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Award Number: DAMD17-02-1-0626

TITLE: Ack-1 Tyrosine Kinase Regulates Integrin Signaling Leading to Breast Cell Migration

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REPORT DATE: March 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2004	Annual Summary (1 Mar 2003 - 28 Feb 2004)

4. TITLE AND SUBTITLE

Ack-1 tyrosine Kinase Regulates Integrin Signaling Leading to Breast Cell Migration

5. FUNDING NUMBERS

DAMD17-02-1-0626

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Appropriate interactions between breast cells and the ECM via $\alpha 2\beta 1$ integrin help to establish normal cellular structure and differentiation. During transformation to a carcinoma, these normal interactions with the ECM are profoundly altered, resulting in cells that lose their specialization and lose control of their growth. Ultimately, these cells become invasive, and then migrate through the connective tissue environment to form distant metastases. We have previously found that Ack-1 tyrosine kinase enhances $\alpha 2\beta 1$ integrin-induced cell migration and regulates signaling components downstream of the integrin. In the course of this research I have determined that Ack-1 interacts with p130Cas and FAK via its SH3 domain, and that the interactions with p130Cas and Crk occur via its proline rich region. I have also determined that the interaction between Ack-1 and Crk occurs in T47D cells in a collagen dependent manner. Additionally, I find that Ack-1 binds to C3G, an exchange factor for the small GTPases Rap and R-Ras. Ack-1 not only binds to these molecules but also modulates their phosphorylation, since over expression of wild type Ack-1 results in hyper-phosphorylation of p130Cas, FAK and C3G. Since phosphorylation of these molecules is crucial for their function and activation of downstream signaling pathways, I am investigating the role of Ack-1 in these processes.

14. SUBJECT TERMS

ACK-1, Non-Receptor Tyrosine Kinase, Integrin, RHO Family GTPASES, Signaling

15. NUMBER OF PAGES

9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Appropriate interactions between breast cells and the extra cellular matrix (ECM) via $\alpha 2\beta 1$ integrin help to establish normal cellular structure and differentiation. During transformation to a carcinoma, these normal interactions with the ECM are profoundly altered, resulting in cells that lose their specialization and lose control of their growth. Ultimately, these cells become invasive, and then migrate through the connective tissue environment to form distant metastases (1,2). We have previously found that Ack-1 (Cdc42 Associated Kinase) tyrosine kinase enhances $\alpha 2\beta 1$ integrin-induced cell migration and regulates signaling components downstream of the integrin. The purpose of this research is to understand the mechanism by which Ack-1 is phosphorylated and how the kinase regulates integrin-dependent signaling leading to cell migration. To do that, I will investigate the role of FAK (Focal Adhesion Kinase) and Src in Ack-1 phosphorylation, as well as screen for Ack-1 binding partners and determine whether these interactions are collagen dependent and whether they play a role in cell migration.

Specific Aims and Research Accomplishments

1) Determine how Ack-1 is phosphorylated upon collagen stimulation and whether this phosphorylation is important in cell migration.

Rationale

Our preliminary results suggested that Ack-1 is phosphorylated upon activation of $\alpha 2\beta 1$ integrin but the mechanism by which this occurs is unknown. It has been established that integrin stimulation leads to activation of FAK and Src. Because Ack-1 participates in a complex with FAK and Src it may be regulated by FAK or Src in a collagen-dependent manner. I will test whether this is accomplished via direct phosphorylation by FAK or by FAK activation of Src, which in turn phosphorylates Ack-1. I will also investigate the importance of Ack-1 phosphorylation in collagen-induced migration.

Research Accomplishments

Since the last report I have not performed additional experiments to address this aim.

2) Define the molecular interactions of Ack-1 and establish their importance in Cdc42-dependent cell migration.

Rationale

In addition to its kinase domain Ack-1 contains a CRIB domain, which is responsible for its interaction with Cdc42 (3), an SH3 domain, and a C-terminus rich in prolines. SH3 domains are known to interact with proline rich motifs. Thus far there are have not been any molecules shown to interact with the Ack-1 SH3 domain. The proline rich region of Ack-1 interacts with clathrin, and is thought to bind to Src kinase (4,5). I will screen libraries of SH3 domains and proline rich domains to identify potential Ack-1 interacting partners. During this stage of my project, I will collaborate with the laboratory of Dr. Brian Kay who is an expert in combinatorial chemistry and specifically protein-protein interactions.

