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13. ABSTRACT *(Maximum 200 Words)*
Award DAMD17-99-1-9367 seeks to understand the role of membrane trafficking in Epidermal Growth Factor Receptor (EGFR) signal transduction. We have been using a tissue culture model system (HeLa cells) to isolate the activated EGFR at distinct stages in the endocytic pathway. Our research has focused on rab5, a small molecular weight GTPase, implicated in the biogenesis of the early endosome. Mutations to modulate the guanine nucleotide binding properties of this protein have been reported for constituitvely internalized receptors, but little is known about its role in EGFR endocytic trafficking. In the past year, we have discovered that expression of a 'dominant negative' rab5 (rab5(S34N)), has no effect on EGFR endocytosis, but inhibits entry of the EGFR into the early endosome. The functional consequence of this mutation is slowed rate of EGFR degradation. Additionally, we have found that the guanine nucleotide binding state of the rab5 proteins dictates the ability of the cells to mediate EGFR-dependent cell growth. Cells expressing rab5 that preferentially bindings GDP growth significantly better than cells expressing rab5 that binds GTP.

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Introduction:

The signal transduction specificity that underlies Epidermal Growth Factor (EGF) Receptor (EGFR) physiology is an important component of tissue growth and development. Improper regulation of signal transduction by the EGFR and related family members is characteristic of many mammary carcinomas. Identifying the basic molecular mechanisms that regulate this signaling network is an important step in distinguishing the difference between positive (normal growth) and negative (uncontrolled cell proliferation) EGFR-mediated cell biology. A well described phenomenon that accompanies EGFR signaling is the entry of the activated EGFR into the cell via clathrin coated vesicles (1) Inhibition of EGFR internalization results in the selective inhibition of some, but not all, signaling pathways (2-5). The purpose of this research is to further explore the spatial and temporal components of EGFR signal transduction by examining the signaling properties of the activated EGFR at various points in the endocytic pathway. The scope of this research is to identify membrane trafficking proteins involved in EGFR endocytosis and to use mutant forms of these membrane trafficking proteins to develop a model system that permits isolation of the EGFR at discrete endocytic stages. Our long-term goal is to determine the biochemical characteristics and the signaling properties of the EGFR, and other members of the EGFR family, namely ErbB2, at these discrete endosomal stages.

Body:

Growth Factors and their corresponding cell surface receptors are important components in the maintenance and growth of normal epithelial cells. Overexpression and unregulated signaling of members of the ErbB growth factor receptor family is associated with many mammary carcinomas and a poor prognosis for recovery (6,7). This proposed research seeks to better understand the coordinated regulation of signaling by the ErbB family of cell surface receptors, and more specifically, ErbB1 or the Epidermal Growth Factor (EGF) Receptor (EGFR). The EGFR, like many cell surface receptors, undergoes ligand-mediated endocytosis. This internalization process has historically been thought to be merely a mechanism for attenuating receptor signaling by removing the activated receptor and ligand from the cell surface and dissociating the ligand/receptor complex. It is now appreciated that the role of the endocytic pathways plays a complex regulatory role in EGFR signaling (2-5). Our hypothesis is that each stage in the endocytic pathway uniquely regulates EGFR signaling. The goal of our research is to overexpress proteins involved in endocytic trafficking to disrupt normal endocytic trafficking of the EGFR allowing for analysis of EGFR signaling at distinct cellular locations.

Specific Aim I

Our first Specific Aim was to determine the role of rab5 in the endocytic trafficking of the EGFR. Based on reports in the literature, we had suspected that an activating mutant of rab5 would cause accumulation of the EGFR in the early endosome. That turned out not to be the case (8,9) and we have since examined the role of the inactivating mutant of rab5 to accumulate the EGFR in the pre-early endosomal compartment.

The study of the role of rab5 in regulating EGFR endocytic trafficking has resulted in several important and surprising findings. We have utilized a tissue culture model system to overexpress wild type and mutant rab5 proteins by adenoviral expression. Rab5 is a small molecular weight GTP-binding protein found at the plasma membrane and the early endosome that is functionally important in the biogenesis of the early endosome (10,11). The role of these proteins in regulating

endocytic trafficking has been controversial. Data support a role for rab5 at the plasma membrane regulating endocytosis and at the early endosome regulating vesicular fusion. To determine the role of rab5 in EGFR endocytic trafficking, we utilized to different mutant forms of rab5. A point mutation of glutamine to lysine mutation at residue 79 – denoted rab5(Q79L) reduces the ability of rab5 to hydrolyze GTP thus, leaving the protein in the constituitively active state. Mutation of serine to asparagine at amino acid 34 (rab5(S34N)) results in a protein that preferentially binding GDP, putting the protein in an inactive form. We have studied the consequence of expression of these proteins on EGFR internalization, subcellular localization, and the consequence on cell proliferation. We have found that neither activating and inactivating mutations in rab5 have any effect on EGFR endocytosis. However, expression of rab5(S34N) changes the subcellular localization of the EGFR and results in a slowed kinetics of degradation. The results of these studies has been written up in a manuscript that was submitted to the journal, *Molecular Biology of the Cell* (12). (See appendix for the submitted manuscript).

Specific Aim II

Specific Aim II tests the hypothesis that EGFR signaling is regulated by the cellular location of the EGFR. To answer this question, we have used the mutant forms of rab5 described in Aim I, to examine changes in EGFR signaling from early endosome (rab5(Q79L)) and in a pre-early endosomal compartment (rab5(S34N)). Due to our unexpected findings with rab5 and the additional time required to characterize the rab5(S34N) mutant, we were only able to assess EGFR signaling in a limited number of assays. We were able to compare EGFR-mediated auto-tyrosine phosphorylation, MAPK activity, and cell proliferation (Figure 1). These data indicate that the guanine nucleotide binding of rab5 effects the EGFR's ability to sustain phosphorylation, MAPK activity, and growth. Cells expressing rab5(Q79L) are either rapidly phosphorylated and dephosphorylated or unable to sustain phosphorylation as indicated from phosphotyrosine analysis of the EGFR. This is reflected by a decrease in the EGFR-stimulated MAPK activity in the

presence of rab5(Q79L). Conversely, when rab5(S34N) is expressed there is no significant change in ligand-mediated EGFR tyrosine phosphorylation, however MAP Kinase activity is slightly higher and more sustained. This is likely due to the slowed rate of EGFR degradation (see submitted manuscript in appendix). The changes in phosphorylation are also reflected in changes in EGFR-dependent cell growth/survival. This effects is specific of EGFR-mediated cells growth as cells grown in 5% FBS are similar for all conditions. We are currently planning experiments that will provide additional measures of EGFR signaling.

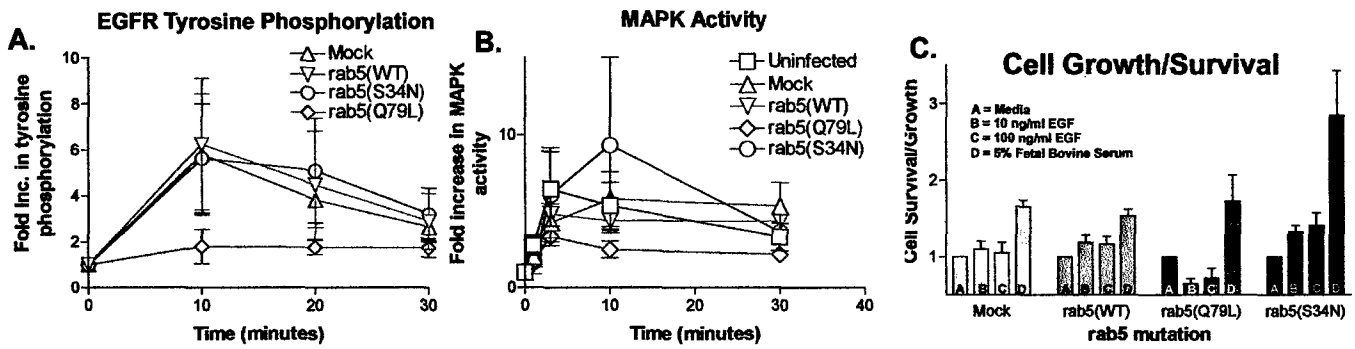

Figure 1. Consequence of expression of wild type and mutant rab5 proteins on EGFR mediated signal transduction. tTA-HeLa cells were infected with the indicated adenoviral constructs. A. Cells were treated with 10 ng/ml EGF for the indicated time, cell lysates were made, run on a gel and the amount of tyrosine phosphorylation was determined by an anti-phosphotyrosine western blot. The amount of tyrosine phosphorylation was determined by analysis of the chemiluminescent intensity using a UV Products Imaging System. B. MAP Kinase activity in the infected and EGF treated cells was determined by immunoprecipitating the MAP kinase and performing an in vitro MAP Kinase assay [Ceresa, 1997 #153]. For figures A and B the data shown are the mean ± S.E.M. from 3-5 experiments. C. Cells were infected with the indicated constructs and then replated at a density of 5,000 cells/well in a 96 well dish. Cells were treated as indicated for 72 hours and assays for the number of living cells using a standard MTT assay [Hansen, 1989 #211]. Data shown are the mean ±

Specific Aim III

This specific aim seeks to examine ht signaling properties of ErbB2, a member of the EGFR family of receptor tyrsoine kinases with no known ligand. The primary focus of this research has been to identify a cell line in which to study ErbB2 signaling. We have examined several candidate cell lines, including HeLa cells, MCF-7 cells, HepG2 cells, and PC12 cells. None of those cell lines had an appreciable number of ErbB2 receptors. Thus, we were limited in our progress in this avenue of research. We are currently making a cell line that will be appropriate for these studies in conjunction with Dr. XiaoHe Yang (Assistant Professor, Department of Pathology, OUHSC).

Key Research Accomplishments – July 1, 2001 - June 30, 2002 :

- Characterization of rab5(S34N) in tTA-HeLa cells. Manuscript submitted to Molecular Biology of the Cell, abstract presented at FASEB Summer Research Conference – "Receptors and Signal Transduction".

- Finding that the guanine nucleotide binding state of rab5 dictates the potential of the EGFR to mediate cell growth. Manuscript submitted to Molecular Biology of the Cell, abstract presented at FASEB Summer Research Conference – "Receptors and Signal Transduction".

- Generation and characterization of GFP-tagged rab5(WT), rab5(Q79L), and rab5(S34N) cDNA constructs.

- Demonstration that expression of rab5(S34N) does not inhibit EGFR endocytosis, but does slow the rate of EGFR degradation, suggesting that rab5 regulates EGFR endocytic trafficking at a location distal from the plasma membrane.

- Initiation of studies that examine EGFR-mediated signal transduction from a pre-endosomal compartment.

- Initial analysis of EGFR tyrosine phosphorylation and MAPK activity in cells with altered EGFR endocytic trafficking.

Key Research Accomplishments - July 1, 2000 - June 30, 2001

- Characterization of rab5(WT) and rab5(Q79L) in tTA-Hela cells.

- Characterization of rab5(WT) and rab5(Q79L) in tTA-HepG2 cells.

- Generation and characterization of rab5(S34N) adenoviruses in tTA-HeLa cells.

- Demonstration that expression of rab5(S34N) does not inhibit EGFR or fluid phase endocytosis, but does inhibit entry into the early endosome.

Key Research Accomplishments - July 1, 1999 - June 30, 2000

- Characterization of stable rab5(WT) and rab5(Q79L) HeLa cells lines.

- Generation of tetracycline regulatable adenoviruses encoding for rab5(WT) and rab5(Q79L).

- Demonstration that the rab5(Q79) formed enlarged endosome is retains its functionality.

- Demonstration that the rab5(Q79) formed enlarged endosome is not an accurate predictor of changes in membrane trafficking.

- **Reportable Outcomes**

Manuscripts:

B. P. Ceresa, B. P. and S. L. Schmid, Regulation of signal transduction by endocytosis (2000) *Curr Opinion in Cell Biology*, p 204-210.

B. P. Ceresa, M. Lotscher, and S. L. Schmid, Receptor and membrane Recycline can Occur with Unaltered Efficiency Despite Dramatic Rab5(Q79L)-induced Changes in Endosome Geometry (2001) *J Biol Chem* 276 p 9649-9654.

