SHAPE AND QUANTITATIVE ANALYSIS OF FACTOR #4 (FILOPODIA) AND FACTOR #7 (MASSIVE PROTRUSIONS) IN TUMORIGENIC CELLS

Santosh Malwade

A Thesis

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Committee:

Dr. Carol A. Heckman, Advisor

Dr. Donald W. Deters

Dr. Zhaohui Xu
ABSTRACT

Carol A. Heckman, Advisor

After activation by guanine triphosphatases (GTPases) like Rac and cell division control protein (Cdc42), the p21-activated kinase (PAK) unfolds and binds tightly with SH3 domain of partner PAK-interacting exchange factor (PIX). PIX binds to G protein coupled receptor kinase interacting protein (GIT1) which binds to paxillin. Paxillin binds to focal adhesion kinase (FAK). The N-terminal region of PAK binds with Nck-α through its SH3 domain and Nck-α binds to FAK. This chain of five proteins is located at the focal contact site and believed to be important for production of filopodia and membrane protrusion. At the regulatory level, the mechanism is not known but could be clarified by further work.

I studied the role of PAK in the formation of these two protrusions, by supplying PAK kinase inhibitor, PAK$^{83-149}$, in the presence or absence of Nck-α. These two constructs were found to have an effect statistically distinguishable from control in previous experiments. Filopodia and larger membrane protrusions were affected differently by Nck-α alone and PAK$^{83-149}$ alone. The large protrusions were more prevalent with Nck-α alone and unaffected by PAK$^{83-149}$. Expression of both constructs together was required to affect the filopodia. When the cells were treated with tyrosine phosphatase inhibitor, phenylarsine oxide (PAO), the results of Nck-α and PAK$^{83-149}$ expression were different. PAK$^{83-149}$ now showed a significant effect on massive protrusion formation which was statistically distinguishable from all other groups. None
of the treatments any longer promoted filopodia formation. Surprisingly the effect of Nck-α in all treated samples was removed by PAO. Maybe the Nck-α alone affects the protrusions by binding PAK molecules to sites on receptors such as phosphorylated focal adhesion kinase (FAK), or transmembrane growth factor receptors. The reversal of this effect by PAO is a novel effect.
I dedicate this work to my parents and advisor.
ACKNOWLEDGEMENTS

I feel very lucky for being able to work with Dr. Heckman. I have learned many precious and wonderful things from my advisor as well as lab mates. I would sincerely like to thank Dr. Heckman for her support, patience and guidance throughout my master’s degree. I also would like to thank Dr. Donald Deters for his precious help in my experiments. It was a nice experience working with him in his lab. I am thankful to Dr. Zhaohui Xu for being my committee member. The help by Dr. Cayer in electron microscopy was valuable in all of my projects and I am thankful to her. Thanks to the Department of Biological Sciences and the Graduate College for their financial support throughout my education. Last but not the least; I am thankful to my wonderful lab mates Surya, Mita, Manisha and John, for their support and friendly help.
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INTRODUCTION

Using high-resolution imaging coupled with computerized shape analysis, this laboratory has found that a large number of cell features can be discriminated mathematically. Some of the cell edge features are represented mathematically by factor #7 (massive protrusions, MPs) and factor #4 (filopodia). We have also found that overexpression of the PAK-PIX binding domain, which prevents PAK binding to focal contact sites, prevents expression of these edge features [12, 15]. The experiment, in which the PAK kinase inhibitory domain (KID) was overexpressed, showed that the focal complexes at the cell edge were enlarged, and KID positively affected the cells’ ability to form or stabilize the larger protrusions [15]. PAK localization was critical to the dissolution of the focal contacts [27], but the question remained whether the arrangement of the dissolved sites in turn might dictate sites of protrusion formation. The adaptor, Nck-α, has been implicated in PAK targeting in experiments where axon path-finding mechanisms have been studied by genetic methods. The results from the developmental genetic studies fail to indicate how PAK and Nck-α interact to guide an axon to its destination.

The current experiments could confirm quantitatively and qualitatively whether Nck-α-PAK interaction played a role in PAK targeting to either the factor #4 or factor #7 features. In our laboratory, I could study the effect of PAK and Nck-α overexpression on these morphological features. The work of previous authors suggests that Nck-α binds to PAK and attaches it to the membrane [20]. The Nck-α in turn could be targeted to the phosphorylated tail domains of tyrosine kinase receptors, through its Src
homology 2 (SH2) domain. Therefore, PAK could be targeted to focal contacts in the region of a nascent protrusion. Because the major role known for PAK is to dissolve focal contacts, this hypothesis made it difficult to rationalize the effect of KID. On the other hand the focal contacts may serve to keep the cell body stationary.

