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14. ABSTRACT

Establishing a role for ACK2 and SH3PX1 in ErbB-2 receptor degradation is appealing based on the predictive property between receptor overexpression and breast cancer. Currently, we are interested in determining the significance of ACK2-dependent phosphorylation of SH3PX1 in cells. To address these objectives, we have carried out deletion analysis studies to delineate the region of the phosphorylation on SH3PX1. Based on these studies, we conclude that SH3PX1 phosphorylation is lost in the truncation mutant ΔC84. In additional binding studies, ACK2 is unable to bind to any of the deletion mutants. Site-directed mutagenesis studies indicate that all conserved point-mutants of SH3PX1 retain tyrosine phosphorylation. Given these findings, we employed mass spectrometry as a means to determine the multiple phosphorylation sites on SH3PX1, recently identified as tyrosine residues 177, 239, 269, and 561. Additionally, recombinant forms of kinase and substrate were generated for *in vitro* kinase screens carried out at the high-throughput facility at Merck& Co., Inc. The pyrido-pyrimidine compound from Park Davis (PD158780) was shown to effectively block ACK2 *in vitro* with an IC50 of 80 pM. Low kinase activity of ACK2 *in vivo* and *in vitro* has been one of the greatest challenges in carrying out the research objectives in our statement of work. As a result, the DOD-sponsored research has led us to new and exciting discoveries, namely identifying two unreported kinases, FAK and Src, for SH3PX1. We believe that the phosphorylation state of SH3PX1 is critical in mediating its binding to a growing number of proteins involved in endocytosis, including the GTPase, dynamin. Our work is now focused on determining the biological outcome of SH3PX1 phosphorylation.

15. SUBJECT TERMS
Tyrosine kinase, EGF receptor, ErbB-2/Neu, signaling, small molecules

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Introduction

Regulation of growth factor receptor expression and kinase activity is critical in several signal transduction pathways, including mitogenic pathways. The normal regulation of cell growth is achieved through a balance between the activation, endocytosis, and degradation of growth factor receptors. As a result, receptor overexpression or mutations altering receptor kinase activity can disrupt this delicate balance, and is often sufficient to cause malignant transformation of the cell. Of the various known growth factor receptors, the epidermal growth factor receptor (EGFR) and other ErbB family members are probably the best studied and most highly characterized. Overexpression of the ErbB family of receptors appears to play a causative role in various forms of cancer [1-6]. In particular, ErbB-2/Neu has received special attention in the clinical field based on the relationship between ErbB-2/Neu gene amplification and human breast cancers, with overexpression correlating with a poor prognosis for breast cancer patients [7,8].

Given the association of breast cancer with ErbB-2/Neu overexpression, much effort has been directed toward identifying therapeutic agents that will down-regulate ErbB-2/Neu activity. Currently, the use of monoclonal antibodies that inhibit ErbB-2/Neu activity, paired with chemotherapy, is the most successful mode of treatment for patients with metastatic breast cancer [9]. Preliminary results attribute the observed reduction in tumor growth to the acceleration of ErbB-2/Neu degradation, promoted by the monoclonal antibody, Trastuzumab (Herceptin^{TM}) [10]. Based on these findings, our studies are now focused on elucidating the molecular machinery underlying growth factor receptor endocytosis and degradation. To do so, we must identify the major proteins involved and develop tools to gain insight into this complex cellular process.

Two such proteins, ACK2 and its substrate, SH3PX1 have been linked to endocytosis and sorting through their ability to associate with various proteins involved in the processes and their effects on receptor degradation. More specifically, we and others have demonstrated that ACK2 and SH3PX1 form complexes with endocytic proteins, including clathrin, dynamin-2 and AP-2 [11,12,13]. The link between SH3PX1 and dynamin is particularly interesting given the proposed regulatory role of SH3PX1 in targeting dynamin to the plasma membrane [16] and stimulation of its GTPase activity by enhancing dynamin assembly [17]. In addition, overexpression of ACK2, SH3PX1, or various combinations of the two or their mutants, results in changes in the processing and trafficking of EGF and transferrin receptors [12,13].

ACK2 is a nonreceptor tyrosine kinase specifically activated by the Rho family GTP-binding protein, Cdc42. The domain structure of ACK2 consists of several signaling domains, including a tyrosine kinase domain, an SH3 domain, a CRIB (Cdc42/Rac interactive-binding) domain, two proline-rich domains, and a clathrin-binding domain [12,14]. SH3PX1 was identified as a binding partner and substrate for ACK2 through a series of GST pull-down assays [13]. SH3PX1 (sorting nexin 9, SNX9), a member of the nexin family of vesicle transport proteins, undergoes an EGF-dependent phosphorylation, mediated via the Cdc42-promoted activation of ACK2 [13]. While the precise role of SH3PX1 phosphorylation remains to be established, various lines of evidence point to a sorting function involving the EGF receptor and related family members. At least one other

member of the nexin family, sorting nexin 1 (SNX1), has been implicated in EGF receptor sorting and degradation [15]. Sorting nexin 1 was found to bind the cytoplasmic domain of the EGF receptor through yeast two hybrid approaches, and has been implicated in directing EGF receptors to the lysosome for degradation [15]. Similarly, data from our laboratory suggests that the ACK2-catalyzed phosphorylation of SH3PX1 stimulates EGF receptor degradation [13].