• J. L. Dinneen and B. P. Ceresa, Dominant Negative rab5 alters post-membrane EGFR trafficking in HeLa cells – *Submitted to Molecular Biology of the Cell* July 2002

Abstracts:

B. P. Ceresa and S. L. Schmid, Expression of rab5(Q79L)-induced enlarged endosomes does not alter Epdermal Growth Factor Receptor Endocytic Trafficking. Abstract #5023 at 2001 Annual meeting of the American Association of Cancer Researchers, New Orleans, LA

J. L. Dinneen and B. P. Ceresa, dominant negative rab5(S34N) inhibits EGFR endocytic trafficking to the early endosome. Abstract # 8 at 2002 FASEB Summer Research Conference – "Receptors and Signal Transduction", Salt Lake City, UT.

cDNA constructs:

We have made chimeric proteins that encode for the green fluorescent protein (GFP) fused to the rab5(WT), rab5(Q79L), and rab5(S34N). These constructs were generated to help us characterize the function of rab5(S34N) in EGFR endocytic trafficking.

Cell lines Developed:

Human hepatoma cells (HepG2 cells) were stably transfected to express the tetracycline transactivator – tTA-HepG2. By stably expressing the tetracycline transactivator, we can express our rab5 adenoviruses that are under the control of a tetracycline inducible promoter. This new cellular environment allows us to examine the consequences of the various rab5 mutants on the endocytic trafficking of endogenous EGFR.

Adenoviruses Generated:

We have generated tetracycline regulatable adenoviruses that encode for HA-rab5(S34N), HA-rab7(WT), HA-rab7(Q67L), and rab7(N125I).

Conclusion

Through the funding of this grant, we have made signficant progress in understand the interplay between endocytic trafficking and growth factor receptor signaling. In particular, we have defined a role for rab5 in EGFR endocytic trafficking. Expression of dominant negative rab5 inhibits entry of the EGFR into the early endosome. We are in the process of assessing the consequence of this mislocalized EGFR in EGFR-mediated signal transduction. The field remains controversial in terms of the molecular role of rab5 in endocytic trafficking with literature supporting a cellular function at the plasma membrane and the early endosome. Our findings have contributed to this field by using a multidisciplinary approach to show rab5 functions to regulate EGFR endocytic trafficking at the level of the early endosome.

Preliminary data indicates that EGFRs from this pre-early endosomal compartment still retain their ability to signal and may in fact have enhanced EGFR-mediated tyrosine phosphorylation, MAPK activity and cell proliferation. These exciting findings are indicative that endocytosis is a regulator of EGFR-mediated signaling. We are continuing to pursue this line of investigation by looking at additional signaling pathways that may be effected by the changes in endocytic trafficking.

We have been disappointed by our inability to identify a cell line in which we can biochemically study the endocytic trafficking of ErbB. Currently, we are collaborating with Dr. XiaoHe Yang (Assistant Professor, Department of Pathology, OUHSC) who is developing a number of cell lines that express varying ratios of the EGFR and ErbB2. Once he has generated and characterized these cell lines, we will use them for studying the endocytic trafficking of ErbB2.

The medical and scientific significance of this study is that we have identified a key regulatory protein, rab5, in the endocytosis of the EGFR. By expressing a dominant negative form of rab5, we can examine the signal transduction properties of the EGFR from a distinct cellular location. Our preliminary experiments have revealed that infact when we express this

protein that we do see changes in the EGFR-mediated signal transduction. We are currently in the process of evaluating those changes. Additionally, we have found when the EGFR is retained in the early endosome, there is dramatic inhibition in EGFR-mediated cell survival. Since this is seen with EGFR stimulation and not growth in 5% FBS, we postulate that this may provide insight into how EGFRs can cause cell death. Both of these findings will be useful in our goal of understand EGFR specificity.

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Appendix to Summary:

Appendix A - Abstracts

B. P. Ceresa and S. L. Schmid, Expression of rab5(Q79L)-induced enlarged endosomes does not alter Epidermal Growth Factor Receptor Endocytic Trafficking. Abstract #5023 at 2001 Annual Meeting of the American Association of Cancer Researchers, New Orleans, LA

J. L. Dinneen and B. P. Ceresa, dominant negative rab5(S34N) inhibits EGFR endocytic trafficking to the early endosome. Abstract # 8 at 2002 FASEB Summer Research Conference – "Receptors and Signal Transduction", Salt Lake City, UT.

Appendix B – Publications

B. P. Ceresa, M. Lotscher, and S. L. Schmid, (2001) *J Biol Chem* 276 p 9649-9654. (3 pages)

• J. L. Dinneen and B. P. Ceresa, *Submitted to Molecular Biology of the Cell* July 2002 (44 pages)

Appendix A
Abstract
2001 Annual Meeting of the American Association of Cancer Researchers

Abstract from the 2001 American Association of Cancer Researchers Meeting

Back
5023

Expression of Rab(Q79L)-Induced Enlarged Endosomes Does Not Alter Epidermal Growth Factor Receptor Endocytic Trafficking

Brian P. Ceresa, Sandra L. Schmid, Oklahoma University Health Sciences Center, Oklahoma City, OK; The Scripps Research Institute, La Jolla, CA.

As part of its molecular physiology, the EGFR enters the endocytic pathway upon ligand binding and subsequent activation. It has been demonstrated by numerous investigators that EGFR entry into the endocytic pathway is required for activation of some, but not all, EGFR mediated signal transduction. In an effort to further explore the role of the endocytic pathway in EGFR signal transduction, we examined EGFR trafficking in to and out of the early endosome with the goal of being able to explore EGFR signal transduction from that intracellular compartment. Rab5 is a small molecular weight guanine nucleotide binding protein involved in the formation of the early endosome. Expression of a constituitively active form of rab5 (glutamine to alanine mutation at amino acid residue 79 - denoted rab5(Q79L)) has been reported to induce the formation enlarged endosomes and result in the net accumulation of the transferrin receptor in the early endosome. Since this phenotype mirrored our desired results with the EGFR, we engineered a tetracycline-regulatable adenoviral expression system to introduce both wild type and constituitively active mutant rab5 into HepG2 cells. Overexpression of rab5(Q79L) produced the characteristic enlarged endosomal morphology. These endosomes could effectively traffic EGFR as evidenced by indirect immunofluorescent staining of the EGFR in the enlarged endosome after EGF treatment. However, there was no net accumulation of the EGFR in the endosomes as indicated by the unaltered rates of 125I-EGF endocytosis and recycling. Thus, rab5 is plays a role in the trafficking of EGFRs, but does not exert the same regulatory component in endocytosis that has been demonstrated with the transferrin receptor.

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Online Publication Date: February 27, 2001.
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Abstract
2002 FASEB Summer Research Conference
Salt Lake City, UT

Characters: 1422.

Dominant Negative rab5(S34N) Inhibits EGFR Endocytic Trafficking to the Early Endosome

Jennifer L. Dinneen, Brian P. Ceresa University of Oklahoma Health Sciences Center

Endocytosis is an important regulatory mechanism in the signaling of epidermal growth factor receptors (EGFR). Not only is endocytosis a mechanism to dissociate ligand/receptor complexes and degrade receptors, it also modulates downstream receptor/effector communication. In this report we examine the role of the small molecular weight guanine nucleotide binding protein, rab5, in the endocytic trafficking of the EGFR. It is clear rab5 functions in the biogenesis of the early endosome, however, its exact molecular role is a subject of debate. Literature exists that supports a role for rab5 in vesicles budding from the plasma membrane as well as fusion of vesicles to the early endosome. Using adenovirally expressed wild type, dominant negative, and constitutively active rab5 (rab5(WT), rab5(S34N) and rab5(Q79L)) in HeLa and HepG2 cells, we have found that rab5 activity has no bearing on the rate of EGFR endocytosis. However, EGFR import into the early endosome is inhibited by expression of rab5(S34N). Additionally, this alteration of EGFR endocytic trafficking results in a slowed rate of EGFR degradation. Thus, rab5 is a regulator of endogenous EGFR endocytic trafficking to the early endosome.

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Appendix B (part 1 of 2)

Receptor and Membrane Recycling Can Occur with Unaltered Efficiency Despite Dramatic Rab5(Q79L)-induced Changes in Endosome Geometry*

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Published, JBC Papers in Press, January 2, 2001, DOI 10.1074/jbc.M010387200

Brian P. Ceresa‡, Marius Lotscher§, and Sandra L. Schmid¶

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Current models for sorting in the endosomal compartment suggest that endosomal geometry plays a significant role as membrane-bound proteins accumulate in tubular regions for recycling, and lumenal markers accumulate in large vacuolar portions for delivery to lysosomes. Rab5, a small molecular weight GTPase, functions in the formation and maintenance of the early/sorting endosome. Overexpression of the constitutively active form, Rab5(Q79L), leads to enhanced endosome fusion resulting in the enlargement of early endosomes. Using an adenoviral expression system to regulate the time and level of Rab5(Q79L) overexpression in HeLa cells, we find that although endosomes are dramatically enlarged, the rates of transferrin receptor-mediated endocytosis and recycling are unaffected. Moreover, despite the enlarged endosome phenotype, neither the rate of internalization of a fluid phase marker nor the rate of recycling of a bulk lipid marker were affected. These results suggest that GTP hydrolysis by Rab5 is rate-limiting for endosome fusion but not for endocytic trafficking and that early endosome geometry may be a less critical determinant of sorting efficiencies than previously thought.

Endocytic vesicles deliver their content of membrane proteins, lipids, and lumenal content to the early or sorting endosomal compartment consisting of tubular and vacuolar portions. Many receptor-ligand complexes dissociate in the mildly acidic environment of the early endosome (1). It has been proposed (1, 2) that endosomal morphology and resulting geometric considerations play a major role in controlling sorting efficiency in the early endosome. In this model, membrane proteins destined for recycling accumulate in long tubular extensions of the early endosome, which have a high surface to volume ratio. Fluid phase content including released ligands is deposited in the vacuolar portions of the early endosome, which, being spherical, approach a minimum surface to volume ratio. These vacuolar portions dissociate from tubular regions to carry their contents to late endosomes and/or lysosomes (3).

Rab5 is a small molecular weight GTPase associated with the plasma membrane and early/sorting endosomes. Rab5 controls homotypic early endosome fusion and thus functions in the formation of early endosomes (4–6). A point mutation in the GTPase domain (glutamine to leucine; denoted as Rab5(Q79L)) reduces Rab5 GTPase activity and results in a mutant Rab5 with an increased propensity to be in the active, GTP-bound state (7, 8). Expression of this constitutively active form of Rab5 enhances homotypic endosome fusion leading to the formation of enlarged early endosomes. It also has been reported that Rab5(Q79L) overexpression increases the rate of transferrin receptor uptake and decreases the rate of transferrin receptor recycling (8) although the mechanism for these effects remains obscure.

Rab5 is preferentially associated with the vacuolar portions of the early endosome (9), and Rab5(Q79L) overexpression leads to the formation of large spherical endosomes as visualized in semithick sections by electron microscopy (10). To test whether the Rab5(Q79L) effects on transferrin receptor endocytosis and recycling can be correlated with these dramatic changes in endosomal size and geometry, we utilized a tetracycline-regulatable adenoviral expression system that allows us to temporally control Rab5(Q79L) expression levels. The early endosomal compartment was dramatically enlarged in adenovirally infected HeLa cells overexpressing Rab5(Q79L). However, the presence of these enlarged endosomes did not alter the kinetics of endocytic membrane trafficking of either cell surface receptors or bulk membrane lipids. These unexpected results argue that geometric considerations may contribute to a lesser extent than previously assumed in determining the sorting and recycling efficiencies of the early endosomal compartment.

MATERIALS AND METHODS

Cell Culture—tTA-HeLa cells were cultured in DMEM[1] supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin (growth medium). Wild type and mutant (canine) Rab5a constructs (a gift of M. Zerial) were tagged with the hemagglutinin epitope on the amino terminus and subcloned into pUHD expression vectors (11). HA-Rab5(WT)- and HA-Rab5(Q79L)-expressing cells were generated by cotransfecting the tTA-HeLa cells with cDNA that encodes HA-Rab5(WT) or HA-Rab5(Q79L) (10 μg) and the plasmid pBSpac (0.5 μg) by calcium phosphate transfection. Positive clones were selected by culture in 200 ng/ml puromycin, 400 μg/ml G418, and 2 μg/ml tetracycline and screened by Western blot for their abilities to express Rab5 48 h after induction by the removal of tetracycline (11).

Stably transfected cells were cultured in the presence of 1 μg/ml tetracycline. Wild type or mutant Rab5 expression was induced by

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[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; PBS, phosphate-buffered saline; WT, wild type; HRP, horseradish peroxidase; Tfn, transferrin; TfnR, transferrin receptor; B-XX-Tfn, biotinylated Tfn; C_6-NBD-SM, N-((6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino) hexanoyl)-sphingosyl phosphocholine.

washing out the existing tetracycline with two PBS (phosphate-buffered saline, pH 7.4) washes and incubating the cells in growth medium without tetracycline for 48 h.