**PAKs**

PAK1, the first of the PAK family members to be discovered, was originally cloned from brain tissue [19]. It is homologous to yeast Ste20. Five other PAK family members were identified and investigators showed that this family of proteins is highly conserved in mammalian cells. PAKs are serine/threonine protein kinases that bind and are activated by small GTPases, Rac and Cdc42 [19]. The PAKs are classified into two groups which contain members PAKα, PAKβ, PAKγ and PAK4, PAK5, PAK6 respectively. The members share significant homology at the amino acid level, although the biological function of each member is distinct [3]. Group I PAKs are composed of an amino-terminal regulatory domain containing a Cdc42/Rac p21-binding domain (PBD) and several Src homology 3 (SH3) binding motifs (Fig. 1). One of these mediates binding to a guanine nucleotide exchange factor called PAK-interacting exchange factor (PIX) and another mediates binding to Src homology 3 (SH3) - SH2 adaptor Nck-α [2, 32]. The carboxyl terminus of both group I and II PAKs contains the kinase domain. PAK is thought to be activated by PIX. This interaction, either directly or through the activation of Rac and subsequent binding of Rac-GTP to the PBD, releases an
intermolecular interaction between the kinase domain and the KID which enables unfolding of the kinase [32]. PIX is bound in turn to GIT1 [1].

![Diagram of PAK domains](image)

**Fig. 1:** Positions of PAK domains. The N terminus (residues 1-69) was identified as interacting with Nck. The autoregulatory regions (residues 70-149) contain the dimerization segment (Di), the PBD, and the KID. The Inhibitory Switch (IS) is defined as a region containing the PBD and part of the KID. The C terminus contains the kinase domain and the Gβγ-binding motif. [29]. The inhibitory switch used in the current work comprises amino acids 83-149.

Since overexpression of PAK had little effect on actin-based features unless a dominant positive Rac or Cdc42 GTPase was also overexpressed, PAK activity in the cell may be regulated by availability of activated GTPases in different locations [14]. As PAK limits accretion of components to focal complexes by its kinase activity and is particularly active in turning over Rho-type focal adhesion plaques [20, 32], it may play a pivotal role in dictating how a protrusion is developed. In this research, I classify and quantify various protrusions to determine the role played by PAK interaction with Nck-
α in forming two different types of protrusions. PAK1 serves as an effector for Cdc42 and/or Rac1 in promoting cell motility. It was originally found to regulate the actin cytoskeleton in mammalian cells by acting through a protein that contains an SH3 domain. This protein, Nck-α, was essential for the localization of PAK to polarized ruffling regions [25]. Nck-α contains three SH3 domains [17]. PAK kinase mediated autophosphorylation blocks binding of PIX to PAKα, thus providing a mechanism to regulate PAK interactions with the focal contact site. One cellular consequence of the GTPase regulatable binding of PAK is facilitation of its cycling between cytosolic and focal complex sites [32]. With the exception of vimentin [10], cortactin [28], and myosin light chain kinase [9], the main substrate of PAK appears to be PAK itself.

From previous studies it has been proven that the Rho family GTPases, Cdc42, Rac and Rho, regulate the reorganization of actin cytoskeleton induced by growth factors. PAK was isolated by virtue of its ability to bind to activated Cdc42 and Rac1. It is implicated in cell shape changes induced by growth factors. Previous studies also found that microinjection of activated PAK1 protein into quiescent Swiss 3T3 cells induced the rapid formation of polarized filopodia and membrane ruffles. Thus PAK1 may serve as an effector of Cdc42 and/or Rac1 in promoting cell motility [26]. Other studies showed that overexpression of βPAK in PC12 cells induced a Rac phenotype, such as cell spreading/membrane ruffling and increased lamellipodia at growth cones and the shafts of nerve growth factor-induced neurites [22]. Furthermore, these effects are observed in cells which express kinase negative or Rac/Cdc42 binding-deficient PAK mutants which clearly indicates that kinase and p21-binding domains are not essential for cytoskeletal
effects. Lamellipodia formation in all cells including those expressing Rac binding-deficient PAK, was also found to be inhibited by dominant-negative RacN17 [22]. During the same studies, equal inhibition was achieved by blocking PAK interaction with PIX using the N-terminal PAK-PIX binding fragment. These studies show that PAK through its N-terminal acts upstream of Rac and mediates lamellipodia formation through interaction with PIX. Also another study showed that PIX, which is widely expressed and enriched in Cdc42-and Rac1-driven focal complexes, is required for PAK recruitment to these sites [21]. The same claim has been made for PAK-Nck-α interaction mediating lamellipodia formation. When the Nck-α binding portion of PAK is mutated, cells appear to lack the leading edge formed by overexpression of other PAK mutants [25].