Investigating the role of ACK2 and SH3PX1 in growth factor receptor degradation may lead to a better understanding of receptor overexpression and subsequent cellular transformation. Determining the significance of tyrosine phosphorylation of SH3PX1 in cells, and developing the ability to regulate this phosphorylation event, perhaps through the identification of specific inhibitors of ACK2, will further our understanding of ACK2 activity in receptor endocytosis and degradation and its contribution to malignancy.

Progress Report

Task 1. Biochemical characterization of the interaction of ACK2 with its phospho-substrate, Months 1-12:

Deletion Mutant Analysis

To delineate the region on SH3PX1 that contains the phosphorylation site(s) for ACK2, a series of C-terminal truncation mutants was designed. Mutants ΔC84, ΔC197, ΔC339, ΔC395, ΔC547 were engineered with BamH1 and EcoR1 restriction sites and generated by PCR (PCR Sprint, Hybaid). The PCR products were directly ligated into the HA-tagged pcDNA3 expression vector using the Topo TA cloning kit from Invitrogen. Phosphorylation of these mutants was measured by co-expression of the ACK2 and SH3PX1 constructs in COS-7 cells, followed by immunoprecipitation and Western blotting using an anti-phosphotyrosine antibody. Loss of phosphorylation, as detected with 4G10 antibody (Upstate), occurs between SH3PX1 *wild-type* and the SH3PX1-ΔC84 mutant (Figure 1). Consequently, the five tyrosine residues in this C-terminal portion of SH3PX1 were mutated to phenylalanine and the resulting mutants were co-expressed in COS-7 cells. The mutants were precipitated and immunoblotted with anti-phosphotyrosine antibody and unexpectedly, the F546/F561/F563/F570/F578 mutant retained a phosphorylation signal (Figure 2). Binding between ACK2 and the SH3PX1 deletion mutants was then studied, and the loss of ACK2 binding to the deletion mutants was observed (Figure 3).

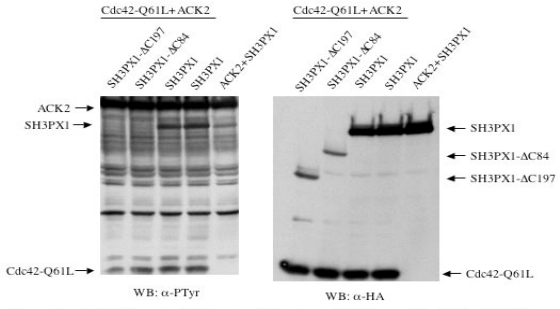

Figure 1. ACK2-dependent Phosphorylation is Lost at Truncation Mutant ΔC84
ACK2 and SH3PX1 truncation mutants were co-expressed in COS-7 cells. Cell lysates were immunoblotted with anti-phosphotyrosine and anti-HA antibodies.

Figure 2. ACK2 Phosphorylates C-terminal Tyrosine-to-Phenylalanine Mutants
ACK2 and SH3PX1 point mutants were co-expressed in COS-7 cells. SH3PX1 mutants were immunoprecipitated from cell lysates with anti-HA antibody and blotted with anti-phosphotyrosine and anti-HA antibodies.

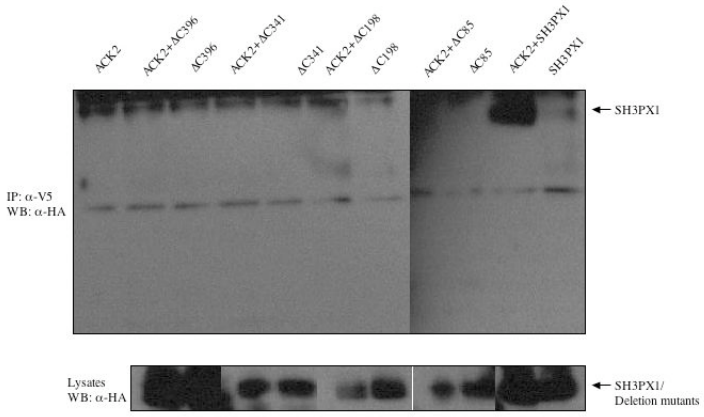

Figure 3. ACK2 does not Bind to SH3PX1 Deletion Mutants
ACK2 and SH3PX1 deletion mutants were co-expressed in COS-7 cells. ACK2 was precipitated with anti-V5 antibody and co-immunoprecipitation was determined by immunoblotting for HA-tagged SH3PX1 and C-terminal deletion mutants.

