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Award Number: DAMD17-99-1-9351

TITLE: Study of the Regulation of erbB Signaling by Receptor-mediated Endocytosis

PRINCIPAL INVESTIGATOR: Anthony Lee, M.S., B.A.
Mark A. Lemmon, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

REPORT DATE: May 2000

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
	May 2000	Annual Summary (1 May 99 - 30 Apr 00)

4. TITLE AND SUBTITLE
Study of the Regulation of erbB Signaling by Receptor-mediated Endocytosis

5. FUNDING NUMBERS
DAMD17-99-1-9351

6. AUTHOR(S)
Anthony Lee, M.S., B.A.
Mark A. Lemmon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-MAIL:
anthonyl@mail.med.upenn.edu

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

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Endocytosis of erbB receptors is an important regulator of erbB receptor signaling. Given that abnormal erbB signaling may lead to cellular transformation, it is important to understand the factors that determine its regulation. ErbB-2, the erbB receptor that forms heterodimers with all other erbB receptors, is widely implicated in the development of breast cancer. ErbB-2 does not have a known ligand and has an intrinsically slow rate of endocytosis/down-regulation. An attractive hypothesis is that erbB-2 associates with other erbB receptors, forming more persistent signaling complexes because they are down-regulated slower than erbB homodimers. Prolonged, potentiated erbB signaling is thought to contribute to cellular transformation and cancer. It is the goal of this project to study and establish a link between regulation of endocytosis/down-regulation and abnormal signaling that may contribute to cancer development.

14. SUBJECT TERMS
endocytosis, erbB-2, heterodimer, down-regulation

15. NUMBER OF PAGES
12

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

The premise of my project is that co-expression of erbB-2 with EGF receptor (EGFR), or erbB-1, decreases ligand-induced down-regulation of EGFR, and boosts its signaling. The effect of erbB-2 overexpression is likely to contribute to the development and progression of breast cancer. It is thought that such a decrease in the down-regulation of EGFR, a normal consequence of EGFR signaling, allows active receptors to persist in cells and leads to more sustained level of mitogenic signaling, which can result in cellular transformation. Since erbB-2 forms heterodimers with the other erbB receptors, and has an intrinsically lower rate of internalization (Baulinda *et al.*, 1996), it follows that an erbB-1/-2 complex should be internalized slower than an erbB-1 homodimer. My plan is to mutate erbB-2's cytoplasmic domain, which in erbB-1 has been shown to mediate erbB-1 trafficking, in order to increase the rate of erbB-2 endocytosis/down-regulation, and to determine how enhancing erbB-2 internalization might reduce its oncogenecity.

Body

The first task in the statement of work is to make deletion mutants of erbB-1, -3 and –4, by removing cytoplasmic domain C-terminal to their kinase domain, and express them in a suitable cell line. The goal is to assess the importance of the cytoplasmic domain in the regulation of endocytosis/down-regulation of each receptor. Once that is established, then erbB-2 is to be co-transfected into each of the cell lines. This will allow me to evaluate the influence of erbB-2 on each of the receptor. I have changed this plan because of a recent paper from Waterman *et al.* (1999) that established the principle I aimed to show: the cytoplasmic domains of the erbB receptors are responsible for regulating down-regulation. These published studies have allowed me to begin mine with a more advanced line of questioning. Waterman *et al.* replaced the cytoplasmic domain of EGFR, with the corresponding part in erbB-3. The chimeric erbB-1/erbB-3 is down-regulated substantially less than EGFR itself in response to EGF. The reason for the decrease in down-regulation is that the chimeric receptor is diverted from degradation in lysosomes to the recycling pathway. ErbB-3 itself is destined primarily to the recycling pathway (Waterman *et al.*, 1998), so the transfer of the cytoplasmic domain of erbB-3 into erbB-1 confers to the chimeric receptor the endocytosis properties of erbB-3. The decrease in down-regulation leads to elevated level of signaling, which mediates cell growth and survival in 32D cells. In view of this evidence, my first task is less essential because the principle has been established.

Jumping my first task, I have immediately focused on developing a quantitative assay to measure internalization of EGF and other ligands. In my previous work in my

lab I have used immunofluorescence to study the endocytosis of transferrin in the presence of normal and mutant dynamin (Lee *et al.*, 1999). This assay suffers from the fact that it is qualitative. The quantitative assay that I have chosen is an ELISA assay, based on the protocol developed by Sandra Schmid's lab (Smythe *et al.*, 1992). At this point I have the assay working reproducibly in my hands. I can reliably show that internalization of ligands occurs when cells are incubated with ligand and shifted to 37 °C. At the end of paper is a representative result showing internalization of ligand (transferring) in a time-course experiment.

