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Role of the Non-Receptor Tyrosine Kinase ACK2 in EGF Receptor Degradation

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Epidermal growth factor receptor and members of the ErbB family of receptor tyrosine kinases have been implicated in several mitogenic pathways. Regulated growth factor-mediated signaling relies upon a balance between receptor activation, endocytosis, and degradation. As a result, overexpression or mutations altering receptor kinase activity is often sufficient to cause malignant transformation. We are interested in the emerging role of the nonreceptor tyrosine kinase, ACK2, and its substrate, SH3PX1, in regulating ErbB family degradation. Establishing a role for ACK2 and SH3PX1 in ErbB-2 receptor degradation is especially appealing based on the predictive property between receptor overexpression and breast cancer. Currently, we are interested in characterizing the ACK2-SH3PX1 interaction and 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 AC339. In parallel site-directed mutagenesis studies, we conclude that all conserved point-mutants of SH3PX1 retain tyrosine phosphorylation. Given these findings, we believe that Mass Spectrometry may provide a more sensitive means to identify the ACK2 phosphorylation site(s) on SH3PX1 and efforts have been made to generate recombinant forms of ACK2 and SH3PX1. In addition, in vitro kinase screens for inhibitors of ACK2 have been carried out at the high-throughput facility at Merck & Co., Inc. The pyrido-pyrimidine compound from Park Davis (PD158780) has been shown to effectively block ACK2 in vitro and additional experiments will be carried out to determine its activity in vivo. The ability to regulate this phosphorylation event will help to determine the importance of ACK2 activity in receptor endocytosis and degradation.
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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™) [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]. 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, recent 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 ACK2-dependent 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 ACK, 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 ACK and SH3PX1 constructs in COS-7 cells, followed by immunoprecipitation and Western blotting using an anti-phosphotyrosine antibody. Loss of phosphorylation, as detected with HRP-conjugated 4G10 antibody (Upstate), occurs between ΔC197 and ΔC339 in an ACK-dependent manner (Figure 1).

**Point Mutant Analysis**

Due to variable expression of the C-terminal truncation mutants and variable sensitivity of anti-phosphotyrosine antibodies, conserved tyrosine-to-phenylalanine point mutants were designed and the tyrosine phosphorylation signal was measured in parallel through the afore-mentioned
studies. Conserved mutants of *Drosophila* and human orthologs: Y9F, Y56F, Y287F, Y496F, Y546F, Y578F, and multiple mutants: F546/F578, F546/F561/F563/F578, and F546/F561/F563/F570/F578 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 ACK and SH3PX1 mutants in COS-7 cells. Lysates were subject to immunoprecipitation, followed by immunoblotting with anti-phosphotyrosine antibody. All single point mutants and multiple point mutants retained a phosphorylation signal (Figure 2). Consequently, we believe that there may be multiple sites of phosphorylation on the substrate as we did not observe a decrease in phosphorylation of the mutants compared to wild-type SH3PX1. We now believe that Mass Spectrometry may be used as a more sensitive method for determining the site(s) of phosphorylation. 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 2. Phosphorylation of SH3PX1 Point Mutants](image)

**Figure 2. Phosphorylation of SH3PX1 Point Mutants:**
SH3PX1 point mutants were co-expressed with Myc-ACK2 in COS-7 cells. Point mutants were immunoprecipitated with anti-HA antibody from cell lysates and immunoblotted with anti-phosphotyrosine or anti-HA.

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

To be initiated in summer 2005

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

**Recombinant Protein Generation**
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. We soon found that 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. However, we found that this had a minimal effect. Despite this setback, we were able to confirm ACK2 kinase activity from our insect cell expression system by co-infecting Sf21 cells with ACK2 and its substrate, SH3PX1. An observed increase in His-SH3PX1 phosphorylation was detected by Western Blotting immunoprecipitated samples with anti-phosphotyrosine antibody (Figure 3).
To overcome the basal phosphorylation of our 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 4). Purification by size exclusion revealed the monomeric nature of the His-tagged SH3PX1 (data not shown).
Kinase Reaction
Preliminary kinase reactions were performed with His-ACK2 and GST-PX1 on glutathione-agarose beads or His-PX1 in 1x HMN (10 mM Hepes [pH 7.4], 5 mM MgCl₂, 150 mM NaCl) in the presence of 1 mM Na₃VO₄ 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 5). Given the complications associated with the GST-fusion protein, it was decided that for the purpose of the in vitro kinase assay and Mass Spec. analysis, His-SH3PX1 was to be used in place of GST-SH3PX1.

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 6).
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 7). 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 8). 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 9).
Ultimately, a $^{33}\text{P}$-ATP filter assay was used to simplify detection (figure 10). Following the kinase reaction, participating proteins were precipitated with trichloroacetic acid. The reaction mixture was then transferred to a filter plate where $^{33}\text{P}$ incorporation of adherent proteins was measured. A comparative time course was carried out under the same conditions as the LANCE™ system. However, incorporation of $^{33}\text{P}$-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 11). 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 \textit{in vitro} with an IC$_{50}$ of 80 pM (figure 12). Further plans include testing this compound for \textit{in vivo} activity.

![Figure 10: 33P-ATP Filter Assay](image)

**33P-ATP Filter Assay**

1. Precipitate with TCA
2. Add to filter plate
3. Wash
4. Add scintillation fluid and read plate

![Figure 11: Overnight Kinase Reaction Measured by Filter Assay](image)

**Figure 11. Overnight Kinase Reaction Measured by Filter Assay**

Conditions: 8.3 µg/mL ACK2 + 200 nM PX1 + 50 µM ATP + 0.2 µCi/well. 18-hour reaction carried out at room temperature.
Research Accomplishments

- Generation and expression of C-terminal truncation mutants of SH3PX1—
The loss of phosphorylation of SH3PX1 occurs between ΔC197 and ΔC339 in an ACK2-dependent manner, as detected by HRP-conjugated anti-phosphotyrosine antibody from Upstate.

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

- Expression and purification of ACK2, kinase-deficient ACK2-K158R, and SH3PX1 viruses—
ACK2 activity confirmed in insect (Sf21) cells
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 Ni²⁺ column

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

- ACK2 high-throughput screen
Tyrosine kinase inhibitor from Park-Davis, PD158780, inhibits ACK2 kinase activity in vitro, IC₅₀ 80 pM
Conclusions

Investigating the role of ACK2 and SH3PX1 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 ΔC197 mutant of SH3PX1 by deletion analysis, and believe that there are multiple phosphorylation sites on the substrate due to mutagenesis studies. We hope to employ Mass Spectrometry in phosphopeptide mapping experiments to address this question. 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 a dominant-negative form of SH3PX1 or by identifying specific inhibitors of ACK2, will further our understanding of ACK2 activity in receptor endocytosis and degradation and its contribution to malignancy.
References