**Nck-α**

Nck-α plays an important role in coupling activated receptors, especially tyrosine kinase receptors, to various signaling pathways [2]. The adaptor Nck-α is rich in potential phosphorylation sites and becomes phosphorylated on serine, threonine, and tyrosine residues in response to activation of a number of growth factor receptors including those for epidermal growth factor, nerve growth factor and platelet derived growth factor [2]. It was shown that Nck-α binds to PAK through its second SH3 domain, while PAK interacts with Nck-α via the first proline-rich SH3 binding motif at its amino terminus. These interactions of active PAK with Nck-α lead to phosphorylation of Nck-α at multiple sites [2]. The interaction between PAK and Nck-α had been strengthened upon platelet derived growth factor receptor stimulation due to
phosphorylation of Nck-α at multiple sites [2]. Since previous results failed to indicate how PAK was targeted to focal contacts by Nck-α, I made up a model of this interaction which is shown in Fig. 2.
Fig. 2: A) Nck-α implicated in stabilizing focal contact. PAK recruitment by Nck-α allows PAK to remain bound to the paxillin scaffold even when the PIX-GIT1 complex was missing.

B) PAK could be activated by one of two possible mechanisms. (Left) GTPases binding to the PBD causes unfolding and allows PIX to bind. (Right) GIT1 binding activates PAK activity in the absence of GTPase binding [18].
Phenylarsine oxide

Phenylarsine oxide (PAO) is a tyrosine phosphatase inhibitor, which does not affect tyrosine kinase activity [7]. It has molecular formula C₆H₅AsO and molecular weight 168. PAO inhibits internalization of cell surface receptors in studies on mammalian brain [4]. PAO cross-links vicinal thiol groups, hence inactivating phosphatases possessing XCysXXCysX motifs. RhoA GTPase, but not Rac1, possesses vicinal cysteines within the guanine-nucleotide-binding region and the phosphohydrolase active site. Treatment of Caco-2 cells with PAO caused a dose dependent reorganization of actin cytoskeleton, which was attributed to involvement of Rho GTPases [8]. It has been shown that PAO inhibits the strengthening of cytoskeletal linkages which indicates the role for dephosphorylation [5]. This mechanism was used to study the dependence of integrin-cytoskeleton linkages on matrix rigidity and its biochemical composition therefore indicating its role in fibroblast mechanotaxis [5].

It was found that the activation of the thiol protease results in lysis of proteins associated with focal adhesions severing cytoskeleton-integrin links [24]. Ironically, another tyrosine phosphatase inhibitor, calpeptin, induces assembly of stress fibers and was inhibited by botulinum toxin C3, indicating that calpeptin is acting on a phosphatase upstream of small GTPase Rho, a protein that controls stress fiber and focal adhesion assembly [24]. It is known that tyrosine phosphorylation regulates the formation of focal
Tyrosine phosphorylation and FAK are also involved in the cytoskeletal changes induced by serum or lysophosphatidic acid (LPA) in quiescent Swiss 3T3 fibroblasts [6]. Tyrosine phosphatase activity in Swiss 3T3 cells was markedly increased after actin stress fibers were disassembled by cell detachment from the substratum, by serum starvation [23]. This activity was blocked by PAO. Thus treatment of serum starved cells with PAO induced increased tyrosine phosphorylation of p125FAK and paxillin, and induced assembly of focal adhesions and actin fibers. This suggests that, inhibition of one or more PAO sensitive tyrosine phosphatases is enough stimulus for triggering focal adhesion and stress fiber formation in adherent cells [23].

PAO might be inhibiting a phosphatase and thereby affecting a major focal contact protein such as FAK, or even be activating actin stress fibers by inhibition of a phosphatase upstream of RhoA, as mentioned above. If PAO worked through the latter mechanism, it would be expected to have the same effect as the activation of RhoA. Since the effects of RhoA could be detected, these alternative mechanisms could be distinguished as shown in Fig. 3.
Fig. 3: Relationship between RhoA and the band of active cytoplasm. When the cells were treated with ACK, Nck-α and dnRho they showed ample production of factor #7 features. As suggested by the diagram, the production of factor #7 was dependent upon RhoA inhibition by dnRhoA.

**Computer solutions**

Factor #7 is a bulky projection on the cell edge. It is also called a massive protrusion and is distinct from a sharp, tapering feature called filopodia or factor #4 [12]. Factor analysis is the mathematical method used to extract theoretical variables from a set of 102 discrete variables representing the entire shape of the cell’s peripheral cytoplasm [14]. It was found that factor #4 values decreased in cancer-type cells, but factor #7 values increased [14]. The primary variables are used to compute these factor values and can be loaded on a factor by adding to or subtracting from that factor’s value. There are a number of primary variables that are loaded on factors #4 and #7 in opposite directions.
including those measuring the number of negative curvature regions (NONC), the fraction of the perimeter in negative curvature (FRNC), and the width of the projections from the cell at the base of the projection (WDTH).
MATERIALS AND METHODS