To demonstrate the soundness of the assay, I have measured the internalization of ligands by cells in which wild type and mutant dynamin have been stably transfected. The mutant dynamin, K44A, is a dominant-negative mutant that inhibits receptor-mediated endocytosis in vivo. The prediction is that one will see normal internalization in cells transfected with normal dynamin, but internalization will be suppressed in K44A-transfected cells. This is what we found.

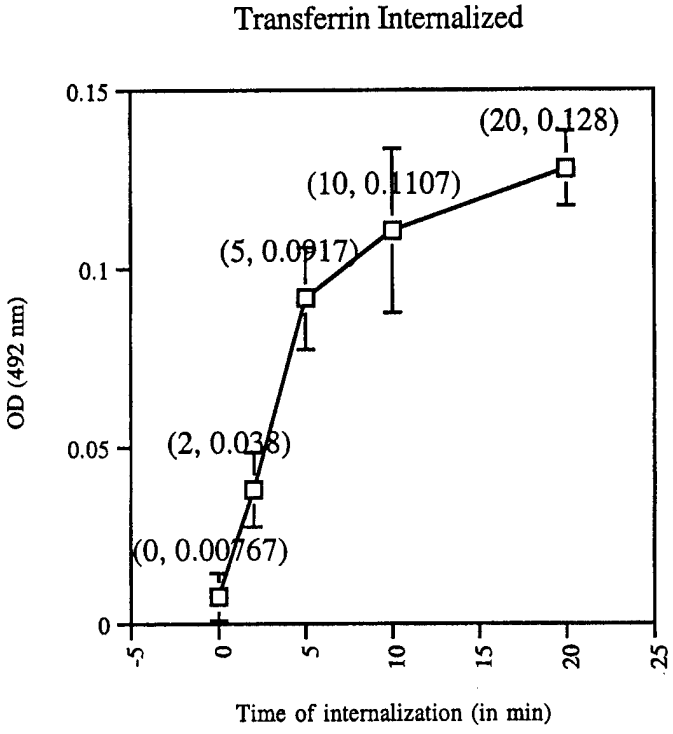
The next phase of my project will be to make mutated erbB-2, by introducing the endocytosis sequence of erbB-1 into erbB-2. I will then co-transfect normal and mutant erbB-2s with other erbB receptors, and study the effects of their overexpression on EGF internalization and signaling in transfected cells. In light of the results of Waterman *et al.*, I shall also do the converse experiment and introduce part of the cytoplasmic end of erbB-2 into that of erbB-1. If, as expected, some or all of the mutations in erbB-2 do change the endocytosis behavior of erbB-2 and that of another erbB receptor that forms heterodimers with erbB-2, then the part of erbB-2 that was replaced can be introduced

into the erbB receptor directly. The prediction is that even in cells without normal erbB-2 the resultant chimeric receptor will behave as if erbB-2 were present.

Internalization of transferrin
In a time-course experiment

time internalization (min)	OD (492 nm) run #1	run #2	run #3	mean	std. dev.
0	0	0.011	0.012	0.00767	0.00665833
2	0.048	0.039	0.027	0.038	0.01053565
5	0.083	0.084	0.108	0.0917	0.01415392
10	0.092	0.103	0.136	0.1107	0.02289833
20	0.135	0.116	0.133	0.128	0.01044031

Internalization of transferrin
In a time-course experiment



Key Research Accomplishments

- development of a quantitative assay that can be used to measure internalization of EGF/EGFR;
- show that the assay is sound with cells that express normal and mutant dynamins.

Reportable Outcomes

None yet.

Conclusions

The principle behind the first task- cytoplasmic domains of erbB receptors are important in the regulation of endocytosis/down-regulation- is shown by Waterman *et al.* (1999). Given that I shifted my focus to the development of a quantitative assay for measuring EGF/EGFR internalization, which will be invaluable later when I begin to introduce normal and mutant erbB-2 into cells expressing other erbB receptors and study the effects of their overexpression on EGF internalization.

"So What"

To show that motifs in erbB receptors that mediate internalization/down-regulation also affect their signaling, because aberrant signaling of erbB receptors can lead to cellular transformation, it is essential that a sensitive assay that measures small difference in endocytosis is developed. This way, one can meaningfully compare behavior of cells expressing normal and mutant erbB-2. If we can establish that the oncogenecity of erbB-2 depends on its internalization characteristics, this would argue that the endocytosis machinery could be a useful drug target.

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