Alexa-Transferrin Labeling and Immunofluorescence—Cells, grown on coverslips to ~70% confluency, were washed twice with room temperature PBS and then incubated with 50 μg/ml Alexa-transferrin (Molecular Probes) in PBS^{++} (PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.2% bovine serum albumin) for the indicated times at 37 °C. Coverslips were moved to 4 °C, washed twice with ice-cold PBS^{++++} and three times with ice-cold citrate buffer, and re-equilibrated with two additional ice-cold PBS^{++++} washes (12). The coverslips were fixed in a 4% formaldehyde/PBS^{++} (PBS, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) solution at room temperature for 5 min and on ice for an additional 15 min. Excess formaldehyde was removed with 3 × 5-min washes in PBS^{++}. Cells were permeabilized in 0.1% saponin/5% goat serum/PBS^{++} for 15 min. After 3 × 5-min PBS^{++} washes, the coverslips were incubated with primary antibody for 1 h. Antibodies used (source in parentheses) were mouse monoclonal anti-HA tag 12CA5 (Ian Wilson, The Scripps Research Institute) and mouse monoclonal anti-Rab5 (Transduction Laboratories). Unbound primary antibody was removed with 3 × 5-min PBS^{++} washes, and the coverslips were incubated with the appropriate secondary antibody (noted in figure legends). Coverslips were subjected to 6 × 5-min PBS^{++} washes, rinsed in Millipore water, and mounted on a coverslip slide using Fluoromount G (EM Sciences).

Western Blot Detection of HA-Rab5—35-mm dishes of cells were washed twice in room temperature PBS and put on ice with PBS to cool to 4 °C for 5 min. Cells were then harvested in 500 μl of ice-cold solubilization buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM phenylmethylsulfonyl fluoride). Lysates were solubilized by gently rocking for 10 min at 4 °C and centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 10 min at 4 °C to pellet the insoluble material. Protein concentration was determined by BCA, and 100 μg of solubilized protein was run on 13% SDS-polyacrylamide gel electrophoresis mini-gel, transferred to nitrocellulose, and immunoblotted using monoclonal antibodies against Rab5 or 12CA5 as above. Proteins were detected using secondary goat anti-mouse antibodies conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence.

Adenovirus Generation—HA-Rab5(WT) or HA-Rab5(Q79L) was put under the control of a tetracycline-regulatable promoter in the pAdlox vector 3′ to the Ψ5 packaging site and 5′ to the poly(A) site. Adenoviruses were generated as previously described (13). Prior to use in experiments, adenoviruses were plaque-purified to a single viral population and then amplified.

Adenoviral Infection—In experiments in which cells expressed Rab5 continuously for 18 h, tTA-HeLa cells at ~70% confluency were infected with adenovirus at an m.o.i. of 10 plaque-forming units/cell. Cells were infected with adenovirus in DMEM with or without 1 μg/ml tetracycline for 2 h at 37 °C. After infection, the viral medium was removed and replaced with growth medium with or without tetracycline.

When Rab5 expression was studied using a bolus concentration, tTA-HeLa cells at ~70% confluency were infected with adenovirus at an m.o.i. of 300 plaque-forming units/cell. Infection took place at 37 °C for 2 h in DMEM, and the adenovirus-containing medium was removed and replaced with growth medium containing 10 μg/ml cycloheximide for 3 h to prevent protein but not mRNA synthesis. Cycloheximide was washed out with three washes of DMEM, and the cells were returned to 37 °C incubation with growth medium for the indicated times, typically 1, 3, or 12 h. At all time points, the cells remained adherent to the dish without any obvious signs of toxicity or cell death.

Transferrin Uptake and Recycling—Infected cells were assayed for transferrin uptake and recycling as previously described (14) with minor modifications. To measure a single round of transferrin receptor endocytosis, harvested cells were preincubated for 1 h with 4 μg/ml B-XX-Tfn. Prior to internalization, unbound ligand was removed by three washes in ice-cold PBS^{++++} (PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 0.2% bovine serum albumin, pH 7.4). Cells were resuspended to a concentration of 3–4 × 10^6 cells/ml and incubated at 37 °C for the indicated times. B-XX-Tfn trafficking was stopped by transferring a 50-μl aliquot of the cell suspension to an Eppendorf tube containing 750 μl of ice-cold PBS^{++++}. Internalized B-XX-Tfn was determined using an enzyme-linked immunosorbent assay-based assay as previously described (14).

Horseradish Peroxidase (HRP) Uptake—Fluid phase uptake was performed on infected 35-mm dishes of cells as previously described (15, 16).

Lipid Recycling—Lipid recycling was performed as described by Hao and Maxfield (17). Quantitation of C_6-NBD-SM in medium or in cell lysates was performed using a PerkinElmer Life Sciences fluorimeter at an excitation wavelength of 465 nm and measuring the peak height between 518 and 558 nm. Data are plotted as the C_6-NBD-SM that remains cell-associated at each time point.

RESULTS AND DISCUSSION

Expression of HA-Rab5(Q79L) Induces Enlarged Endosomes in Stably Transformed Cells—To begin to probe the mechanism of Rab5(Q79L) effects on TfnR endocytosis and recycling, we generated stably transformed cell lines expressing HA-tagged wild type Rab5 (HA-Rab5(WT)) and constitutively active HA-Rab5(Q79L) under the control of a tetracycline-responsive expression system. Using this system, stable cell lines can be generated while avoiding any deleterious effects that may result from continuously altering cellular membrane trafficking (11). Stable cell lines generated in this manner express either HA-Rab5(WT) or HA-Rab5(Q79L) in a tetracycline-regulated manner as determined by Western blotting using either the 12CA5 anti-HA antibody (not shown) or antibodies against Rab5, which reveal both the more slowly migrating recombinant HA-tagged protein and the endogenous Rab5 (Fig. 1*A*). In these stably transformed cells, recombinant HA-Rab5 is expressed at roughly equimolar levels compared with endogenous protein. Expression of Rab5(WT) at these levels did not affect endosome morphology (Fig. 1*B*, *upper left*), whereas expression of Rab5(Q79L) at these levels was sufficient to cause the expected morphological phenotype—enlarged endosomes (*upper right*). Expression of HA-Rab5(Q79L) was suppressed when cells were cultured in the presence of tetracycline (*upper middle panel*). In all cases, the endosomes were functional in that fluorescently labeled Tfn was internalized and delivered to them (Fig. 1*B*, *lower panels*). Unexpectedly, examination of the single-round uptake and recycling kinetics of Tfn revealed that despite formation of enlarged endosomes there was no perturbation of Tfn uptake in cells induced to express either WT or mutant Rab5 compared with uninduced control cells (Fig. 1*C*). Similarly, there was no change in the steady state accumulation of Tfn receptors in this endosomal compartment. These data suggested that the enlarged endosome phenotype is not predictive of defects in endocytic membrane trafficking.

Adenovirus-mediated, Tetracycline-regulated Expression of Rab5(Q79L)—Given that a threshold level of Rab5(Q79L) expression may be required to cause changes in membrane trafficking (8), it remained possible that the lack of changes in TfnR trafficking was due to low levels of exogenous protein expression. Consequently, we elected to employ an adenoviral expression system to obtain reproducibly and uniformly higher levels of overexpression. In addition, adenovirus allows for the rapid induction of high levels of protein, thus circumventing problems that may occur as a result of chronic exposure of a foreign protein to the cell. The adenoviral expression system was designed to retain the tetracycline regulation so that any potential adenovirus effects could be controlled by infection of cells in the presence of tetracycline.

When cultured under inducing conditions for 18 h in the absence of tetracycline, tTA-HeLa cells infected with recombinant adenoviruses encoding either WT or mutant Rab5 expressed 50–100-fold higher levels of the desired protein compared with endogenous Rab5 (Fig. 2*A*). Importantly, WT and mutant Rab5 expression was not detectable when infected cells were cultured in the presence of tetracycline. As expected at these high levels of overexpression, the characteristically enlarged endosomal morphology was readily apparent in cells expressing HA-Rab5(Q79L) (Fig. 2*B*). These enlarged endosomes remained accessible to internalized Tfn as indicated by the colocalization of fluorescently labeled transferrin (Alexa-Tfn) and Rab5 containing vesicles stained with an antibody

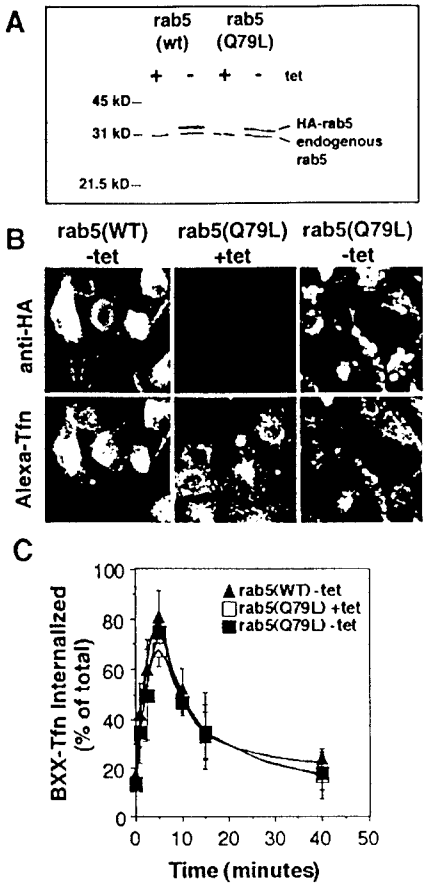

FIG. 1. **HA-Rab5(Q79L) expression in stably transformed tTA-HeLa cells causes enlarged endosomes but does not effect Tfn endocytosis or recycling.** tTA-HeLa cells stably expressing either HA-Rab5(WT) or HA-Rab5(Q79L) under control of a tetracycline (*tet*)-regulatable promoter were clonally selected as described under "Materials and Methods." Cells were incubated in the absence (induced) or presence (uninduced) of 1 μg/ml tetracycline for 48 h. *A*, immunoblots of cell lysates probed with antibodies against Rab5 showing expression of endogenous and HA-tagged Rab5. *B*, transformed tTA-HeLa cells uninduced or induced to express either HA-Rab5(WT) or HA-Rab5(Q79L) as indicated were incubated with Alexa-Tfn (*lower panels*) for 20 min at 37 °C before fixation, permeabilized in 0.1% saponin, and processed for indirect immunofluorescence using the 12CA5 anti-HA antibody (*upper panels*), as described under "Materials and Methods." *C*, single-round kinetics of uptake and recycling of prebound biotinylated Tfn in tTA-HeLa cells uninduced (□) or induced to express either HA-Rab5(WT) (▲) or HA-Rab5(Q79L) (■) for 48 h as described under "Materials and Methods." Results are average ± S.D. of three independent experiments.

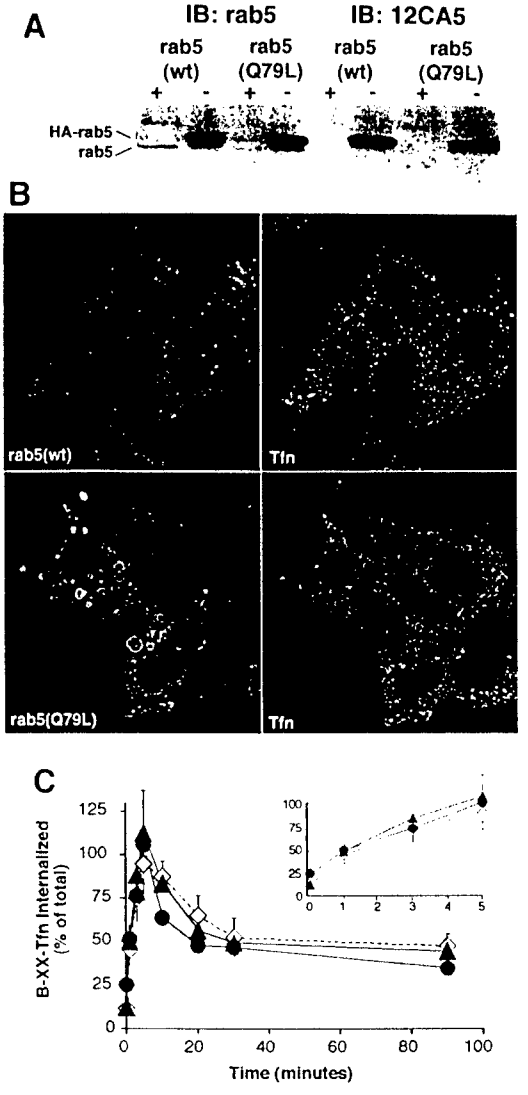

FIG. 2. **High levels of Rab5(Q79L) expression cause enlargement of endosomes without perturbing endocytic trafficking of transferrin.** tTA-HeLa cells infected with adenoviruses expressing either HA-Rab5(WT) or HA-Rab5(Q79L) under the control of a tetracycline-regulatable promoter were incubated for 18 h in the presence or absence of tetracycline as described under "Materials and Methods." *A*, immunoblots of cell lysates probed with either anti-Rab5 antibodies (*right panel*) or anti-HA antibodies (*left panel*). *B*, immunofluorescence images of adenovirally infected tTA-HeLa cells expressing either HA-Rab5(WT) or HA-Rab5(Q79L) incubated with Alexa-Tfn and subjected to indirect immunofluorescence with anti-HA monoclonal antibody as described under "Materials and Methods." *Left panels* show HA-Rab5 distribution visualized with a goat anti-mouse antibody conjugated to Texas Red; *right panels* show internalized Alexa-Tfn. *C*, single-round kinetics of uptake and recycling of prebound biotinylated Tfn in uninfected tTA-HeLa cells (◇) or in cells infected with either HA-Rab5(WT)-encoding adenoviruses (▲) or HA-Rab5(Q79L)-encoding adenoviruses (●) assayed 18 h after infection as described under "Materials and Methods." *Inset* shows expanded axis for early time points. Results are average ± S.E. of two independent experiments.

that recognizes the HA epitope (Fig. 2*B*). Despite the fact that transferrin receptors were trafficking through these dramatically enlarged endosomes when the kinetics of transferrin receptor trafficking was measured, there were no changes in the uptake of the transferrin receptor (Fig. 2*C*) compared with control uninfected cells or cells infected with HA-Rab5(WT). Like the stably transfected Rab5 tTA-HeLa cell lines, adenovirus expression of wild type and mutant Rab5 did not alter the steady state accumulation of Tfn within the cell.