Research Accomplishments

Phage display

The phage display screen resulted in a peptide sequence that did not point me to any obvious candidates that might interact with Ack-1 and mediate its effects on cell migration. I decided not to pursue it any further and concentrate my efforts on the binding partners identified thus far.

Yeast two hybrid

The results from the yeast two hybrid screen generated several potential leads of which I have started to investigate one, E-cadherin, a receptor that mediates cell-cell interactions and affects cell migration. In my attempt to determine whether E-cedherin binds to Ack-1 in T47D cells, I found that it does not, at least not in response to collagen.

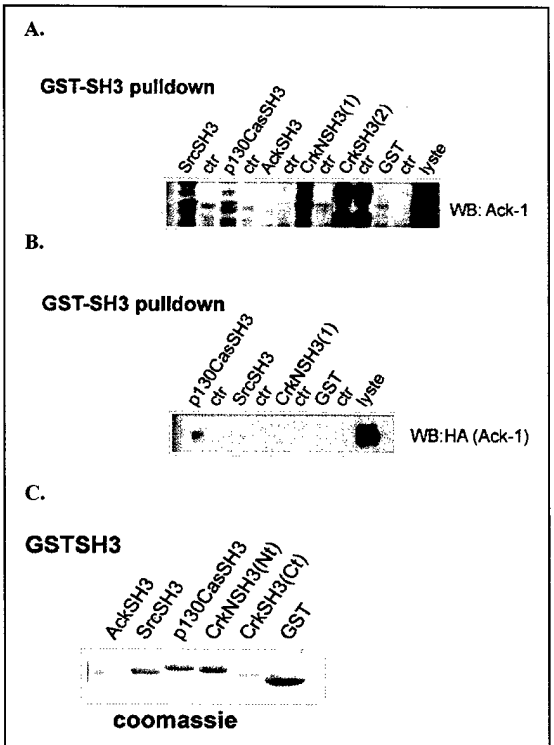

Figure 1 . p130Cas and Crk SH3 domains associate with Ack-1. (A) T47D cells were lysed and incubated with GST fused to the SH3 domains of Src, Ack-1, p130Cas, or Crk. Association with Ack-1 was determined by immunoblotting anti Ack-1 antibody. (B) HEK293 cells transiently transfected with wild type HA-Ack-1 were lysed and incubated with GST-SH3 domain of p130Cas, Src, or Crk. Ack-1 association was determined by immunoblotting with anti-HA antibody. (C) Recombinant GST-SH3 domains adjusted for protein concentration.

SH3 domain screen

I have determined that p130Cas and Crk SH3 domains associate with Ack-1 and that the SH3 domain of Ack-1 binds to p130Cas and FAK.

I have previously determined that p130 Cas co-immunoprecipitates with Ack-1 in a collagen dependent manner. I have also, shown that the SH3 domain of p130Cas can be immunoprecipitated with Ack-1 from cells. Here, I demonstrate that the GST fusion of p130Cas SH3 domain can pull down endogenous Ack-1 (Figure 1 A) or over expresses HA-Ack-1 (Figure 1 B) from cell lysates. In addition, the SH3 domain of Ack-1 fused to GST can also pull down p130Cas. These results suggest that the interaction between Ack-1 and p130 Cas can be mediated through both SH3 domain and the proline rich region of Ack-1.

During the SH3 screen, I also saw an interaction between the N-terminal domain of the adaptor molecule Crk and Ack-1 (Figure 1A). To determine whether this interaction is relevant *in vivo*, I investigated whether they interact in whole cells. I was able to co-immunoprecipitate Crk with Ack-1 from T47D cells in a collagen dependent manner (Figure 3). This suggests that Crk is an additional component of a protein complex that associates with Ack-1 and which is enhanced by collagen stimulation. This finding is relevant because Crk, in addition to p130Cas, has been show to play a key role in cell migration.