Point Mutant Analysis

Due to variable expression and lack of ACK2 binding to the C-terminal truncation mutants, tyrosine-to-phenylalanine point mutants of conserved residues were designed and the tyrosine phosphorylation signal of these mutants was measured. Conserved mutants of *Drosophila* and human orthologs: Y9F, Y56F, Y287F, Y496F, Y546F, Y578F were generated by PCR using the Quick-change site-directed mutagenesis kit from Stratagene. Phosphorylation of the point mutants was carried out by co-expression of ACK2 and SH3PX1 mutants in COS-7 cells. Lysates were subject to immunoprecipitation, followed by immunoblotting with anti-phosphotyrosine antibody. All single point mutants retained a phosphorylation signal (Figure 4). Consequently, we believe that there are multiple sites of phosphorylation on the substrate as we did not observe a decrease in phosphorylation of the mutants compared to *wild-type* SH3PX1. Mass spectrometry is thought to be a very sensitive method for determining post-translational modifications and we have taken steps to prepare a sample for submission. More specifically,

we have generated recombinant sources of kinase and substrate for the purpose of phosphopeptide mapping and *in vitro* kinase assays (task 3).

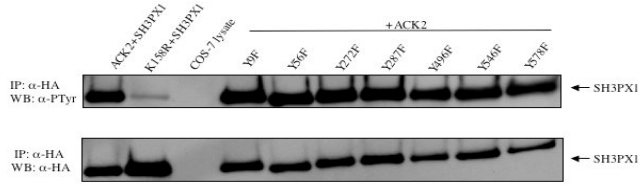

Figure 4. ACK2 Phosphorylates Conserved SH3PX1 Tyrosine-to-Phenylalanine Point Mutants
ACK2 and SH3PX1 and point mutants were co-expressed in COS-7 cells. SH3PX1 and point mutants were precipitated with anti-HA antibody and phosphorylation was detected by Western blotting with anti-phosphotyrosine antibody.

Mass Spectrometry

Our initial mass spectrometry sample was submitted from a kinase reaction with recombinant sources of His-ACK2 and His-SH3PX1 (protein expression and purification detailed below). The kinase reaction was carried out for 7 hours at 30°C in Hepes/$MgCl_2$/NaCl buffer and quenched with 5X SDS-PAGE sample buffer. The sample was boiled, run on an SDS-PAGE gel, and stained with colloidal blue stain. The protein band was extracted, rinsed with acetonitrile and submitted to the Harvard Microchemistry Facility. The stoichiometry of the sample was below the levels of detection, also observed in previous *in vivo* and *in vitro* ACK2 experiments. Given these results, we focused on methods to increase our pool of phosphorylated substrate. After unsuccessfully using phospho-enrichment columns, we identified two unreported kinases for SH3PX1, FAK and Src. In COS-7 cells transfected with FAK and SH3PX1, SH3PX1 phosphorylation was largely enhanced over ACK2-dependent phosphorylation (Figure 5). Based on reported cooperation between Src and FAK, Src phosphorylation of SH3PX1 was measured in HEK 293 cells and found to be greatly enhanced even over FAK-dependent phosphorylation. A second mass spectrometry sample was prepared by immunoprecipitating SH3PX1 out of HEK 293 cells co-expressing Src and SH3PX1. The precipitated sample was boiled, run on an SDS-PAGE gel, stained with Coomassie, and excised. Aliquots from this sample were set aside and tyrosine phosphorylation was confirmed by Western Blotting (Figure 6). This sample was then submitted to the Cornell Biotechnology Resource Center Proteomics and Mass Spectrometry Core Facility. The sample was successfully run and four tyrosine phosphorylation sites were detected, Y177, Y239, Y269, Y561. Future steps will involve mutating the tyrosine sites and verifying loss of phosphorylation with ACK2.

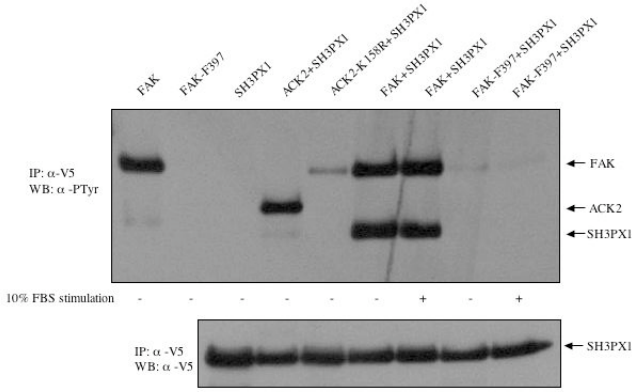

Figure 5. FAK Phosphorylates SH3PX1 *In Vivo*
COS-7 cells were transfected for 24-72 hours, serum starved in 1% FBS overnight, and designated samples were stimulated with 10% FBS prior to cell lysis. SH3PX1 was precipitated with anti-V5 antibody and subjected to Western blotting with anti-phosphotyrosine antibody and anti-V5 antibody.