DNA stock expansion and agarose gel electrophoresis

The first phase was to expand the stock of Nck-α and KID plasmids, which were a gift from Sohail Ahmed and Edward Manser respectively. The KID plasmid was engineered in pXJ-40 vector containing CMV (Cytomegalovirus) enhancer and promoter sequences and Nck-α in pGEX. The enhancer-promoter unit drives strong expression of the inserted gene sequences. The size of the DNA was determined using the standard marker DNAs like λ Hind III and λ Bst II. The pXJ40 eukaryotic expression vector was generated by Xiao [30]. The pXJ40 vector is composed of fragments from plasmids pSG5 and pCMVcat. The pSG5 vector was constructed by Green [11]. It combines the eukaryotic expression vector pKCR2 and the high copy plasmid vector Bluescribe M13+ (Stratagene). The pSG5 vector contains the early SV40 promoter sequence, the rabbit β-globin gene intron II, the T7 promoter, the SV40 polyadenylation sequence and the Bluescribe M13+ (BSM13+) sequence, which includes the ampicillin resistance gene and a bacterial origin of replication. The structural elements of this vector are-

1. The hCMV enhancer promoter unit, which drives very strong expression of the inserted gene sequence
2. The rabbit β-globin intron II which facilitates splicing of the expressed transcript
3. The T7 bacterial promoter which enables in vitro transcription of cloned insert
4. A multiple cloning site (MCS) which enables easy insertion of gene sequences
5. The SV40 polyadenylation which greatly increases the level of protein expression by stabilizing protein synthesis

6. BSM13+ sequence which includes a bacterial origin of replication and the ampicillin resistance gene

7. The N-terminal HA or GFP tag enables easy detection of expressed proteins with commercially available HA and GFP antibodies

For expansion of Nck-α and KID plasmids and bacterial transformation, the BL-21 cells (DE3), strain of Escherichia coli was used. I used BL-21 competent cells from Invitrogen Life Technologies. To transform the cells with plasmids, Nck-α and KID, aliquots of cells were taken in four microcentrifuge tubes, 50 µl in each tube. Then 1 µl of KID was added in first tube and 1 µl of Nck-α in second one, so the first and second were the experimental tubes. As a negative control instead of plasmid, 1 µl of water was used and as a positive control 1 µl of pUC 19 plasmid was used, so the third and fourth tubes were negative and positive controls respectively. Then all 4 microcentrifuge tubes were kept on ice (4°C) for 30 minutes and DNAs were disinfected with 80% ethanol. After this, the tubes were kept in a water bath at 42°C for 1 minute. At 42°C they start self-preserving process. Again the tubes were kept on ice (4°C) for 2 minutes. Then 500 µl of SOC (super optimal broth with catabolite repression) media was added in each tube. Putting nutrients improves chances of the cell survival. Then all tubes were kept at 37°C for an hour. The chances of getting them transformed are 1 in 1000. Then those transformed cells were plated on Luria-Bertani (LB) medium and incubated at 37°C.
DNA isolation protocol was done with a QIAGEN Plasmid Midi Kit 25 (contains 25 tips). The exchange chromatography technique was used for DNA extraction and purification according to QIAGEN protocol as follows-

1. The transformed cells were spun down at 6000 rpm (revolutions per minute). The supernatant was discarded and the cells at the bottom were put into Corex tubes.
2. RNase A and Lyse blue were added to Buffer-1. RNase disrupts the RNA in the cells, so that it can be discarded.
3. 8 ml of Buffer-1 was added to each tube containing the transformed cells with Nck-α and KID plasmids.
4. Then add 8 ml of Buffer-2 in each tube. Buffer-2 has NaOH and sodium dodecylsulfate (SDS) in it which helps in lysing the cell membranes. Shake the tubes but gently so that DNA does not disrupt.
5. Finally 8 ml of Buffer-3 was added to the tubes. Buffer-3 has potassium acetate at pH 5.5; it neutralizes the NaOH of Buffer-2. At this stage the denatured proteins are precipitated, and care is taken not to vortex the tubes, which might disturb precipitated protein. Tubes were put on the ice (4°C) for 15 minutes.
6. Then tubes were rotated at 12000 rpm for 15 minutes.
7. Then 4 syringes were taken from the QIAGEN kit and 4 ml of Buffer QBT (Equilibration Buffer) was added in each syringe.
8. Supernatant was transferred to tubes, one for Nck-α and other for KID. Then supernatant from tubes were poured into columns.
9. Then 10 ml of QC Buffer (Wash Buffer) was added in each Nck-α and KID columns. Again 10 ml of QC Buffer was added to wash the column.

10. Then 5 ml of QF Buffer (Elution Buffer) was added and columns immediately transferred to empty Corex tubes.

11. When 3.5 ml of propanol was added to the tubes containing Nck-α and KID plasmids, the DNA will be precipitated. Shake carefully and centrifuge tubes at 7000 rpm for 30 minutes.