Transferrin Endocytosis and Recycling Are Unaffected by HA-Rab5(Q79L) Overexpression—Our findings are inconsistent with previous results showing effects of both Rab5(WT) and Rab5(Q79L) overexpression on endocytosis and recycling of TfnR (5, 8). One trivial explanation for these differences is that previous studies were performed on adherent cells following internalization of ^{125}I-Tfn, whereas our assay follows B-XX-Tfn uptake in nonadherent cells. However, similar results were obtained when we assayed endocytosis and recycling of ^{125}I-Tfn in adherent adenovirally infected HeLa cells using the methodology of others (Refs. 5, 8, and data not shown). A second methodological difference was that previous studies employed a protocol that ensured a rapid bolus of Rab5 overexpression (5, 8). In contrast, the persistent overexpression of Rab5(Q79L) in our system may enable induction of a compensatory mechanism(s) that restores transferrin receptor trafficking to normal steady state rates. Therefore, we adapted our expression system for rapid induction of a bolus of protein expression. For these experiments, cells were infected with a 30-fold higher

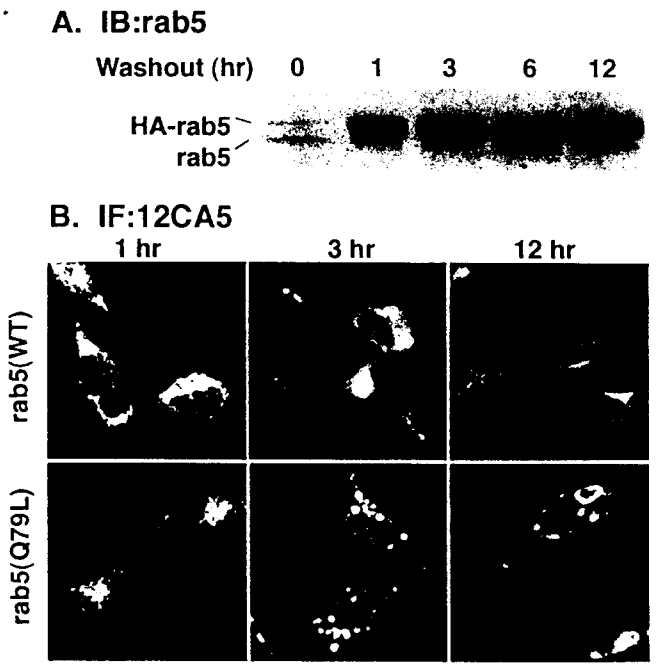

FIG. 3. **Bolus of Rab5 expression in adenovirally infected tTA-HeLa cells.** Rapid, high level expression of HA-Rab5(Q79L) in adenovirally infected tTA-HeLa cells was induced after release from a cycloheximide block as described under "Materials and Methods." *A*, immunoblot probed with an anti-Rab5 monoclonal antibody showing rapid induction of Rab5 expression detectable in 100 μg of whole cell lysates after cycloheximide wash out for the indicated times. *B*, adenovirally infected tTA-HeLa cells induced to express HA-Rab5(WT) or HA-Rab5(Q79L) by release from cycloheximide block for the indicated times were fixed and processed for indirect immunofluorescence (*IF*) using anti-HA antibody 12CA5. Results shown are representative of at least three independent experiments.

m.o.i. of adenovirus (see "Materials and Methods" for details), and Rab5 expression was controlled using cycloheximide. Briefly, tTA-HeLa cells were infected with adenovirus (m.o.i. of 300) for 2 h. Cells were then treated with 10 μg/ml cycloheximide for 3 h to accumulate mRNA in the absence of protein expression. The cycloheximide was washed from the cells, and protein was expressed for the indicated periods of time before experiments were performed. As shown in Fig. 3*A*, this protocol allows for tight control of protein synthesis while permitting a regulatable, high level of Rab5 expression. Within 1 h of HA-Rab5(Q79L) expression, exogenous HA-Rab5(Q79L) levels were estimated to be 50-fold over endogenous Rab5 (Fig. 3*A*). The level of HA-Rab5(Q79L) expression continued to increase with increased time of incubation in the absence of cycloheximide, plateauing at 6–12 h postinfection. A similar expression pattern was seen when cells were infected with HA-Rab5(WT) adenovirus (data not shown). After only an hour of protein synthesis, the enlarged endosomal phenotype could be detected (Fig. 3*B*). Increased duration of HA-Rab5(Q79L) synthesis caused a successive increase in the size of early endosomes, whereas increased HA-Rab5(WT) expression caused only minor increases in endosome size.

At each time point, we performed a kinetic analysis of transferrin uptake and recycling (Fig. 4). Consistent with our findings thus far, despite the dramatic changes in endosomal morphology seen at even the earliest time points of HA-Rab5(Q79L) expression (1 h), we were unable to detect changes in endocytosis or steady state accumulation of TfnR within the cell. Although endosome size continued to enlarge at 3 and 12 h of HA-Rab5(Q79L) expression, there was similarly no effect on the rates or efficiency of TfnR uptake and intracellular accumulation compared with uninfected cells or cells infected in the

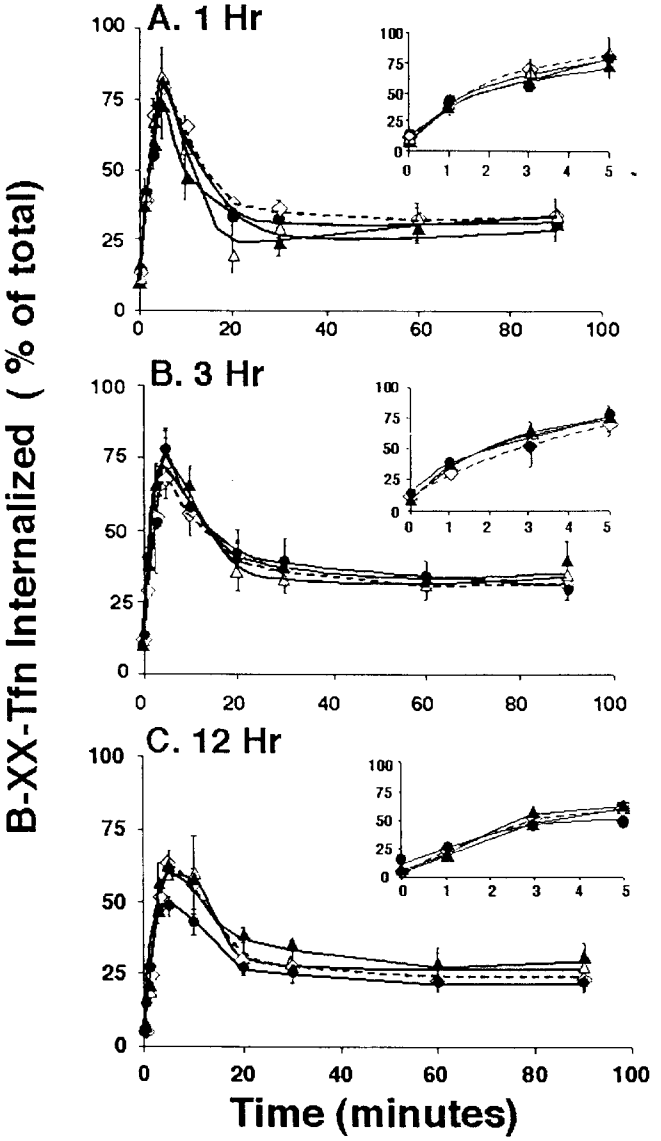

FIG. 4. **Endocytic trafficking of transferrin is unaffected even by rapid induction of high levels of Rab5(Q79L) expression.** Kinetics of uptake and recycling of prebound biotinylated Tfn were measured in uninfected tTA-HeLa cells (◇), tTA-HeLa cells infected with recombinant adenovirus that encode HA-Rab5(Q79L) but maintained in the presence of 1 μg/ml tetracycline (△), or tTA-HeLa cells infected with adenovirus in the presence of cycloheximide and induced to express either HA-Rab5(WT) (▲) or HA-Rab5(Q79L) (●) after release from cycloheximide for 1, 3, and 12 h as described under "Materials and Methods." Transferrin endocytosis and recycling were examined using an enzyme-linked immunosorbent assay-based assay, which monitors the total level of internalized, prebound, and biotinylated transferrin as described under "Materials and Methods." Data are plotted as a percentage of the total biotinylated transferrin initially bound to the cells. The same data are shown over a shorter time course (*inset*).

presence of 1 μg/ml tetracycline, which served as controls. In all cases, Tfn uptake was maximal at 5 min, and recycling occurred with a half-time of ~7–8 min, consistent with results of others (18–20).

Rates of Bulk Phase Endocytosis and Lipid Recycling Are Unaffected by Trafficking through Enlarged Endosomes—It has been proposed (1, 2) that sorting in the early endosome occurs, at least in part, by a default mechanism based on the geometry of the tubulovesicular early endosome. In this model, the high surface area of the tubular portions of the endosome facilitates recycling of membrane-associated components, perhaps through an iterative process (19, 21). Others have argued

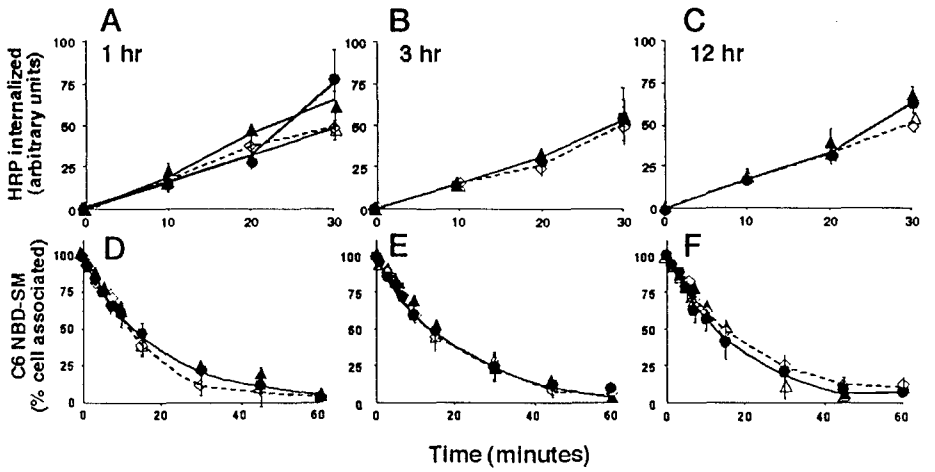

FIG. 5. **Overexpression of Rab5(WT) or Rab5(Q79L) for short or longer periods of time does not effect fluid phase uptake or lipid recycling.** Bolus expression of HA-Rab5(WT) (▲) or HA-Rab5(Q79L) (●) was induced for the indicated times in tTA-HeLa cells as in Fig. 3. As controls, cells were uninfected (◇) or infected with HA-Rab5(Q79L) adenovirus and cultured in the presence of tetracycline (△). *A–C*, the kinetics of fluid phase uptake of HRP at 37 °C measured as described under "Materials and Methods" and expressed in arbitrary units normalized to cellular protein and relative to control uninfected cells (n = 3, average ± S.E.). *D–F*, C_6-NBD-SM was internalized for 10 min and surface-associated lipid was removed. Shown are the kinetics of recycling of internalized lipid during subsequent incubation at 37 °C.

that more directed sorting mechanisms are required for the highly efficient endocytic trafficking of recycling receptors such as the TfnR (22). The appearance of coated buds containing TfnR on early endosomes (22) and the sorting motif-dependent inhibition of TfnR recycling by bafilomycin (23) support this latter hypothesis. Thus, our inability to detect an effect on the kinetics and efficiency of TfnR uptake and recycling in cells despite dramatic alterations in early endosome size and geometry may reflect the involvement of Rab5-independent, directed sorting events. Consistent with this possibility, one notable difference in our experiments compared with others is that in previous studies human TfnR vectors were introduced in parallel with the Rab5(WT) and Rab5(Q79L) constructs (8), whereas we are studying transport kinetics of endogenous receptors. Thus, it is possible that at higher levels of expression TfnR endocytic trafficking becomes more sensitive to alterations in endosomal morphology and/or Rab5 function than that of endogenous Tfn receptors. Importantly, we obtained similar results when examining endogenous TfnR endocytosis and recycling kinetics using adenovirally infected HepG2 cells expressing Rab5(Q79L) (data not shown).