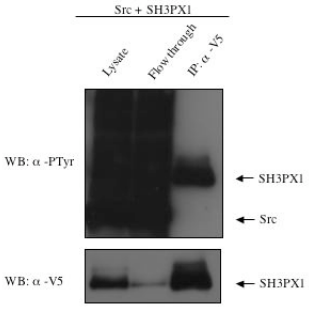

Figure 6. SH3PX1 Sample for Mass Spectrometry
Src and SH3PX1 were co-expressed in HEK 293 cells. SH3PX1 was precipitated from cell lysates with anti-V5 antibody and stained with Coomassie blue. Samples from the cell lysate, flow through and immunoprecipitation were saved for western blotting with anti-phosphotyrosine and anti-V5 antibodies.

Task 2. Evaluation of ACK2-dependent phosphorylation of SH3PX1 on the accumulation of receptors in breast cancer cells, Months 24-36:

Incomplete due to low expression and catalytic activity of ACK2.

Task 3. Combinatorial screen for ACK2 inhibitors/activators, Months 13-24:

<u>Recombinant Protein Generation</u>

Due to the size and complexity of ACK2 and SH3PX1, 83 kDa and 77 kDa, respectively, we initially set out to express these proteins in insect cells. His-tagged and untagged viruses of ACK2 and SH3PX1 were developed for insect cell expression using the Invitrogen Bac-to-Bac kit. However, SH3PX1, when expressed as a His-tagged protein in insect cells, retains a basal level of phosphorylation. We attributed this observation to the *Drosophila* orthologue of ACK2, DACK, and sought to eliminate this phosphorylation signal by co-expressing kinase-deficient ACK2-K158R with SH3PX1. This had a minimal effect. Despite this setback, His-ACK2

kinase activity was confirmed by co-infecting Sf21 cells with ACK2 and SH3PX1. An observed increase in His-SH3PX1 phosphorylation was detected by Western Blotting immunoprecipitated samples with anti-phosphotyrosine antibody (Figure 7).

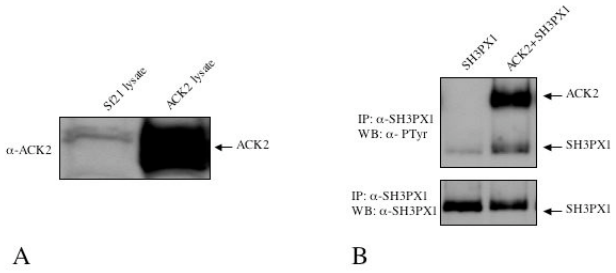

Figure 7. ACK2 Expression and Activity in Sf21 Cells
His-ACK2 and His-SH3PX1 viruses were generated according to the instruction manual for the Bac-to-Bac Baculovirus expression system (Invitrogen). The resulting viruses were used to infect Sf21 cells for 24-72 hours prior to cell lysis. His-ACK2 expression was shown by Western blotting whole-cell lysates with anti-ACK2 antibody (A). Kinase activity of recombinant ACK2 was measured by co-infecting ACK2 and SH3PX1 in Sf21 cells and measuring SH3PX1 phosphorylation of immunoprecipitated samples (B).

To overcome the basal phosphorylation of the substrate, we expressed SH3PX1 as a GST-fusion protein in *E. coli*, where the likelihood of basal phosphorylation was small. Full-length SH3PX1 was cloned into the pGEX-KG vector for recombinant expression. Expression of the GST-SH3PX1 fusion protein was carried out in the BL21 *E. coli* strain. One-liter cultures were grown to an OD_{600} of 0.8 in super broth and induced with 200 µM IPTG overnight. Cells were harvested by centrifugation at 4000 rpm for 10 min in a JLA9.1 rotor (Beckman) and frozen at −80°C. All subsequent purification steps were carried out at 4°C. The bacterial pellet was resuspended in 1x TEDA (20 mM Tris [pH 7.9], 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 150 mM NaCl, 10% glycerol) supplemented with protease inhibitors (1 mM PMSF, 10 µg/mL each of aprotinin and leupeptin, 10 µM benzamidine) and lysed by three passages through a French Pressure Cell (SLM Aminco), followed by sonication for 5 minutes (550 Sonic Dismembrator, Fisher Scientific). The lysate was clarified by ultracentrifugation in a Ti45 rotor (Beckman) at 40,000 rpm. The clarified lysate was incubated for 30 min with glutathione-agarose beads (Sigma) pre-equilibrated in 1x TEDA. The GST-SH3PX1 fusion protein was then eluted with 50 mL of 10 mM glutathione in 1x TEDA and subsequently purified by size exclusion.