12. Get rid off supernatant and add 4 ml of 80% ethanol in each Corex tube and centrifuge at 8000 rpm for 10 more minutes. Get rid off supernatant and air dry tubes for 10 minutes to get rid of excess of ethanol.

13. Take 350 µl of Tris EDTA and add to each of the tubes containing Nck and KID plasmids. Then vortex and transfer the DNAs to microcentrifuge tubes.

The DNAs obtained were confirmed using agarose gel electrophoresis. For this 0.8% agarose gel was used. To prepare 0.8% agarose gel 0.28 gm of agarose was mixed with 35 ml of 5XTBE [Tris Borate EDTA (ethylenediamine tetra acetic acid)] buffer and then microwaved for 50 seconds. The gel was poured in an electrophoresis tray and run at 80 mV for 1 hour. Using the standard markers \(\lambda\ Hind\ III\) and \(\lambda\ Bst\ II\), the size of Nck-α was found to be 5.9 kbp (kilobasepairs) and for KID was 4.5 kbp. Similarly the concentrations of Nck-α and KID were determined using standard markers. Nck-α was 100 ng/µl and KID was 400 ng/µl.
For plating the bacteria on LB, 5 gm bactotryptone, 2.5 gm bacto yeast extract, 5 gm NaCl, and 400 ml distilled water were mixed together and the pH was adjusted to 7.5 using 0.1M NaOH. Then 7.5 gm agar was added, and the plates were autoclaved. The LB media was poured into plates without any bubble formation. For LB plating with ampicillin, we took 0.3 gm/ml of ampicillin from the stock of 20 mg/ml. The ampicillin resistant colonies were grown on LB agar. Two well isolated and circular colonies were selected from each experimental and control group with Nichrome wire. The reason behind using well isolated and well rounded colony is that those colonies are derived from single transformed cell. These samples were put into liquid LB medium, without agar, at pH-7.5. They were kept in a shaker at 37°C overnight.

**Cell culture and chemical treatment**

To generate the cultured 1000W cell line, a heterotopic tracheal transplant was done from a Fisher rat with exposure to 7, 12-dimethylbenzanthracene. 1000W cells at passage 49 were thawed in 37°C water bath for 4 minutes and added into the polystyrene culture dishes with 3 ml of Waymouth’s medium in each. This cell line was cultured at 37°C with 5 % CO2 in air. It was maintained in Waymouth’s medium containing 10 % fetal bovine serum, supplemented with 0.1 µg/ml insulin and 0.1 µg/ml hydrocortisone. The line tested negative initially for tumorigenicity but became tumorigenic by 16 months in vitro. To assay the cell shape features, cells were subcultured onto polystyrene nonpyrogenic tissue culture dishes from Becton Dickinson Labware. The media was changed every two days to meet the nutritional requirements of the cells. Cells were
subcultured after attaining the confluency about 90-95%. Trypsin EDTA was used to remove the cells from the culture dish.

**Transfection experiment**

Firstly the culture dishes were taken out of the $37^\circ$ C incubator and medium was removed. Then 2 ml of trypsin EDTA was added to the cells. The culture dishes were kept at $37^\circ$ C for 5 minutes. After removing dishes from the incubator, the cells were taken off the dish surface using pipette force. Then 2 ml of preincubated ($37^\circ$ C) Waymouth’s medium was added to the centrifuge tube and the cells were added to this tube. Tubes were centrifuged for 5 minutes and the supernatant was removed. Then 4 ml of Waymouth’s medium was added to the tube containing cells and mixed homogenously. The number of cells per ml was counted using Neubauer’s chamber. For transfection experiments, $2.6 \times 10^5$ cells/ml were plated in the Lab-Tek chamber slide with RS (a coating which helps cells to adhere well) coating and having 50mmx20mm sized single well. The slides were marked with alphabetical characters, with a diamond scribe for easy localization of cells under the scanning electron microscope. Lab-Tek chambers were sterilized under ultraviolet light for 45 minutes. Then the cells were uniformly distributed onto chamber slides with 1 ml of Waymouth’s medium. After letting cells attach for 24 hours, transfection experiments were carried out. The transfection reagent used was Lipofectamine 2000. Green fluorescent protein (GFP) in pXJ40 plasmid was used for tagging purposes. One control and three experimental transfections were done. For control GFP only was used. For experimental, Nck-$\alpha$ + GFP, KID + GFP, Nck-$\alpha$ +
KID + GFP combinations were used. After incubation of plasmids with reagents for 20 minutes, transfection was carried out in chambers as follows-

Table1. (Experiment 1)