To determine whether the dramatic changes in early endosome morphology affect the bulk sorting properties of early endosomes as predicted by current models, we examined the kinetics of endocytosis of a bulk fluid phase marker and the kinetics of recycling of a bulk membrane lipid marker. To focus on the rates of volume endocytosis rather than the extent of volume accumulation, we analyzed the initial rate of fluid phase HRP uptake. As can be seen (Fig. 5, *A–C*), we were unable to detect differences in the rate of HRP endocytosis at either 1, 3, or 12 h after bolus induction of expression of WT or mutant Rab5 compared with either uninduced or uninfected controls. Previous studies on HRP uptake in Rab5-expressing cells (7) focused on later time points of uptake when changes in endosomal volume will be reflected by increased accumulation of HRP at steady state. Our results suggest that GTP hydrolysis by Rab5 is not rate-limiting for bulk or receptor-mediated endocytosis in HeLa cells.

We next measured the rates of bulk membrane recycling in cells overexpressing Rab5(Q79L), expecting that membrane lipids would accumulate in the enlarged vacuolar portions of the early endosome slowing their recycling. For these experiments we used C_6-NBD-SM, a readily extractable, fluorescently labeled membrane lipid (17). Briefly, cells were labeled with C_6-NBD-SM for 10 min at 37 °C to allow the C_6-NBD-SM to traffic to the early endosomes. After extracting the plasma membrane C_6-NBD-SM through a series of backwashes in a fatty acid-free bovine serum albumin solution, dissociable C_6-NBD-SM was measured by the fluorescence in the medium. Unexpectedly, there was no appreciable difference in the rate or extent of C_6-NBD-SM recycling from the endosome to the plasma membrane at any time point after induction of Rab5 WT or mutant overexpression (Fig. 5, *D–F*). Thus, in these cells efficiency of recycling of either bulk membrane or TfnR was not affected by dramatic changes in endosome geometry.

Conclusions—We find that the early endosomal compartment significantly expands in cells overexpressing the constitutively active Rab5 mutant, Rab5(Q79L). This finding is consistent with previous work of others (8, 24) and with the model that Rab5 plays a critical role in early endosome biogenesis and morphogenesis by controlling the rate of endosome fusion events while in the Rab5/GTP-bound form (4, 5). Unexpectedly, and in contrast to previous reports (7, 8), in the presence of Rab5(Q79L) overexpression we observed no detectable acceleration in the kinetics of Tfn receptor or fluid phase uptake. Further, there were no changes in steady state, intracellular accumulation of the Tfn receptor or lipid recycling despite the appearance of these morphologically altered early endosomes. Our results were obtained at a variety of levels of Rab5(Q79L) overexpression, which caused varying degrees of change in endosome morphology and after even brief exposure to mutant Rab5 provided little opportunity for the induction of compensatory mechanisms.

Overexpression of dominant-negative Rab5 mutants (*e.g.* Rab5(N133I) or Rab5(S34N)) has been shown by several groups to inhibit TfnR and fluid phase endocytosis and endosome fusion (5, 8). These mutations block Rab5 function and can exert their inhibitory effects independently of downstream effectors. By contrast, the activating mutant studied here, Rab5(Q79L), will require interaction with downstream effectors to manifest its effects. Cell type and other variables may determine whether specific downstream effectors of Rab5 are limiting and therefore whether overexpression of Rab5(Q79L) will alter the kinetics of membrane trafficking along the endocytic recycling pathway. Thus, our results do not rule out a function for Rab5 in controlling membrane trafficking through the early/sorting endosome.

Regardless, the important findings from these studies are

2-fold. First, our results clearly establish that Rab5-induced changes in early endosomal morphology are not predictive of defects in endocytic membrane trafficking. Second, our results argue that geometric considerations may contribute to a lesser extent than previously assumed in determining the bulk sorting and recycling efficiencies of the early endosomal compartment. Morphometric measurements made of the tubular and vesicular portions of early endosomes in baby hamster kidney cells (25) show that 50–70% of total endosomal volume and 55–90% of total surface area are associated with the tubular portions of the endosome. Although there is considerable inherent error in these measurements (25), they also suggest that endosomal geometry would not be sufficient to account for the observed efficiency of sorting and recycling.

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Dominant Negative rab5 alters post-membrane EGFR trafficking in HeLa cells

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Abstract:

Endocytosis is an important regulatory mechanism in the signaling of epidermal growth factor receptors (EGFR). Not only is endocytosis a mechanism to dissociate ligand/receptor complexes, degrade receptors, and attenuate signaling it also modulates the specificity of downstream receptor/effector communication. In this report, we examine the role of the small molecular weight guanine nucleotide binding protein, rab5, in the endocytic trafficking of the EGFR. It is well-established that rab5 functions in the biogenesis of the early endosome, however, its exact molecular role is a subject of debate. Literature exists that supports a role for rab5 in vesicle budding from the plasma membrane as well as fusion of vesicles to the early endosome. Using adenovirally expressed dominant negative and constitutively active rab5 (rab5(S34N) and rab5(Q79L)) in HeLa and HepG2 cells, we have found that rab5 activity has no bearing on the rate of EGFR endocytosis. However, EGF and EGFR localization to the early endosome is inhibited by expression of rab5(S34N). This alteration of EGFR endocytic trafficking results in a slowed rate of EGFR degradation and alters the cell's EGF-dependent growth/survival. Taken together, rab5 regulates EGFR endocytic trafficking distal to the plasma membrane.

Introduction:

Epidermal growth factor (EGF) receptors (EGFRs) are members of the receptor tyrosine kinase (RTK) family of cell surface receptors that regulate cellular growth, development, and homeostasis (Carpenter and Cohen, 1978; Carpenter and Cohen, 1979). Binding of EGF to the EGFR initiates dimerization of the receptor and activation of the receptors' intrinsic kinase domains, which in turn phosphorylate a number of carboxyl-terminal tyrosines. These phosphotyrosines then serve as docking sites for downstream signaling proteins that are activated, and through a coordinated process, initiate multiple intracellular biochemical signaling pathways that activate the appropriate cellular physiology (Schlessinger and Ullrich, 1992; van der Geer et al., 1994). Concomitant with the ligand-induced activation of this receptor, the ligand:receptor complex becomes internalized. This process, termed endocytosis, has been demonstrated by numerous labs to be an important regulatory component of EGFR signaling (Haugh et al., 1999a; Haugh et al., 1999b; Vieira et al., 1996; Wells et al., 1990). Blockade of EGF internalization has been shown to enhance the signaling of some signaling pathways, while inhibiting others (Vieira et al., 1996). This spatio-temporal component of EGFR signaling has been supported by kinetic analysis of receptor internalization and effector activation (Haugh et al., 1999a; Haugh et al., 1999b). The physiological consequence of a perturbation in EGFR endocytosis is enhanced DNA synthesis and mitogenicity (Vieira et al., 1996; Wells et al., 1990). Additionally, the endocytic pathway has been implicated as a regulatory component of other cell surface receptor signal transduction pathways (for reviews see (Ceresa and Schmid, 2000; Wiley and Burke, 2001)).

Rabs are small molecular weight guanine nucleotide binding proteins (G-proteins) that specifically regulate different stages of cellular membrane trafficking based on their interacting

proteins and cellular location (Rodman and Wandinger-Ness, 2000; Woodman, 2000). Rab5 is a 35 kDa protein localized to the plasma membrane and the early endosome, and functions in regulating the biogenesis of the early endosome (Chavrier et al., 1990; Woodman, 2000). Like all small molecular weight G-proteins, rab5 cycles between an active, GTP bound state and an inactive, GDP state. Mutants that selectively trap rab5 in "active" (rab5(Q79L)) and "inactive" (rab5(S34N)) states (Bucci et al., 1992; Li and Stahl, 1993; Stenmark et al., 1994), have greatly facilitated the study of rab5. The exact molecular mechanism used by rab5 to coordinate early endosome biogenesis remains unclear. *In vitro* data support a role for rab5 at the early endosome. In reconstitution assays using isolated vesicle preparations, vesicle fusion is facilitated by the addition of "active" rab5 and inhibited by "inactive" rab5 (Barbieri et al., 1994; Gorvel et al., 1991; Rybin et al., 1996; Stenmark et al., 1994). However, studies in intact cells indicate that rab5 may also play a role in the removal of the clathrin-coated vesicles from the plasma membrane. Numerous reports indicate that expression of activating or inactivating mutations positively or negatively regulate the kinetics of fluid phase and receptor mediated endocytosis, respectively (Barbieri et al., 2000; Bucci et al., 1992; Chen and Wang, 2001; Iwata et al., 1999; Li and Stahl, 1993; Li et al., 2000; Schmidlin et al., 2001; Seachrist et al., 2000; Stenmark et al., 1994). Conversely, there are also a number of reports where overexpression of rab5 mutants with modulated activity have no consequence on the endocytic process (Ceresa et al., 2001; Seachrist et al., 2002).

In this report, we explore the role of rab5 in the endocytic trafficking of endogenous EGFR. Using adenovirally infected HeLa and HepG2 cells, we examine the role of activating and inactivating rab5 mutants on EGFR endocytosis and entry into the early endosome. Biochemical data and confocal microscopy indicate that rab5 does not regulate endocytosis, but expression of

a dominant negative rab5, can alter EGFR endocytic trafficking. The physiological consequence of rab5(S34N) expression is a slowed rate of EGFR degradation. Expression of rab5 mutants alters EGFR-mediated cell growth/survival. Thus, rab5 functionally regulates EGFR endocytic trafficking at cellular locations that are distal from the plasma membrane.

Materials and Methods

Cell Culture

tTA-HeLa cells (HeLa cells stably transfected with the tetracycline transactivator – tTA) were a gift of Dr. Sandra Schmid (The Scripps Research Institute, La Jolla, CA). Cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine, 5% fetal bovine serum and 400 mg/ml G418(Gibco BRL), to provide selection pressure for the tTA gene (Damke et al., 1995).

tTA-HepG2 cells were generated by LipofectAMINE (Gibco BRL) transfection of the pUHD neo vector that encodes for tTA and contains the neomycin selection marker (gift of H. Bujard, ZMBH Heidelberg, Germany). Cultures were grown in the presence of 400 μg/ml G418 to select for those that had been transfected. Single colonies of cells were isolated, and screened for their ability to express the tetracycline regulatable adenoviruses in a tetracycline dependent manner. Parental and stable cell lines were grown in DMEM supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine, 10% FBS, and 400 μg/ml G418.

HEK293 were cultured in DMEM supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM glutamine and 10% fetal bovine serum.

All three cells lines were grown at 37°C in 5% CO_2.

Generation and usage of adenoviruses.