GST-SH3PX1 exists in monomeric and non-uniform oligomeric complexes as determined by purification on a size exclusion column (the oligomeric complex eluting as a broad peak just after the void volume). To determine if the oligomeric complexes of SH3PX1 were due to the intrinsic nature of the protein, we expressed SH3PX1 as a His-tagged protein. SH3PX1 was first cloned into the Pet28A vector and transformed into BL21 cells. One-liter cultures were grown to an OD_{600} of 0.8 in super broth at 37°C and induced with 200 µM IPTG overnight at room temperature. Cells were harvested as described in the GST-SH3PX1 prep. Bacterial pellets were resuspended in Ni^{2+} binding buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 20 mM imidazole, 10% glycerol) supplemented with protease inhibitors. Cell lysis and centrifugation was carried out as described above. The clarified lysate was incubated for 30 minutes with Ni^{2+} beads (Amersham) at 4°C. The beads were then washed with binding buffer and the protein eluted with 50 mL of elution buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 200 mM imidazole, 10%

glycerol, Figure 8). Purification by size exclusion revealed the monomeric nature of the His-tagged SH3PX1 (data not shown).

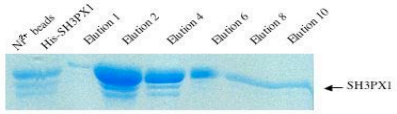

Figure 8. Expression of Recombinant SH3PX1 in E. coli
BL21 cells were induced at O.D. 0.8 with 200 μM IPTG for 16 hours at room temperature. His-SH3PX1 was purified on a Ni^{2+} column and dialyzed into HEPES/$MgCl_2$/$NaCl_2$ buffer.

Kinase Reaction

Preliminary kinase reactions were performed with His-ACK2 and GST-SH3PX1 on glutathione-agarose beads or His-SH3PX1 in 1x HMN (10 mM Hepes [pH 7.4], 5 mM $MgCl_2$, 150 mM NaCl) in the presence of 1 mM Na_3VO_4 and 1 mM ATP for 30 minutes at 30°C. The kinase reaction was quenched with 5X SDS loading buffer and substrate phosphorylation was measured by immunoblotting with anti-phosphotyrosine antibody (Figure 9). Given the complications associated with the GST-fusion protein, we decided that for the purpose of the *in vitro* kinase assay, His-SH3PX1 was to be used in place of GST-SH3PX1.

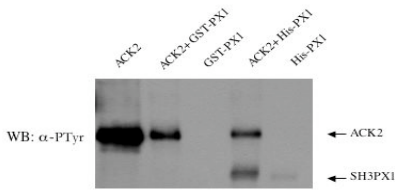

Figure 9. *In Vitro* Kinase Reaction
Kinase reaction was carried out for 30 minutes at 30°C. The reaction was quenched with 5X SDS-PAGE sample buffer and substrate phosphorylation was detected with anti-phosphorylation antibody.

ACK2 High-throughput Screen

For the purpose of screening for small molecule inhibitors of ACK2 activity, the detection system initially used was the LANCE™ time-resolved fluorescent resonance energy transfer system from Perkin Elmer. LANCE is based on the energy transfer between donor (europium chelate) and acceptor (allophycocyanin—APC) reagents bought together by a specific binding event. In the case of the kinase reaction, europium is conjugated to an anti-phosphotyrosine antibody and APC is conjugated to strephavidin beads. Energy transfer occurs in the presence of a tyrosine phosphorylated, biotinylated substrate (Figure 10).

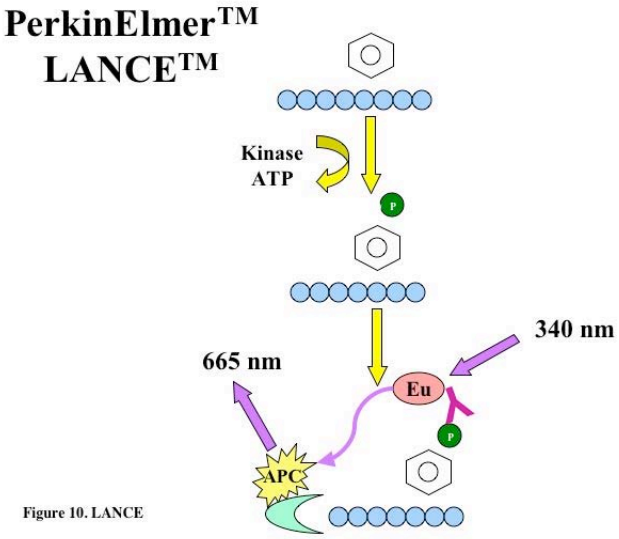

Figure 10. LANCE

Optimal reaction conditions were determined by carrying out a time course for the ACK2 kinase reaction. The most considerable challenge was in generating signal to monitor the reaction. This obstacle was addressed by altering reaction conditions including, increasing the kinase concentration and changing the buffer salinity, while at the same time, running positive controls with recombinant insulin receptor. Through these adjustments, optimal buffer conditions were determined, 50 mM Hepes/10 mM MgCl2/0.1% triton, and sodium chloride was found to severely inhibit ACK2 activity. Under these conditions, the reaction reached completion after 5-10 minutes at room temperature (Figure 11). To slow the reaction for high-throughput purposes, enzyme titrations were carried out, however the decreased concentration of kinase led to a decrease in the total signal (Figure 12). To inhibit potential nonspecific interactions between the kinase and the reaction plate, detergents and BSA were included in subsequent enzyme titrations. The addition of detergent and BSA not alter the results therefore a staurosporine dose response was carried out to confirm that the signal was due to kinase activity (Figure 13).