<table>
<thead>
<tr>
<th>Tube #</th>
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<th>2 (experimental)</th>
<th>3 (experimental)</th>
<th>4 (experimental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waymouth’s medium</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>GFP</td>
<td>0.64 µl (conc. 3.125 mg/ml)</td>
<td>0.64 µl (conc. 3.125 mg/ml)</td>
<td>0.64 µl (conc. 3.125 mg/ml)</td>
<td>0.48 µl (conc. 3.125 mg/ml)</td>
</tr>
<tr>
<td>Nck-α</td>
<td>40 µl (conc. 100 ng/µl)</td>
<td></td>
<td></td>
<td>30 µl (conc. 100 ng/µl)</td>
</tr>
<tr>
<td>KID</td>
<td></td>
<td>10 µl (conc. 400 ng/µl)</td>
<td></td>
<td>7.5 µl (conc. 400 ng/µl)</td>
</tr>
</tbody>
</table>

At 24 hours, the transfected cells were fixed using 3% paraformaldehyde (preincubated at 37°C in water bath) with phosphate buffered saline (PBS), pH 7.3.

Similarly, a second transfection experiment was carried out but before fixing them, the cells were treated with PAO. The cells were treated with 5 µM PAO for 1 hour and then fixed with 3% paraformaldehyde for 10 minutes.
### Table 2. Experiment 2

<table>
<thead>
<tr>
<th>Tube #</th>
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<th>2 (experimental)</th>
<th>3 (experimental)</th>
<th>4 (experimental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waymouth’s medium</td>
<td>50 µl</td>
<td>50 µl</td>
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</tr>
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<td>GFP</td>
<td>0.64 µl (conc. 3.125 mg/ml)</td>
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<td>0.64 µl (conc. 3.125 mg/ml)</td>
<td>0.48 µl (conc. 3.125 mg/ml)</td>
</tr>
<tr>
<td>Nck-α</td>
<td>40 µl (conc. 100ng/µl)</td>
<td></td>
<td>30 µl (conc. 100ng/µl)</td>
<td></td>
</tr>
<tr>
<td>KID</td>
<td></td>
<td></td>
<td>10 µl (conc. 400ng/µl)</td>
<td>7.5 µl (conc. 400ng/µl)</td>
</tr>
</tbody>
</table>

**Fluorescence microscopy**

The chamber slides containing fixed cells were kept in PBS at 4°C. Using the Zeiss Axiophot microscope, pictures were taken with 20X Plan-Neofluar lens and GFP filter set. The pictures were saved in Metamorph 4.6r5 software (Molecular Devices Corp.). The inscription marks made on the slides were used for localization and identification of cells. Single, well isolated and fluorescent cells (positive expression) were photographed. The exposure time used for taking pictures was 15 seconds for each at binning 1. For each sample, approximately 20 to 25 pictures were taken.

**Scanning electron microscopy**

The cells were prepared for scanning electron microscopy using the thiocarbohydrazide osmium tetroxide (OTO) protocol. Firstly the samples were fixed in
2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), post fixed with OsO₄ for 30 minutes and rinsed in buffer 5-10 times. After that the samples were incubated in fresh 1% aqueous thiocarbohydrazide for 10 minutes. After rinsing them 5-10 times again with buffer they were reincubated in 1 % OsO₄ and rinsed 5-10 times in distilled water. Then cells were dehydrated through a series of ethanol (40%-50%-60%-80%-95%-100%). Then, the samples were critical point dried in Samadri-780A critical point drier. The principle of critical point drying is that the samples are dried at specific temperature (33°C -39°C) and pressure (1200 psi-1500 psi) for 4 minutes. After that the cell samples were coated with 1 nm thickness of gold–palladium in Hummer VI-A sputter coater. Then the samples were observed in the Hitachi S-2700 scanning electron microscope. The exact same cells were identified, pictures of which were taken in the light fluorescence microscope. The micrographs were saved using Quartz PCI software.

**Tracing and computerized morphometry**

The cell images were manually traced on a Dell microcomputer running Adobe Photoshop 7. Using Adobe Photoshop allows one to adjust contrast and brightness of the picture, which helps in better visualization of cell edges while tracing them. Then the tracings were analyzed using software which gave each individual cell area and values for factor #4 and factor #7. The edge contours traced from the prints were scanned into an Apple Color One Scanner 600/27 running under One Scanner Dispatcher and saved in TIFF format. The TIFF files were transferred to an anonymous SGI-INDY server <elvis.bgsu.edu> where contour extraction was performed. The software gave the
dimensioned values for cell area, perimeter, and length of the major axis, as well as values for 33 dimensionless shape variables. Primary shape variables were analyzed further in a Sun 400 server. Samples within each experiment were autoscaled by use of a C++ program, "autoscal.cxx." Autoscaled values were converted to values of factor #4 (filopodia) and factor #7 (MPs) as previously described [14]. The programs are publicly available by anonymous FTP to <elvis.bgsu.edu>. Differences between the means of different samples were determined as described previously [13].
RESULTS

