponding to the expected proteins, other bands were repeatedly observed on the gels with the capture experiments. In this experiment it was speculated that if the tyrosine residues in some of these bacterial proteins were phosphorylated then the anti-phosphotyrosine antibody would complex with the phosphotyrosine moieties making them unavailable to the SH2 domain of Nck-α. After analysis of the bands obtained from the gel, it can be inferred that the bacterial proteins being pulled down along with Nck-α or PAK-GST did not associate with them via the SH2 domains.

Discussion on the proposed construction of nanodevices

The study of gold-adsorbed Nck-α captured on gold-adsorbed PAK1 by TEM demonstrated some interesting patterns. After analyzing several images, it was observed that 10

nm gold particles adsorbed on PAK-GST regularly associated with either a single, double, or triad of 2-nm gold particles adsorbed on Nck-α and sometimes even a halo of 2-nm particles was observed around the bigger particles. It can be speculated from the images collected that probably two to three PAK molecules associate with a single 10-nm particle and each of the PAK molecules then associate with either a single Nck-α molecule adsorbed on a 2-nm particle, as illustrated in Fig. 17. This speculation can explain as to why we sometimes observe a halo of 2-nm nanoparticles around 10-nm nanoparticles as illustrated in Fig. 16.

Through this experiment, conditions for the construction of nanodevices using proteins have been established. By following this method, in principle an even more extensive construction is possible by exploiting the interaction of proteins in the FCs.

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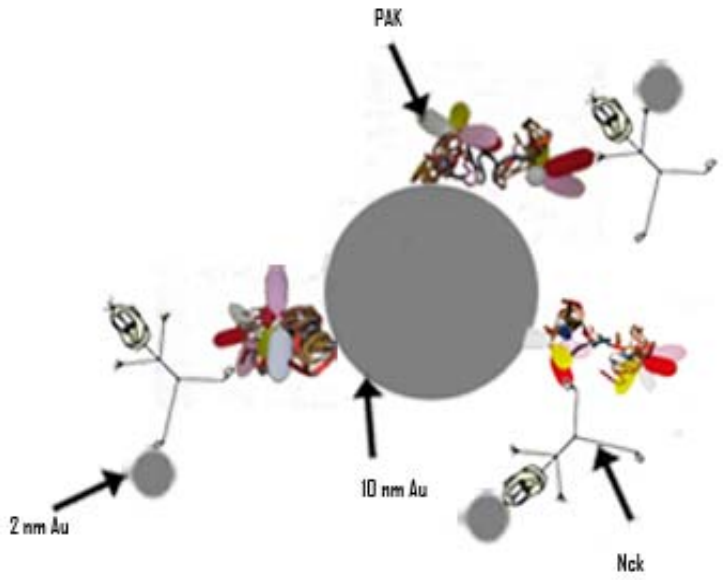

Fig. 18. Graphic speculation showing the arrangement of proteins around the gold particles when there is excess of PAK proteins.

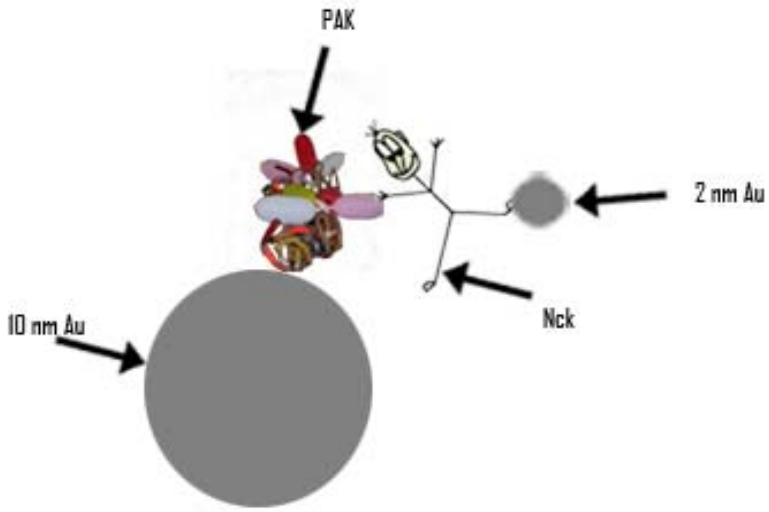

Fig. 19. Graphic speculation showing the arrangement of proteins around the gold particles when the ratio of protein to nanoparticles is 1:1.

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