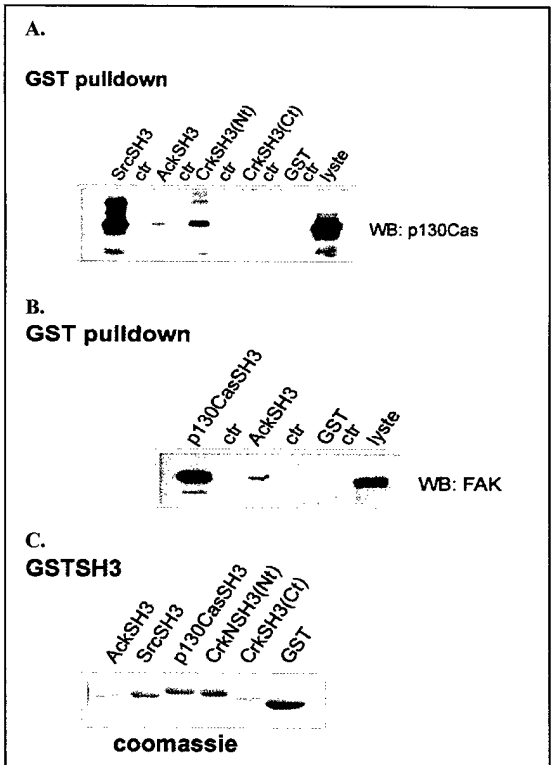

Figure 2 . Ack-1 AH3 domain associates with p130Cas and FAK. T47D cells were lysed and incubated with GST fused to the SH3 domains of Src, Ack-1, p130Cas, or Crk. Association with p130Cas (A) or FAK (B) was determined by immunoblotting with appropriate antibodies. (C) Recombinant GST-SH3 domains adjusted for protein concentration.

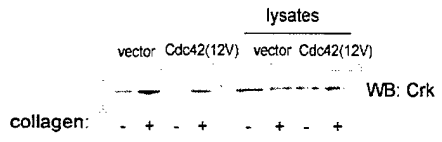

Figure 3. Crk immunoprecipitates with Ack-1 in a collagen dependent manner. T47D cells expressing control vector or activated Cdc42 (12V) were stimulated with collagen or control buffer for 10 minutes in suspension and lysed. Ack-1was immunoprecipitated and association with Crk was determined by immunoblotting with anti-Crk antibody.

In addition, I have previously shown that FAK co-immunoprecipitates with Ack-1 in a collagen dependent manner. Here I demonstrate that this interaction is mediated through the SH3 domain of Ack-1 (Figure 2B).

To obtain the above results, the SH3 domains were expressed as GST fusion proteins and purified from bacteria Figure 2 & 3 (C). Pull down assays were performed with each domain from T47D or 293 HEK cell lysates and association with p130Cas, FAK or Ack-1 was determined by immunoblotting with the appropriate antibody.

3) Determine whether p130Cas is a substrate for Ack-1 and identify whether additional binding partners of Ack-1 are substrates for the kinase.

Rationale

Our preliminary data suggests that Ack-1 interacts with p130Cas and also that p130Cas is phosphorylated in a collagen-dependent manner. The substrate domain of p130Cas is composed of 15 YXXP motifs that are potential phosphorylation sites. I will test whether p130Cas substrate domain serves as a substrate for the Ack-1 kinase. I will also investigate whether any other binding partner of Ack-1 identified during the library screens is a substrate for Ack-1 kinase.

I have determined that over expression of Ack-1 leads to a substantial increase in the phosphorylation levels of p130Cas, FAK and C3G.

To investigate the effect of Ack-1 on signaling molecules implicated in modulating cell migration I have made use of the 293 HEK cells, which are easier to transfect than T47D cells. Over expression of wild type Ack-1 in 293 cells resulted in hyper-phosphorylation of p130Cas, FAK and C3G (Figure 4). This suggests that Ack-1 either directly phosphorylates these molecules or that its kinase activity is required to activate another kinase that in turn phosphorylates these molecules. At this point I do not have a clear idea which one is the case. I have performed initial experiments using PP2, the inhibitor of Src kinases but my results are inconclusive.