The Nck-α and PAK<sup>83-149</sup> plasmids expanded and purified

When the BL-21 cells were transformed and colonies were grown overnight at 37°C, I got an ample number of ampicillin resistant colonies on LB plates indicating the transformation was successfully done. Basically the plasmid of interest enters the BL-21 cells, breaking their plasma membrane, and multiplies itself without affecting the DNA of the cell. Well isolated and well rounded colonies were picked up and were grown in liquid LB medium at 37°C overnight. The medium became murky which showed I successfully grew the transformed cells. Using a QIAGEN kit, I successfully obtained DNAs and confirmed them by using an agarose gel electrophoresis technique. I used λ <i>Hind</i> III and λ <i>Bst</i> II as the standard markers on the agarose gel. Fig. 4 shows the molecular sizes of each plasmid, as determined by agarose gel electrophoresis.
Fig. 4: Agarose gel electrophoresis of plasmids obtained by molecular cloning techniques. The standard markers $\lambda$ Hind III and $\lambda$ Bst II were used as shown in the 1st and 7th wells respectively. The 2nd, 3rd and 4th wells show the restriction digestion of Nck-$\alpha$ by restriction enzymes EcoRI, $\lambda$ Hind III and Pst I and the 5th and 6th wells show the restriction digestion of plasmid KID by EcoRI and $\lambda$ Hind III.
Transfection experiments, fluorescence and electron microscopy

Data were recorded by GFP fluorescence in order to flag cells that were overexpressing KID and/or Nck-α. When the same cells were imaged by SEM, it was clear that many of their edge details were only visible at the higher magnification. An analysis of the results showed that the cells had variations in factor #4 and factor #7 values. The variations in the two indexes were independent. Cells with a high index on either measure are shown in Figs. 5 and 6.

Fig. 5: Frames (a) and (b) show the characteristic appearance of a cell viewed by the fluorescence microscope and SEM respectively. This cell was overexpressing Nck-α and showed a value of 2.56 for factor #4.
Fig. 6: Frames (a) and (b) show the characteristic appearance of a cell viewed by the fluorescence microscope and SEM respectively. This cell was overexpressing KID and showed a value of 5.06 for factor #7.

Surprisingly Nck-α forced the formation or stabilization of the large protrusions and may have synergized with other mechanisms in filopodia formation (Tables 3 and 4). KID overexpression had no significant effect on the large protrusions (Table 3).

Table 3. Effects of PAK^{83-149} and Nck-α on the prevalence of factor #7 (MPs)\(^a\)

<table>
<thead>
<tr>
<th>Tukey grouping</th>
<th>Mean (sample size=23)</th>
<th>Combination of agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.80</td>
<td>Nck-α</td>
</tr>
<tr>
<td>A</td>
<td>0.71</td>
<td>Nck-α + PAK^{83-149}</td>
</tr>
<tr>
<td>B</td>
<td>-0.53</td>
<td>PAK^{83-149}</td>
</tr>
<tr>
<td>B</td>
<td>-1.05</td>
<td>Control (GFP alone)</td>
</tr>
</tbody>
</table>

\(^a\)Means with the same letter are statistically indistinguishable at the level P < 0.05
Nck-α required synergism with KID to enhance the formation of filopodia, since in the absence of KID, the Nck-α effect was statistically indistinguishable from control (Table 4).

Table 4. Effects of PAK^{83-149} and Nck-α on the prevalence of factor #4 (filopodia) ^a

<table>
<thead>
<tr>
<th>Tukey grouping</th>
<th>Mean (sample size=23)</th>
<th>Combination of agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.72</td>
<td>Nck-α + PAK^{83-149}</td>
</tr>
<tr>
<td>A B</td>
<td>-0.10</td>
<td>Nck-α</td>
</tr>
<tr>
<td>A B</td>
<td>-0.15</td>
<td>PAK^{83-149}</td>
</tr>
<tr>
<td>B</td>
<td>-0.47</td>
<td>Control (GFP alone)</td>
</tr>
</tbody>
</table>

^aMeans with the same letter are statistically indistinguishable at the level P < 0.05

Cells were pretreated with the PAO for 1 hour before fixation. PAO inhibits the strengthening of cytoskeletal linkages as mentioned above (see Introduction). When cells were treated with PAO, Nck-α overexpression had no significant effect on factor #7 formation. Nck-α along with PAK^{83-149} also didn’t show any synergy, in fact their effect was statistically indistinguishable from the control. PAK^{83-149} alone was effective in forcing the formation of MPs (Table 5). Surprisingly, Nck-α and PAK^{83-149} together failed to affect factor #4, which was indistinguishable from control. In fact, none of the effects on filopodia formation were statistically distinguishable from control (Table 6).
Table 5. Effects of PAK\textsuperscript{83-149} and Nck-\(\alpha\) on the prevalence of factor #7 (MP) in PAO treated cells \(a\)