This study uses canine rab5 constructs that are 99% homologous to the human rab5a (Genebank). Tetracycline-regulatable rab5(WT) and rab5(Q79L) adenoviruses have been described previously (Ceresa et al., 2001). The rab5(S34N) construct was made by mutation of the existing HA- tagged rab5(WT) construct using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). The resulting construct was placed under the control of a tetracycline-regulatable

promoter in the pAdlox vector 3' to the ψ5 packaging site and 5' to the poly(A) site. Adenoviruses were generated as previously described (Hardy et al., 1997). Prior to use in experiments, a single population of adenovirus was generated by plaque purification and amplified in HEK293 cells. Briefly, approximately 70% confluent 15 cm dishes of cells were infected with adenoviruses at a multiplicity of infection (m.o.i.) of 10/cell in 12 ml of growth media. Cells were incubated at 37°C in 5% CO_2 for 48 hours. Cells and media were collected and subjected to three rounds of freezing (in liquid nitrogen) and thawing (at room temperature) to lyse the cells. Cellular debris was separated with a 2,000 x g spin for 10 minutes at 4°C and the resulting supernatant was then snap frozen in 500 µl aliquots. Prior to use in experiments, the titer of the adenovirus was determined by infection of 35 mm dishes of HEK293 cells with serial dilutions of the adenovirus. Following infection, cells were overlayed with 1% agarose made in growth media. Titer was determined as the number of adenovirus plaque forming units per ml of adenovirus.

For experiments, 70% confluent dishes of tTA-HeLa or tTA-HepG2 cells were infected at a m.o.i. of 10 pfu/cell for 2 hours in serum free DMEM at 37°C in 5% CO2, followed by overnight incubation in the appropriate growth media. Adenoviral expression was verified by immunoblotting and/or indirect immunofluorescence with a dish of cells treated in parallel.

Immunoblotting

Cell lysates were generated by washing the cells twice with phosphate buffered saline (PBS) and solubilizing cells in detergent buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM tris, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, and 2 mM PMSF). The protein concentration of the solubilized protein was assessed using BCA assay (Pierce), and

samples were diluted in SDS-sample buffer. Equivalent amounts of protein were separated by the indicated percentage SDS-PAGE, transferred to nitrocellulose, probed using monoclonal antibodies against the HA-epitope (12CA5) (Roche), rab5 (Transduction Laboratories), or a rabbit polyclonal antibody against EGFR (Santa Cruz biotechnology), followed by probing with a horseradish peroxidase conjugated goat anti-mouse or goat-anti-rabbit secondary antibody. Detected proteins were visualized using enhanced chemiluminescence.

GTP loading assays

GTP loading experiments were conducted as previously described (Stenmark et al., 1994). Briefly, infected 60 mm dishes of tTA-HeLa cells were washed twice with phosphate-free DMEM and incubated for 30 minutes at 37°C to reduce the levels of phosphate. Cells were then incubated for 3 hours in 0.5 mCi/ml of 32P-orthophosphate in phosphate –free DMEM. Cells were washed twice with PBS and equilibrated to 4°C. Cells were harvested in lysis buffer (50 mM HEPES, 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 0.1 mM GTP, 1 mM ATP, 10 mM Na-phosphate, pH7.4), collected, and insoluble material was removed by 2 minutes of centrifugation. Lysates were incubated with 12CA5 antibody pre-conjugated to protein G agarose (Santa Cruz Biotechnology) for 30 minutes at 4°C with constant agitation. The resulting immunoprecipitations were washed three times in wash buffer I (50 mM HEPES, 1% Triton X-100, 500 mM NaCl, and 5 mM $MgCl_2$ pH 7.4), and equilibrated for thin-layer chromatography with three washes in wash buffer II (50 mM HEPES, 0.1% Triton X-100, 100 mM NaCl, and 5 mM $MgCl_2$, 0.005 % SDS, pH 7.4). Samples were then eluted in 8 ml TLC sample buffer (2 mM EDTA, 2 mM DTT, 0.2% SDS, 5 mM GDP, 5 mM GTP) for 5 minutes at 70°C. Three microliters of the eluate was spotted on a cellulose PEI-F TLC plate (J.T. Baker), and GTP-

bound rab5 was separated from GDP-bound rab5 by TLC chromatography using 0.6 M sodium phosphate buffer pH 3.4. Incorporation of 32P was visualized and quantified by a Molecular Dynamic Phosphoimager.

Indirect Immunofluorescence

Cells were fixed in 4% p-formaldehyde/PBS++(PBS/ 0.5 mM $MgCl_2$/ 0.5 mM $CaCl_2$) solution at room temperature for 5 minutes and on ice for 15 minutes. Excess formaldehyde was removed with 3x5 minute washes in PBS++. Cells were then permeabilized 20 minutes in 0.1% saponin/5% FBS/PBS++ and washed 3x5 minutes with PBS++. After washing, cells were incubated for 1 hour with the indicated antibody. Unbound antibody was removed with 3x5 minute washes in PBS++ and cells were incubated 1 hour with an Alexa568 or Alexa 647 conjugated goat anti-mouse secondary antibody (Molecular Probes). After 6x10 minute washes with PBS++, coverslips were rinsed in Millipore water and mounted on a slide with Prolong Antifade (Molecular Probes). Cells were visualized at the Warren Medical Research Institute Flow and Image Cytometry Laboratory (on the University of Oklahoma Health Sciences Center Campus) using a Leica TCS NT Confocal microscope. Collected images were analyzed using the Leica TCS NT software.

Generation and Expression of GFP–tagged rab5 and rab7 constructs

GFP-tagged rab5 constructs were generated by standard PCR techniques to introduce the desired restriction sites in the flanking sequences of the GFP and rab5 or rab7 constructs. The resulting PCR products were serially introduced in the pcDNA-3 mammalian expression vector (enhanced GFP followed by rab5/7). The resulting construct is enhanced GFP linked to rab5/7 by the amino acid sequence - alanine-serine-arginine. Plasmids were confirmed by sequencing at the Oklahoma Medical Research Foundation DNA Sequencing Facility.

HeLa cells were transfected with the cDNA constructs using calcium phosphate transfection (Mortensen, 1994).

^{125}I-EGF Uptake

Infected cells grown on 35 mm dishes were washed with room temperature PBS pH 7.4 and then incubated with 0.5 ml ^{125}I-EGF(NEN) at specific activity of 50,000 cpm/ng and a concentration of 10 ng/ml for 2 hours on ice in washing media (DMEM, 20 mM HEPES pH 7.4, 0.1% BSA). Cells were washed twice with ice cold PBS to remove free radioligand, and then incubated at 37°C with 37°C washing media for the indicated times. After 37°C incubation, cells were washed twice with ice cold PBS, followed by two 6 minute washes in 0.75 ml of 0.2 M Acetic Acid pH 2.8/0.5 M NaCl. Washes were pooled to count. Cells were solubilized with 0.75 ml of 1M NaOH and collected to count. Radioactivity was measured using a Beckman 5500B Gamma counter.

Fluid Phase (HRP) Uptake

Fluid phase uptake was performed on infected 35 mm dishes of cells as previously described (Damke et al., 1994; Marsh et al., 1987).

Fluorescent-EGF labeling

Cells were loaded with 10 μg/ml Alexa-488 or Texas Red EGF (Molecular Probes) for 10 minutes at 37°C and washed twice with cold PBS++++(PBS, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, 0.2%BSA), three times with cold citrate buffer (25.5 mM Citric Acid, 24.5 mM Sodium Citrate, 280 mM sucrose), and twice again with PBS++++. Cells were fixed in 4% p-formaldehyde and processed for indirect immunofluorescence.

EGFR Degradation

Sixty millimeter dishes of infected cells were washed twice in PBS pH 7.4 and incubated serum-free DMEM for 2 hours at 37°C. Cells were treated with 10 ng/ml EGF for the indicated amounts of time. Cell lysates were prepared as described in "Immunoblotting". For each time point, equivalent amounts of proteins were loaded on a 7.5% SDS-PAGE, separated, transferred to nitrocellulose, and immunoblotted with a rabbit polyclonal antibody against the EGFR (Santa Cruz Biotechnology) and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody. Protein bands were visualized using enhanced chemiluminesence and quantified using a UV Products imaging system. Bands were plotted as a percentage of degradation relative to unstimulated EGFR.

Cell Proliferation

Sixty millimeter dishes of infected cells were infected with the indicated adenoviruses in serum-free DMEM for 2 hours at 37°C. Cells were allowed to recover in serum containing media for 3 hours. Cells were washed in PBS, harvested, washed in serum-free media and replated at a density of 5,000 cells/well in a 96 well dish (6 wells per condition). Cells were incubated with either serum free media, 100 ng/ml EGF, or 5% FBS for 72 hours. Cells were assayed using MTT assay (Hansen et al., 1989). Data are plotted as the ratio of cells under the described treatment, relative to the number of cells in serum-free media. Statistics were calculated using Prism software (GraphPad Software, Inc).

Results

Functional expression of wild type and mutant rab5 adenoviruses

Rab5 is a small molecular weight G-protein involved in the proximal stages of endocytic trafficking, namely early endosome formation. Endogenous rab5 exists both at the intracellular face of the plasma membrane as well as at the early endosome, but its exact molecular function remains poorly understood. Literature exists supporting rab5's role in endocytosis as well as early endosome fusion and formation. To determine the role of rab5 in the endocytic trafficking of the endogenous EGFR, we utilized wild type, activating, and inactivating mutants of rab5 (Figure 1A). Substitution of glutamine to leucine at amino acid 79 (denoted 'rab5(Q79L)') results in protein that can not hydrolyze GTP, leaving the protein in the active state. In order to generate an inactive rab5, serine was mutated to an asparagine at residue 34 (denoted 'rab5(S34N)') causing the protein to have an affinity for GDP that is 100X greater than that of GTP. Detailed biochemical characterization of these mutants has been described elsewhere (Bucci et al., 1992; Li and Stahl, 1993; Stenmark et al., 1994).

HeLa cells were used to study the endocytic trafficking of the EGFR because they express endogenous EGFRs at a physiological concentration – approximately 50,000 EGFRs/cell (Berkers et al., 1991). More importantly, in HeLa cells, EGFRs traffic and communicate with downstream signaling pathways in a manner similar to that of endogenous EGFR in intact tissues (Damke et al., 1994; Vieira et al., 1996). Thus, any observed changes in trafficking will likely be an accurate reflection of what is occurring *in vivo*.

To circumvent the problems accompanying the time-consuming and unpredictable generation of stable cell lines, tetracycline-regulatable adenoviruses were used to express wild type and mutant forms of rab5 (Damke et al., 1995). Adenoviral expression offers the advantage of being

able to rapidly introduce high levels of exogenous proteins, homogenously in a variety of cell lines. The tetracycline-regulatable promoter allows manipulation of the extent of protein expression and is a useful mechanism to control for any potential adenoviral effects. Adenoviruses have proven to be a useful tool in the study of a number of proteins that perturb endocytic trafficking events (Baldeon et al., 2001; Ceresa et al., 2001; Damke et al., 2001; Fish et al., 2000; Newmeyer and Schmid, 2001).

HA-epitope tagged wild-type and mutant rab5 (HA-rab5(WT), HA-rab5(Q79L), and HA-rab5(S34N)) adenoviruses under a tetracycline-inducible promoter were generated using standard techniques (Altschuler et al., 1998; Hardy et al., 1997). Adenoviruses were plaque purified to a single colony, verified by DNA sequencing, and amplified for use in subsequent experiments. Figure 1B demonstrates the robust nature with which the wild type and mutant rab5 proteins can be expressed. tTA-HeLa cells were infected as described in "Materials and Methods" and immunoblotted with antibodies against the HA-epitope (12CA5) (Figure 1B, upper panel) or the rab5 (Figure 1B, lower panel) protein itself. Both immunoblots demonstrate how tetracycline inhibits protein expression. The rab5 immunoblot shows that at a m.o.i. of 10 we can easily obtain 30-50 fold overexpression. Immunofluorescence using the 12CA5 to detect infected cells reveals that at this level of infection greater than 90% of the cells are infected (Data not shown) (Fish et al., 2000; Newmeyer and Schmid, 2001). Guanine nucleotide binding experiments were performed to determine whether adenoviral overexpression of the rab5 proteins perturbed their predicted ability to bind guanine nucleotides. In cells infected as described above, and labeled with 32P, HA-tagged proteins were immunoprecipitated, and 32P-GTP vs 32P-GDP was separated by thin layer chromatography. The binding of the radiolabeled nucleotides was visualized and quantified using a Molecular Dynamics Imaging System. Figure

1C is a representative experiment and shows that HA-rab5(WT), HA-rab5(Q79L), and HA-rab5(S34N) are 28%, 68%, and 3% GTP bound. These numbers are consistent with data from the same rab5 mutants expressed in BHK cells by vaccinia virus (Stenmark et al., 1994).