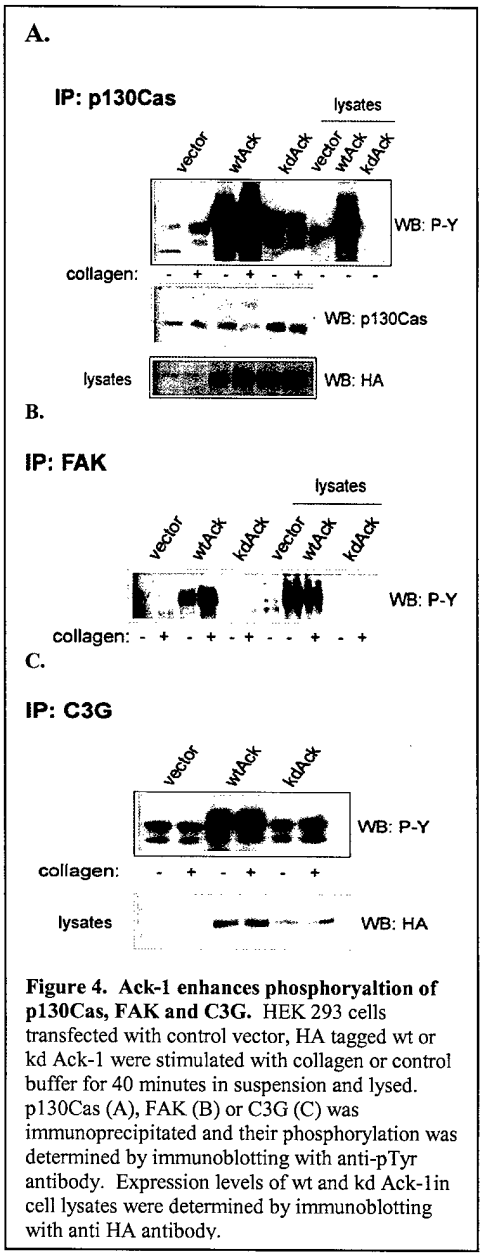

Figure 4. Ack-1 enhances phosphoryaltion of p130Cas, FAK and C3G. HEK 293 cells transfected with control vector, HA tagged wt or kd Ack-1 were stimulated with collagen or control buffer for 40 minutes in suspension and lysed. p130Cas (A), FAK (B) or C3G (C) was immunoprecipitated and their phosphorylation was determined by immunoblotting with anti-pTyr antibody. Expression levels of wt and kd Ack-1in cell lysates were determined by immunoblotting with anti HA antibody.

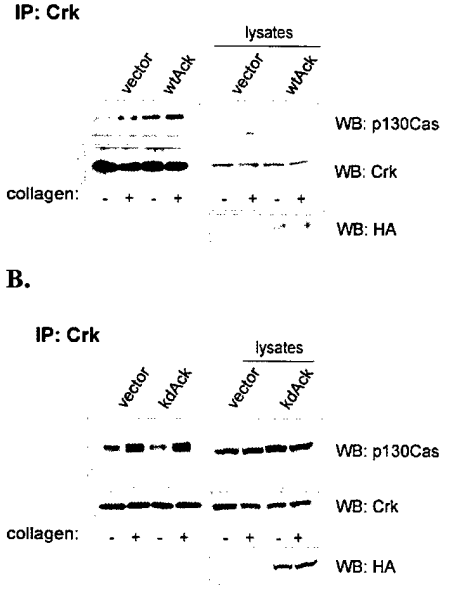

Figure 5. wtAck-1 enhances association of Crk with p130Cas. HEK 293 cells transfected with control vector (A& B), HA tagged wt (A) or kd (B) Ack-1 were stimulated with collagen or control buffer for 40 minutes in suspension and lysed. Crk was immunoprecipitated and its association with p130Cas was determined by immunoblotting with anti-p130Cas antibody. Expression levels of wt and kd Ack-1in cell lysates were determined by immunoblotting with anti HA antibody.

Because Ack-1 affects the phosphorylation of several key molecules implicated in cell migration, I wanted to know whether the kinase plays a role in their downstream signaling. What I found is that over expression of Ack-1 not only leads to hyper-phosphorylation of p130Cas but it also enhances the complex between p130Cas and Crk (Figure 5). This interaction is often described as "a molecular switch" for cell migration, thus this result is consistent with the enhancement of cell migration in cells expressing Ack-1. Crk mediates the interaction between p130Cas and several other molecules involved in cell migration. One such molecule is C3G, and exchange factor for Rap and R-Ras. C3G is activated upon binding to p130Cas-Crk complex as well as by upon phosphorylation. I have found that Ack-1 enhances its phosphorylation (Figure 4) and currently I am in the process of determining whether Ack-1 affect its binding to the p130Cas-Crk complex and the activation of rap and R-Ras.

Key Research Accomplishments

- Found that Crk interacts with Ack-1 in cells in collagen dependent manner
- Determined that Crk and p130Cas SH3 domains interact with Ack-1
- Determined that Ack-1 SH3 domain interacts with p130Cas and FAK
- Established that p130Cas, FAK and C3G phosphorylation are enhanced by Ack-1
- Found that Ack-1 over expression enhances Crk binding to p130Cas

Reportable Outcomes

Guest review article in BBA

Focal adhesion regulation of cell behavior

Michele A. Wozniak, Katarzyna Modzelewska, Lina Kwong and Patricia J. Keely

Poster presentation

The American Society for Cell Biology
43rd Annual Meeting
December 13-17, 2003
San Francisco, CA