<table>
<thead>
<tr>
<th>Tukey grouping</th>
<th>Mean (sample size=22)</th>
<th>Combination of agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.31</td>
<td>PAK\textsuperscript{83-149}</td>
</tr>
<tr>
<td>B</td>
<td>-0.10</td>
<td>Nck-(\alpha)</td>
</tr>
<tr>
<td>B</td>
<td>-0.61</td>
<td>Nck-(\alpha)+PAK\textsuperscript{83-149}</td>
</tr>
<tr>
<td>B</td>
<td>-0.64</td>
<td>Control (GFP alone)</td>
</tr>
</tbody>
</table>

\(a\)Means with the same letter are statistically indistinguishable at the level P < 0.05

Table 6. Effects of PAK\textsuperscript{83-149} and Nck-\(\alpha\) on the prevalence of factor #4 (filopodia)
in PAO treated cells\(a\)

<table>
<thead>
<tr>
<th>Tukey grouping</th>
<th>Mean (sample size=22)</th>
<th>Combination of agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.26</td>
<td>PAK\textsuperscript{83-149}</td>
</tr>
<tr>
<td>A B</td>
<td>0.18</td>
<td>Nck-(\alpha)</td>
</tr>
<tr>
<td>A B</td>
<td>0.07</td>
<td>Control (GFP alone)</td>
</tr>
<tr>
<td>B</td>
<td>-0.52</td>
<td>Nck-(\alpha) + PAK\textsuperscript{83-149}</td>
</tr>
</tbody>
</table>

\(a\)Means with the same letter are statistically indistinguishable at the level P < 0.05
DISCUSSION

Brief introduction to the research question

Nck-α involvement in formation of both small and large protrusions has been demonstrated. Its function may be implemented through the agency of other proteins, not PAKs, which exist in a multiprotein complex that includes Nck-α. In the current research, I sought to determine the role of PAO, a tyrosine phosphatase inhibitor, on the filopodia and membrane protrusion formations and also to elucidate the pathways involved in the formation of same. The triangular or strap-shaped protrusions from cultured cells are of great interest, because they are characteristically elevated in cells from neoplastic lines. In previous work, this laboratory found only two proteins which could enhance the prevalence of the protrusions when expressed as a single construct. One of the aims of this work was to determine whether the route affecting their action on protrusions was the same, i.e. one of the constructs acted upstream or downstream of other. The other aim was to determine whether their effect on filopodia was similar or identical to that on the large protrusions. Both of these dynamic structures were attached to the plastic surface of the culture dish by focal contacts. If the constructs, namely the KID and Nck-α, had similar effects on both structures, this might suggest that the effect was operating at the level of the focal contact itself.
Discussion of Tables 3 and 4

When expressed alone, KID had no significant effect on the prevalence of the large protrusions. Since its overexpression in combination with Nck-α had no effect exceeding that supplied by Nck-α alone (Table 3), KID may be working upstream or downstream of Nck-α. This suggests that both treatments could have a common mechanism, for example, stabilizing the focal contact. The mechanism could be as simple as PAK-mediated phosphorylation of Nck-α releasing PAK from a nascent focal contact site on the plasma membrane. However, the treatment clearly synergized to enhance filopodia (Table 4), since, in the absence of KID, the Nck-α effect on the prevalence of these features was statistically indistinguishable from control. This implied that the effect of combining of two constructs was not due to stabilization or destabilization of the PAK-Nck-α complex.

Discussion of Tables 5 and 6

When the cells were treated with PAO, definitely, it changed the way KID affected large protrusions in first experiment, which was without PAO treatment. When KID was expressed alone, it affected the large protrusion formation, but it did not show any synergy with Nck-α in forming the protrusions. It also reversed the effect of Nck-α on large protrusions (Table 5). Surprisingly, none of the treatment groups showed any significant effect on filopodia formation (Table 6).
MP formation

In current studies, I worked on deciding the role played by Nck-α and PAK^{83-149} on the formation of MPs. The aim also was to look at; if Nck-α and PAK^{83-149} act in synergy with each other or cancel each others effect in MP formation. My results showed that combination of Nck-α + PAK^{83-149} have a greater effect on MP formation than PAK^{83-149} alone and less than Nck-α alone. When the same treatments were performed and cells were treated with PAO after transfection, it showed that the effect of PAK^{83-149} alone was now greatest on MP formation and it did not show any synergy in combination with Nck-α in formation of MPs. In other words, the effect of Nck-α was erased by PAO treatment.

Filopodia formation

My results showed that only the overexpression of combination of agents Nck-α + PAK^{83-149} was able to produce filopodia, in an effect that was statistically distinguishable from the control group. But when the cells were treated with PAO, there was no significant production of filopodia with any single agents or combination of agents. Surprisingly, Nck-α + PAK^{83-149} combination showed an effect even lower than the control. Again, as in the case of the MPs, the effect of Nck-α was erased, and none of the treatments showed a difference from control.
References