Expression of rab5(Q79L) and rab5(S34N) does not perturb EGFR endocytosis

Confident that the adenoviruses expressed in a high percentage of cells, at the desired expression levels, and bound guanine nucleotides as predicted, we next examined the consequence of wild type and mutant rab5 expression on 125I-EGF endocytosis. In these experiments, infected cells were assayed for the ability to internalize 125I-EGF. Cells were incubated with 125I-EGF for the indicated amounts of time and assayed for cell surface versus internalized 125I-EGF (see " Materials and Methods" for details). Despite the adenoviral expression of three biochemically distinct forms of rab5 (rab5(WT), rab5(Q79L), and rab5(S34N)), there is no change in the rate of EGFR endocytosis when compared with uninfected cells or cells infected in the presence of tetracycline (Figure 2A). As a control, cells were infected with an adenovirus encoding dominant negative dynamin, which blocks clathrin-mediated endocytosis of the EGFR. Only 34% of the 125I-EGF was internalized after 6 minutes (data not shown). To determine whether this lack of an effect on endocytosis was specific to the EGFR or a more general effect, fluid phase endocytosis was assayed in identically treated tTA-HeLa cells. Again, despite the presence of the different forms of rab5, there was no change in rate of horseradish peroxidase uptake (Figure 2B). Thus, in HeLa cells, neither expression of activating or inactivating mutants of rab5 have a consequence on receptor mediated or fluid phase endocytosis.

Fluorescently labeled EGF co-localizes with rab5(WT) and rab5(Q79L), but not rab5(S34N)

Since endogenous rab5 is localized to the plasma membrane and at the early endosome and has no effect on EGFR endocytosis, we hypothesized that rab5 may regulate EGFR trafficking at the early endosome. To test this hypothesis, HeLa cells were infected with the wild type and mutant adenoviral constructs, incubated for 10 minutes with fluorescently labeled EGF (Alexa 488-EGF, Molecular Probes), fixed in para-formaldehyde, and processed for indirect immunofluorescence with an antibody against the HA-epitope. Cells were then visualized using confocal microscopy.

The distribution of wild type and mutant rab5 is consistent with previous reports (Figure 3) (Bucci et al., 1992; Gorvel et al., 1991; Stenmark et al., 1994). Cells infected with adenoviruses encoding for rab5(WT) distribute the protein in the plasma membrane and endosomal compartments. Rab5(Q79L) expression results in the formation of the characteristic enlarged early endosome (up to 4 μm in size), while rab5(S34N) expression generates vesicles approximately half the size of vesicles formed by rab5(WT) (approximately 0.5 μm versus 1 μm).

Fluorescently labeled EGF localizes in both rab5(WT) and rab5(Q79L) generated vesicles, while residing next to, but not within rab5(S34N) containing vesicles (Figure 3, 'merge'). An analysis of the pixel intensity of 8-10 μm long slice through the rab5(WT) and rab5(Q79L) expressing cells, shows co-localization of exogenous rab5 (red) and fluorescent EGF (green) (Figure 3B). Pixel intensities of rab5A(S34N) expressing cells indicate the fluorophores are next to one another, but do not overlap (Figure 3B). This experiment served as a secondary method for determining whether endocytosis occurs in the presence of the rab5 mutants, as well as examining whether rab5 and EGF co-localize.

To make sure this change in EGF localization was not an artifact of adenoviral expression, we generated cDNAs encoding for GFP-tagged rab5 proteins that were under the control of a mammalian promoter. When these constructs were transiently transfected into HeLa cells and incubated with fluorescently labeled EGF, we observed the same pattern of distribution of the rab5 and EGF– co-localization of GFP-rab5(WT) and GFP-rab5(Q79L) with the fluorescently labeled (Texas Red) EGF, but the GFP-rab5(S34N) did not co-localize with the fluorescently-labeled EGF (Figure 4A).

The use of GFP-tagged constructs allowed us to examine the localization of the wild type and mutant rab5 proteins in relationship to another endocytic marker – early endosome antigen 1, EEA1 (Mu et al., 1995). EEA1 is a rab5 effector protein that is necessary to confer rab5 mediated fusion in vitro (Christoforidis et al., 1999). It has been shown previously that when endosomal fusion is perturbed, EEA1 redistributes to a non-early endosomal compartment and does not integrate into the early endosome (Mills et al., 1998). We found that expression of rab5(S34N) caused of a similar change in rab5:EEA1 localization (Figure 4). When the pixel intensities along the xy-plane of the cell are compared, there is strong co-localization of all three proteins (rab5, EGF, and EEA1) in cell expressing rab5(WT) and rab5(Q79L). This colocalization is particularly striking when one considers the signal to noise ratio. Only in rab5(WT) and rab5(Q79L) expressing cells do the high GFP pixel intensities correspond to EGF and EEA1. Cells expressing rab5(S34N) only co-localize EGF and EEA1, but not rab5, particularly at pixel intensities above 50 (arbitrary units) (Figure 4B).

To determine whether this these changes in EGF trafficking were specific to dominant negative rab5, we examined the subcellular localization of fluorescently labeled EGF in cells expressing cDNAs that encode for GFP-tagged wild type and dominant negative rab7 (GFP-

rab7(WT) and GFP-rab7(N125I)); rab7 is a protein that regulates early to late endosomal trafficking and dominant negative rab7 disrupts this stage in endocytic trafficking (Feng et al., 1995; Press et al., 1998). We were able to determine that this altered cellular location was specific for rab5(S34N). Expression of either mutant had no effect on the ability of EEA1 and Texas Red EGF to co-localize (Figure 4), indicating that the alterations in trafficking were specific for rab5(S34N).

Cells were transiently transfected with the expression vectors encoding for the GFP-tagged proteins, treated with EGF, and processed for indirect immunofluorescence using a mouse monoclonal antibody against the EGFR (13A9 Genentech), and examined using confocal microscopy. HeLa cells expressing GFP-rab5(WT) or GFP-rab5(Q79L) produced vesicles in which the EGFR localized, whereas GFP-rab5(S34N) transfected cells did not produce vesicles that co-localized with the EGFR (data not shown). These data are consistent with those generated by examining fluorescent EGF/rab5 localization and eliminate the possibility that the pattern of rab5(S34N) and EGF localization was not an artifact of ligand/receptor dissociation.

Expression of rab5 adenoviruses in HepG2 has no effect on EGFR endocytosis.

To determine whether these observations are a cell specific phenomena or reflect the true in vivo function of rab5, we examined the effect of rab5 on EGFR trafficking in another cell line that expresses endogenous EGFR. HepG2 cells are a human liver carcinoma cells that express endogenous EGFRs at density of approximately 180,000 receptors/cell (Yang et al., 1996). In order to use our tetracycline regulatable adenoviruses in these cells, the HepG2 cells had to first be stably transfected with the tetracycline transactivator (tTA). tTA-HepG2 cells were generated as described in " Materials and Methods". Three different cell lines were generated and assayed for their ability to express the adenoviruses in a tetracycline dependent manner (data not shown).

All showed comparable levels of adenovirus expression and response to tetracycline. A single cell line was then used for subsequent experiments. Infection of the tTA-HepG2 cells with adenovirus at a m.o.i. of 10 yields a 30-fold overexpression of the exogenous rab5 (Figure 5A) and results in >90% of the cells infected (data not shown).

Our studies in HepG2 cells confirmed our findings in HeLa cells. Uptake of 125I-EGF was assayed in the wild type and mutant rab5 expressing tTA-HepG2 cells, the kinetics of internalization were indistinguishable from control (Figure 5B). Thus, in two different cell lines we find that the internalization of EGFR is not perturbed by expression of activating or inactivating mutants of rab5.

rab5(S34N) blocks entry of the EGFR into the early endosome in HepG2 cells.

We next examined the subcellular localization of the wild type and mutant rab5 with respect to the internalized fluorescently labeled EGF. Indirect immunofluoresence using the 12CA5 antibody reveals the same rab5-induced vesicular morphology in HepG2 cells as was seen in HeLa cells (Figure 6 – 'rab5'). Further, fluorescently labeled EGF co-localizes with rab5(WT) and rab5(Q79L), but not with rab5(S34N) (Figure 6 – 'Merge' and Figure 6B). These data are consistent with rab5 blocking the entry of EGF into the early endosome in HepG2 cells.

Alteration of EGFR trafficking by rab5(S34N) expression slows the rate of ligand mediated EGFR degradation.

One fate of internalized EGFRs is lysosomal degradation. To determine if expression of rab5(S34N) was truly altering EGFR endocytic trafficking, we examined the kinetics of EGFR degradation in the presence of wild type and mutant rab5. A slowed rate of EGFR degradation would provide biochemical evidence that endocytic trafficking had truly been disrupted. HeLa cells were infected with the rab5 adenoviruses and after overnight recovery, cells were treated

with EGF for 0 - 30 minutes. Cell lysates were prepared and after separation by 7.5% SDS-PAGE were immunoblotted for the EGFR with an polyclonal antibody against the carboxyl terminus of the EGFR (Santa Cruz Biotechnology). EGFR was detected by western blot analysis, visualized by enhanced chemiluminescence, and quantified with a UV Products Imaging System (Figure 7A). Rates of EGFR degradation in control, mock infected, rab5(WT), and rab5(Q79L) infected cells are consistent with previous reports (Zheng et al., 2001). In cells infected with rab5(S34N), the rate of EGFR degradation is slowed dramatically, consistent with rab5(S34N) inhibiting the normal endocytic trafficking of the EGFR.

Guanine nucloetide binding of rab5 effects EGFR-mediated HeLa cell growth/survival.

To determine whether the changes in EGFR localization and degradation had any effect on EGFR signal transduction, we assessed the EGFR-mediated cell growth in HeLa cells in the presence of wild type and mutant rab5 (Figure 7B). Infected cells were treated for 72 hours with 100ng/ml EGF in DMEM (Figure 7A) or 5% fetal bovine serum in DMEM (Figure 7C) and assayed for cell number using an MTT Assay (Hansen et al., 1989). Data are plotted as the fold cell growth as compared to cells maintained in DMEM alone. Expression of rab5(WT) and rab5(S34N) had a trend of EGFR-mediated, increased number of cells over mock infected cells, whereas, expression of rab5(Q79L) caused a statistically significant, EGF-dependent decrease in cell number (Figure 7B). Overexpression of the rab5 proteins had no effect on the ability of the cells to grow, as in the presence of 5% fetal bovine serum, all cells retained the ability to grow (Figure 7C).

Discussion

Ligand mediated endocytosis is an important regulatory component of EGFR signaling and multiple lines of evidence indicate that disruption in EGFR internalization alters activation of EGF-stimulated biochemical pathways and whole cell physiology (Damke et al., 1994; Haugh et al., 1999a; Haugh et al., 1999b; Vieira et al., 1996; Wells et al., 1990). Understanding the proteins involved in regulating EGFR endocytic trafficking is one of the first steps in dissecting the underlying molecular mechanism of EGFR-mediated signal transduction that leads to receptor specific physiology.

In this report, we examine the role of the small molecular weight G-protein, rab5, and its ability to regulate EGFR endocytosis. There is nearly uniform agreement that rab5 functions in the proximal stages of the endocytic pathway (Rodman and Wandinger-Ness, 2000; Woodman, 2000), however, the exact molecular mechanism remains obscure. It is clear that rab5 does play a role in the endocytic trafficking of the EGFR, but whether it functions at the plasma membrane or the early endosome remains controversial. When partially purified vesicle preparations are assayed for their ability to fuse in the presence of wild type and mutant rab5, the rab5(S34N) mutant inhibits vesicle fusion (Gorvel et al., 1991; Stenmark et al., 1994). However, when the function of rab5 is studied in some cellular contexts, particularly with cell surface receptors that are transiently overexpressed, rab5 functions to regulate receptor entry into the cell.

Our data are consistent with rab5 functioning primarily as a regulator of vesicle:early endosome fusion. In the presence of rab5(S34N), we observe no alteration in EGFR endocytosis as assayed by radioligand internalization (Figures 2 and 5), uptake of fluorescently-labeled EGF (Figures 3, 4, and 6), or the plasma membrane to cytosol redistribution of EGF stimulated EGFRs (data not shown). There is a marked change in the subcellular compartmentalization of

the fluorescently-labeled EGF and the EGFR in the cytosol. When cells express rab5(WT) or rab5(Q79L), EGF and the EGFR reside in the endosomes containing these proteins, whereas when rab5(S34N) is expressed the EGF and EGFR reside next to but not within the rab5(S34N) containing vesicles (Figures 3, 4, and 6). The physiological consequence of this change in EGF/EGFR trafficking is a slowed rate of EGFR degradation in cells expressing rab5(S34N) (Figure 7A).