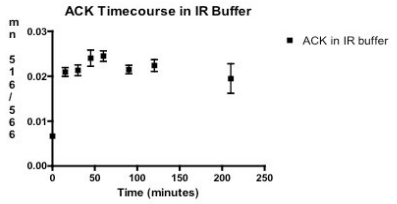

Figure 11. ACK2 Activity Optimized in the Absence of Salt
Kinase reaction was carried out with 4.15 µg/mL ACK2, 200 nM SH3PX1, and 2mM ATP in HEPES/$MgCl_2$ buffer.

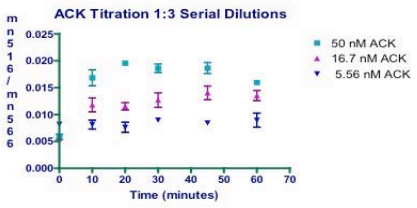

Figure 12. ACK2 Loses it's Activity at Lower Enzyme Concentrations
Kinase reactions were carried out with 4.15, 1.38, 0.46 µg/mL ACK2, 200 nM SH3PX1, 2 mM ATP in HEPES/$MgCl_2$ buffer.

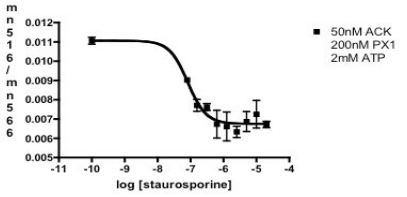

Figure 13. The LANCE Signal is Kinase-dependent
5-minute kinase reaction was carried out with 4.15 µg/mL ACK2, 200 nM SH3PX1, and 2 mM ATP in the presence of 20 µM-80nM staurosporine.

Ultimately, a ^{33}P-ATP filter assay was used to simplify detection (Figure 14). Following the kinase reaction, participating proteins were precipitated with tricholoroacetic acid. The reaction mixture was then transferred to a filter plate where ^{33}P incorporation of adherent proteins was measured. A comparative time course was carried out under the same conditions as the LANCE^{TM} system. However, incorporation of ^{33}P -ATP was very low. The reaction time was extended and in the end, overnight conditions were used to maximize the signal-to-background ratio to 2.5:1, within the range of screening (Figure 15). The next step was to automate the assay, with each reaction component distributed by an automated instrument. Unfortunately, the DMSO control plate showed several spikes in signal, most likely due to the low signal-to-background ratio as well as to a manual step necessary in the process. We did not proceed to an automated screen because we felt that the artifacts would skew any true hits. Despite this setback, the signal-to-background ratio was high enough to screen compounds in replicates. Dose responses were carried out using the tyrphostin EGF receptor inhibitor, AG1478, the Park-Davis pyrido-pyromidine, PD158780, and the SUGen compound, SU6656. We found that the Park-Davis compound inhibited ACK2 kinase activity *in vitro* with an IC_{50} of 80 pM (Figure 16).

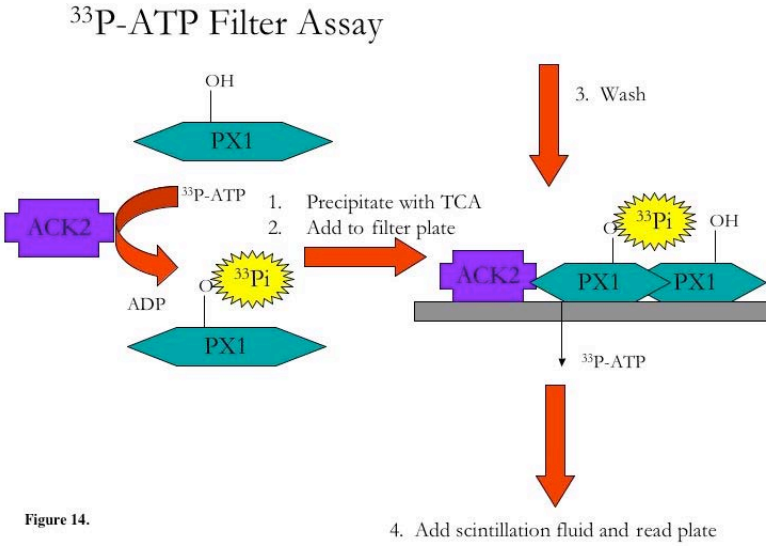

Figure 14.

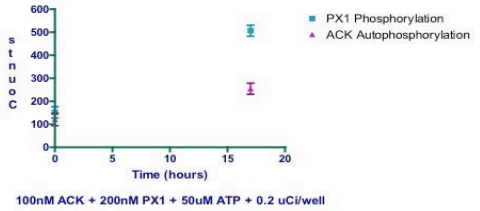

Figure 15. Overnight Kinase Reaction Measured by Filter Assay
Kinase reaction carried out with 8.3 μg/mL ACK2, 200 nM SH3PX1, 50 μM ATP, 0.2 μCi/well for 18-hour reaction at room temperature.