Abstract

Ack-1 complexes with FAK and p130Cas to mediate Cdc42-induced cell migration

Katarzyna Modzelewska, Radhika Desai, Laura Newman, Melanie Lo, Luv Javia, and Patricia Keely
Department of Pharmacology, University of Wisconsin, Madison

The loss of epithelial polarization and the acquisition of a migratory phenotype is a key determinant of breast cancer progression ultimately leading to invasion and metastasis. We have previously found that activation of Cdc42, a member of the Rho family of small GTPases, promotes integrin-mediated migration and invasion of breast epithelial cells across collagen matrices (Keely et al, 1997. Nature 390: 632). To elucidate the molecular basis for the effects of Cdc42, we tested whether Cdc42 regulates integrin signaling pathways that lead to cell migration. We found that Cdc42 becomes activated by and is a necessary component of the alpha2beta1 integrin signaling pathway leading to focal adhesion kinase (FAK) and p130Cas activation. Signaling from Cdc42 to FAK and p130Cas was functionally relevant, since dominant negative FAK (FRNK) and p130Cas (p130Cas-SH3) blocked Cdc42-induced cell migration. Moreover, we find a role for the Cdc42 effector, the tyrosine kinase Ack-1. Ack-1 became tyrosine phosphorylated following collagen stimulation, but this phosphorylation was surprisingly independent of Cdc42 activation. Over expression of wild-type Ack-1 significantly enhanced p130Cas and FAK phosphorylation and the kinase dead Ack-1 lead to a small increase in p130Cas and FAK phosphorylation. Consistent with these results, both wild type and kinase dead Ack-1 over expression enhanced Cdc42-induced migration, suggesting a novel role for Ack-1 in regulating cell migration. Finally, co-immunoprecipitation studies suggest the presence of a signaling complex between Ack-1, p130Cas, PI3-kinase, FAK, and Cdc42. Importantly, the formation of this complex was collagen-dependent, even in Cdc42(12V)-expressing cells. The interactions of Ack-1 with its binding partners p130Cas and FAK appear to be direct, since the SH3 domain of p130Cas could be co-immunoprecipitated with Ack-1 in cells and GST-p130CasSH3 could pull down Ack-1 from cell lysates. Conversely, the SH3 domain of Ack-1 pulls down both p130Cas and FAK. Our results suggest a signaling pathway

downstream of the alpha2beta1 integrin by which collagen stimulates the activation of Cdc42, the phosphorylation of Ack-1, and their association with p130Cas and FAK. These signaling events ultimately enhance cell migration.

Conclusions

I have found that in addition to p130Cas, FAK and Src, Ack-1 interacts also with the adaptor molecule Crk and that this interaction occurs in a collagen dependent manner. Additionally, I determined that Ack-1 associates with Crk via its proline region, with FAK via its SH3 domain and with p130 Cas through both the SH3 domain and the proline rich region. Furthermore, I could co-immunoprecipitated ack-1 with C3G and exchange factor for Rap and R-Ras, small GTPases involved in cell migration. Ack-1 not only forms a complex with these molecules but also enhances their phosphorylation. One functional outcome of such hyper-phosphorylation is an increase in binding of Crk to p130Cas in cells over expressing Ack-1. These results suggest that Ack-1 modulates phosphorylation of molecules involved in cell migration thereby affecting the downstream signaling pathways. I am in the process of elucidating the key signaling events modulated by Ack-1.

References

1. Owens, L., Xu, L., Craven, R., Dent, G., Weiner, T., Kornberg, L., Liu, E., and Cance, W. (1995) Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors.*Cancer Research* **55,** 2752-2755
2. Tavassoli, F. A. (1992) *Pathology of the Breast*, Elsevier Science Pub Co, New York
3. Manser, E., Leung, T., Salihuddin, H., Tan, L., and Lim, L. (1993) A non-receptor tyrosine kinase that inhibits the GTPase activity of p21cdc42. *Nature* **363,** 364-367
4. Teo, M., Tan, L., Lim, L., and Manser, E. (2001) The Tyrosine Kinase ACK1 Associates with Clathrin-coated Vesicles through a Binding Motif Shared by Arrestin and Other Adaptors.*J Biol Chem* **276**(21), 18392-8.
5. Hopper, N. A., Lee, J., and Sternberg, P. W. (2000) ARK-1 inhibits EGFR signaling in C. elegans.*Mol Cell* **6**(1), 65-75.