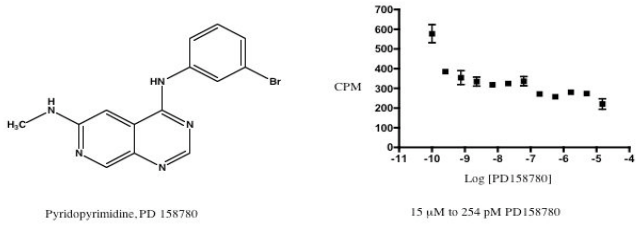

Figure 16. PD158780 Inhibits ACK2 Activity with an IC_{50} of ~80 pM
Kinase Reaction carried out with 8.3 μg/mL ACK2, 200 nM SH3PX1, 50 μM ATP, 0.2 μCi/well for 18 hours at room temperature in the presence of PD158780.

Protein Partners of SH3PX1

As mentioned previously, many of our problems stemmed from the low catalytic activity of ACK2, both in cells and *in vitro*. Consequently, we were interested in means for increasing phosphorylation of the sorting nexin. At a departmental seminar involving research on FAK-mediated phosphorylation of endophilin A2, we recognized several similarities between endophilin A2 and SH3PX1. When we co-expressed FAK with SH3PX1 in COS-7 cells, we found the sorting nexin to be strongly phosphorylated (Figure 5). In HEK 293 cells, we observed stronger Src-mediated phosphorylation of SH3PX1 over FAK, ultimately dictating the conditions for our mass spectrometry sample (Figure 17). This Src-dependent phosphorylation of SH3PX1 was not diminshed by the introduction of FAK-F397. We were also able to show phosphorylation of SH3PX1 by FAK and Src, independently, through *in vitro* kinase experiments with His-purified recombinant kinase and substrate (Figures 18, 19).

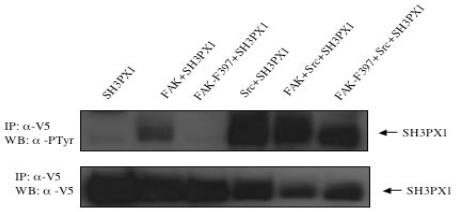

Figure 17. Src is a More Catalytic Kinase for SH3PX1
Src, FAK and SH3PX1 were co-expressed in HEK 293 cells. SH3PX1 was immunoprecipitated with anti-V5 antibody and blotted with anti-phosphotyrosine and anti-V5 antibodies.

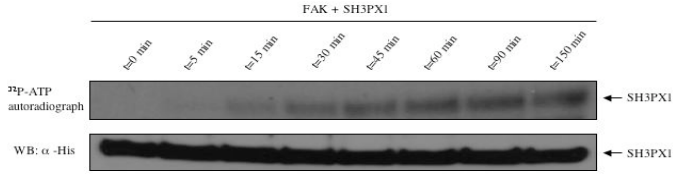

Figure 18. FAK Phosphorylates SH3PX1 *In Vitro*
Time course was carried out with recombinant FAK and SH3PX1 in the presence of ATP and ^{32}PATP. Reaction was quenched with 5X SDS sample buffer and ^{32}P incorporation was measured by autoradiograph, later blotted with anti-His antibody. 5X SDS sample buffer was added to t=0 sample before the addition of kinase.

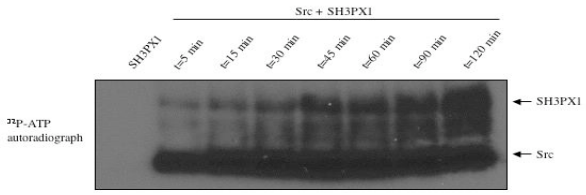

Figure 19. Src Phosphorylates SH3PX1 *In Vitro*
Time course was carried out with recombinant Src and SH3PX1 in the presence of ATP and ^{32}PATP. Reaction was quenched with 5X SDS sample buffer and ^{32}P incorporation was measured by autoradiograph, later blotted with anti-His antibody.

More recently, we have become interested in the emerging role of SH3PX1 in dynamin binding and regulation. Based on our original proposal, the observed changes in EGF receptor levels under conditions where SH3PX1 is phosphorylated are key. Dynamin is a 100 kDa GTPase involved in the fission of the clathrin-coated pits from the cell membrane to form clathrin-coated vesicles. We have verified SH3PX1 as a binding partner for dynamin and demonstrated binding through the SH3 domain of SH3PX1 through co-immunoprecipitation studies involving the SH3PX1-W39A mutant (Figure 20). We were then interested to see if the binding is mediated through ACK2 and found that ACK2 does not appear to bind to dynamin (Figure 21). We believe that tyrosine phosphorylation plays an important role in dynamin binding and regulation and are currently carrying out experiment to try to address the mechanistic details of this complicated process.

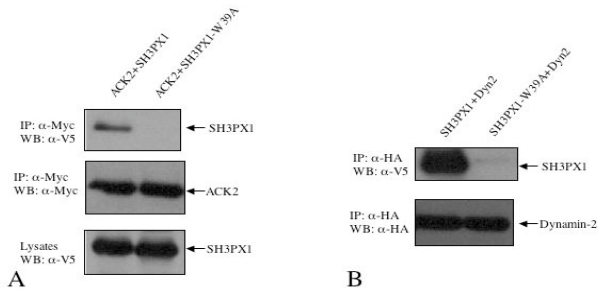

Figure 20. ACK2 and Dyamin-2 Bind SH3PX1 through its SH3 Domain
ACK2 or Dynamin-2 were co-expressed with SH3PX1 or the SH3PX1 tryptophan mutant in COS-7 cells. Binding studies were carried out by precipitating through ACK2 (A) or Dynamin-2 (B) and blotting for SH3PX1 and SH3PX1-W39A with anti-V5 antibody.

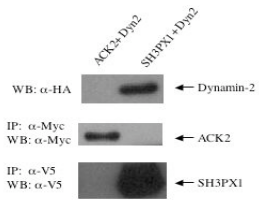

Figure 21. ACK2 Does Not Bind to Dynamin-2
ACK2 or SH3PX1 were co-expressed with Dynamin-2 in COS-7 cells. Binding studies were carried out by precipitating through ACK2 or SH3PX1 and blotting for Dynamin-2 with anti-HA antibody.

Research Accomplishments

- Generation and expression of C-terminal truncation mutants of SH3PX1—
    1. The loss of phosphorylation of SH3PX1 occurs between *wild-type* and ΔC84 in an ACK2-dependent manner, as detected Western blotting with anti-phosphotyrosine antibody from Upstate.
    2. Mutating all of the C-terminal tyrosine residues to phenylalanine in the C-terminal region does not change the phosphorylation state in ACK2-expressing cells.
    3. ACK2 is unable to bind to the SH3PX1 deletion mutants.

- Generation and expression of SH3PX1 point mutants—
  All single, conserved point mutants retain a phosphorylation signal comparable to *wild-type* SH3PX1.

- Preparation of mass spectrometry samples and subsequent identification of tyrosine residues 177, 239, 269 and 561 as Src-phosphorylated sites.

- Expression and purification of ACK2, kinase-deficient ACK2-K158R, and SH3PX1 viruses—
    1. ACK2 activity confirmed in insect (Sf21) cells.
    2. Basal phosphorylation of SH3PX1 detected from Sf21 cells.

- Expression and purification of GST-SH3PX1—
  GST-SH3PX1 is expressed in *E. coli* (BL21) cells and purified on glutathione-agarose beads.

- Expression and purification of His-SH3PX1—
  His-SH3PX1 is expressed in *E. coli* (BL21) cells and purified on a Ni^{2+} column.

- Kinase Reaction—
  ACK2, purified from Sf21 cells, is able to phosphorylate GST-SH3PX1 on beads and recombinant His-SH3PX1 *in vitro*.

- ACK2 high-throughput screen
  Tyrosine kinase inhibitor from Park-Davis, PD158780, inhibits ACK2 kinase activity *in vitro*, IC_{50} 80 pM.

- Identification of FAK and Src as unreported kinases for SH3PX1, both *in vivo* and *in vitro.*

- Determination of Dynamin binding to SH3PX1
  1. Dynamin binds to SH3PX1 through its SH3 domain.
  2. Dynamin binding to SH3PX1 is not mediated through ACK2, as ACK2 does not bind to Dynamin-2.

Reportable Outcomes

Characterization of the ACK2-SH3PX1 Interaction and its Role in Receptor Endocytosis
Carrie J. Stearns and Richard A. Cerione, Book of Abstracts, 43rd Annual ASCB Meeting, San Francisco, December 13-17, 2003

Conclusions

Investigating the role of ACK2 and SH3PX1, and more recently, FAK and Src, in growth factor receptor degradation may lead to a better understanding of the mechanisms underlying normal cell growth and proliferation and how these controls are disrupted in transformed cells. Based on the ties between overexpression of ACK2 and SH3PX1 and changes in the processing and trafficking of EGF and transferrin receptors, we are interested in further characterizing the ACK2-SH3PX1 interaction, and determining the significance of ACK2-dependent phosphorylation of SH3PX1 in cells. To date, we have demonstrated the loss of phosphorylation in the ΔC84 mutant of SH3PX1 by deletion analysis, and have confirmed multiple phosphorylation sites on the substrate through mass spectrometry. Specifically, tyrosine residues 177, 239, 269 and 561 are phosphorylated by Src in HEK 293 cells. We will make this quadruple mutant and test for ACK2 activity in the near future. In addition, we have identified PD158780 as an *in vitro* inhibitor of ACK2 kinase activity. Improving our ability to regulate this phosphorylation event by developing dominant-negative forms of SH3PX1 will help to further our understanding of the mechanism of action in SH3PX1 phosphorylation and its effects on receptor endocytosis and degradation.

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