Despite the dramatically slower rate of EGFR degradation in cells expressing rab5(S34N), there was only a trend of increase in EGFR-mediated cell growth. Differences in the rate of cell growth may be obscured by the fact the HeLa cells only exhibit weak EGFR-mediated cell growth (Figure 7B). Surprisingly, in cells expressing rab5(Q79L), cells exhibited a EGFR-dependent decrease in cell number that was not seen in cells maintained in 5% FBS (Figure 7B-C). Explanations of this EGFR-dependent effect include improper EGFR recycling, thereby permitting only one round of receptor activation, or sequestration of the activated EGFR to a subcellular compartment which enhances 'negative' signaling for cell survival.

Our observations that rab5 is functioning in the cell differ from those of Barbieri et al., who report a 50% decrease in the kinetics of EGFR internalization when rab5(S34N) is co-expressed with EGFRs in NR6 cells (Barbieri et al., 2000). The ability of rab5(S34N) to attenuate receptor internalization has also been reported for a number of cell surface receptors (Barbieri et al., 2000; Bucci et al., 1992; Chen and Wang, 2001; Iwata et al., 1999; Li et al., 2000; Schmidlin et al., 2001; Seachrist et al., 2000; Stenmark et al., 1994), as well as being able to decrease fluid phase endocytosis (Li and Stahl, 1993).

A number of explanations can reconcile the apparently discrepant cellular functions of rab5. First, our studies examine the internalization of endogenous EGFR, rather than those transiently

transfected. Overexpression of cell surface receptors can cause hyperactivation and/or abnormal activation of downstream effector systems. Therefore, inhibition in EGFR endocytosis in the presence of rab5(S34N) may be a consequence of perturbed signal transduction arising from altered receptor/effector coupling and ultimately causing changed membrane trafficking. Alternatively, the overexpressed EGFRs may mislocalize in the cell causing them to utilize trafficking proteins they may not normally use. Data that argue against these possibilities are the observation that fluid phase endocytosis, which is not dependent on co-expression of a receptor, is altered by wild type and mutant rab5 expression in BHK cells (Li and Stahl, 1993).

A second explanation is that early endosome import may be more tightly coupled to internalization in the other cell lines than in HeLa and HepG2 cells. If this hypothesis is correct, in other cell lines rab5(S34N) may still block entry into the early endosome and in effect cause a "membrane traffic jam". The result would be seen as slowed receptor internalization because clathrin-coated vesicles and intermediate vesicles can not transport their cargo into the early endosome. In the absence of the ability of a receptor to properly internalize, previous studies have not been able to draw conclusions about downstream trafficking events.

Alternatively, rab5 may have two functions as suggested by the subcellular localization and the summation of literature. The first function would be to regulate budding from the plasma membrane and the second to regulate fusion to the early endosome. Exogenous rab5 may not be properly post-translationally modified when expressed at such high levels in HeLa and HepG2 cells. Rab5 is geranylated at its C-terminus, facilitating its association with the plasma membrane (Quellhorst Jr. et al., 2001). If high-levels of rab5 are made but can not be properly geranylated, then the protein may not be appropriately localized in the HeLa and HepG2 cells. If

this is the case, the distribution of exogenous rab5 may be altered in HeLa and HepG2 cells and the function of rab5 may be dictated by its cellular locale.

In summary, we have found that expression of activating and inactivating mutants of rab5 have no effect on endogenous EGFR endocytosis in HeLa and HepG2 cells, but these mutants do effect EGFR degradation and EGFR-mediated cell proliferation. Thus, rab5 serves as a regulator of EGFR endocytic trafficking distal from the plasma membrane and directly or indirectly modulates EGFR function.

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Footnotes:

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Abbreviations:

EGFR – epidermal growth factor receptor; EGF – epidermal growth factor; FBS – fetal bovine serum; RTK – receptor tyrosine kinase; tTA – tetracycline transactivator; HA – hemagluttin; GFP – green fluorescent protein; HRP – horseradish peroxidase, TLC – thin layer chromatography

Figure Legends

Figure 1. Functional expression of tetracycline-regulatable HA-rab5 adenoviral constructs. A. Description of hemagluttin (HA)-epitope tagged rab5 proteins used in this paper. B. Expression of HA-tagged rab5 adenoviruses in tTA-HeLa cells. 70% confluent dishes of tTA-HeLa cells were infected with nothing (no virus), the indicated adenoviruses alone (m.o.i. = 10), or the rab5(S34N) adenovirus in the presence of 1 μg/ml tetracycline as described in "Materials and Methods". Cell lysates were prepared, separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against rab5 (αrab5, Transduction Labs) or the HA-epitope (12CA5, Roche Laboratories). Immunoblotting using the rab5 antibody allows detection of the endogenous rab5 protein. C. Guanine nucleotide binding of HA-tagged rab5 adenoviruses in tTA-HeLa cells. tTA-HeLa cells were infected with the indicated HA-tagged rab5 adenoviruses. Eighteen hours after infection, cells were incubated with 0.5 mCi/ml 32P in phosphate-free, serum free DMEM for 2 hours. Cells were immunoprecipitated with the 12CA5 antibody, washed, and equilibrated in thin-layer chromatography (TLC) buffer. Guanine nucleotides associated with the immunoprecipitates were separated by TLC and visualized by autoradiography. Percentage GTP bound was calculated using the Molecular Dynamics Phosphoimaging System. Shown is a representative experiment repeated three times.

Figure 2. Over-expression of wild-type or mutant rab5 have no effect on the rates of 125I-EGF or fluid phase endocytosis. tTA-HeLa cells were infected as described in Figure 1. A. 125I-EGF Uptake. Cells were incubated with 125I-EGF for 2 hours on ice to achieve steady-state binding. Free radioligand was removed by a series of washes in ice-cold PBS and 37°C media was added to the cells and they were placed at 37°C. At the indicated times, cells were assayed for the

percent internalized radioligand. Cell surface ligand was collected by incubation in high salt/low pH buffer (0.5M NaCl/0.2 M acetic acid pH 2.8); internalized radioligand was obtained by solubilizing the remaining cells in 1M NaOH. Radioactivity in each fraction was determined by counting on a Beckman Gamma counter. Data are plotted as the percentage of 125I-EGF internalized/ (internalized + cell surface). Data shown are the average ± S.E.M, N=3-4 experiments. B. Fluid phase endocytosis was measured in rab5 adenovirus infected cells incubated with 4 mg/ml horseradish peroxidase (HRP) at 37°C for the indicated amounts of time as described in " Materials and Methods". HRP uptake was normalized to the amount of cellular protein, and is plotted relative to maximal HRP uptake in uninfected cells. Data shown are the average ± S.E.M, N=3-4 experiments.

Figure 3. Fluorescently labeled EGF co-localizes with adenovirally expressed rab5(WT) and rab5(Q79L), but not rab5(S34N) in HeLa cells. A. Infected, serum starved tTA-HeLa cells were incubated with Alexa488-EGF for 10 minutes at 37°C. External Alexa488 EGF was removed by a series of PBS and low pH washes as described in " Materials and Methods". Cells were fixed in 4% p-formaldehyde and processed for indirect immunofluorescence using the 12CA5 (anti-HA) antibody and visualized with Alexa 568 conjugated to a goat anti-mouse secondary antibody. Images were obtained using a Leica TCS NT confocal microscope (bar = 10 um). B. Pixel intensities along the XY-plane were collected from the corresponding image in 'Figure 3A' and analyzed using Leica TCS NT Imaging and ImageJ software. Plotted are the pixel intensities along a 8-12 um section of the XY-plane. Red represents the 12CA5 immunoreactive proteins and green represents the Alexa488-labeled EGF.

Figure 4. Fluorescently-labeled EGF co-localizes with GFP-tagged rab5(WT) and rab5(Q79L), but not GFP-tagged rab5(S34N), rab7(WT) or rab7(N125I). A) HeLa cells were calcium phosphate transfected with the indicated constructs. After overnight recovery, the transfected cells were incubated with Texas Red-conjugated EGF for 10 minutes. Cells were fixed 4% p-formaldehyde and processed for indirect immunofluorescence using an EEA1 mouse monoclonal antibody (Transduction Laboratories) and visualized with Alexa647 conjugated to a goat anti-mouse secondary antibody (Molecular Probes). The distribution of rab5/rab7 is shown in green, the EGF in red, and the EEA1 in blue. Images were collected as described in Figure 3. B. Pixel intensities of 'Figure 4A' were processed as described in Figure 3B. Data are plotted as GFP-rab5 (green), Texas Red-EGF (red), and EEA1 (blue).

Figure 5. Expression of tetracycline regulatable rab5 adenoviruses express in tTA-HepG2 cells, but do not alter the rate of 125I-EGF endocytosis. A. tTA-HepG2 cells express high levels of the rab5 constructs. Seventy percent confluent dishes of tTA-HepG2 cells were infected with nothing (none), the indicated adenoviruses alone (m.o.i. = 10), or adenovirus in the presence of 1 μg/ml tetracycline (+) as described in "Materials and Methods". Cell lysates were prepared, separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against rab5. B. Cells infected as described above were assayed for their ability to internalize 125I-EGF, as described in Figure 2.

Figure 6. Fluorescently labeled EGF co-localizes with rab5(WT) and rab5(Q79L), but not rab5(S34N) in HepG2 cells. A. tTA-HepG2 cells were infected with the wild type and mutant adenoviral constructs as previously described and assessed for Alexa488-EGF uptake. Cells

were further processed for indirect immunofluorescent localization of HA-tagged rab5 using the 12CA5 antibody, and visualized with Alexa568 conjugated to a goat anti-mouse secondary antibody. Images were collected with a Leica TCS NT confocal microscope (bar = 10 um). B. Pixel intensities along the XY-plane were collected from the images in 'Figure 6A' and collected as previously described.

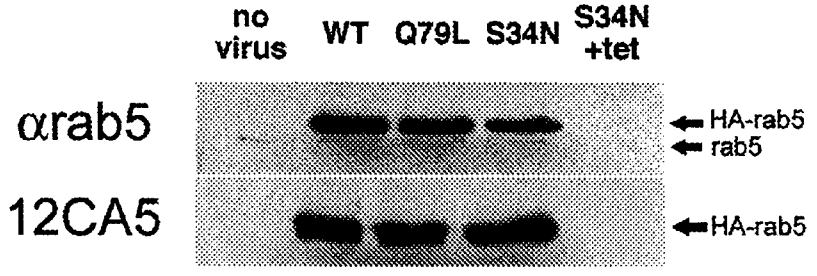
Figure 7. Expression of rab5 mutants alters the rate of EGFR degradation and differentially effects EGFR-mediated cell growth. A. Infected cells were incubated with 10 ng/ml EGF for the indicated amounts of time. Cell lysates were collected, resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a rabbit polyclonal antibody against the EGFR. After incubation with a horseradish peroxidase conjugated goat anti-rabbit antibody, proteins were visualized by enhanced chemiluminescence and quantified on a UV Productions Imaging System. Data are plotted as the fraction of EGFR detected as compared to infected cells not incubated with EGF. Data points are the average ± S.E.M, N=3-4 experiments. B and C. tTA-HeLa cells were infected as described, allowed to recover, and plated at a density of 5,000 cells/well. Cells were incubated in DMEM, (B) 100 ng/ml EGF, or (C) 5% FBS for 72 hours and assayed for the number of viable cells using standard MTT assay (Hansen et al., 1989). Data are plotted as the fraction of cell growth as compared to DMEM alone. Data points are the average ± S.E.M, N=3-8 experiments; *p* values were calculated using a paired t-test (Prism software). * = no statistical difference; ** = statistically different. There is no statistical difference between the cell growth with 5% FBS with expression of any of the rab5 mutants.

Figure 1

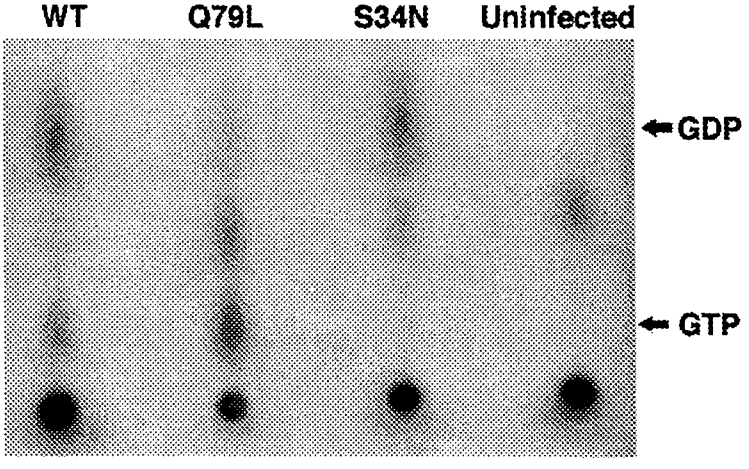
A.

Construct	Mutation	Guanine Nucleotide
HA-rab5(WT)	None	GTP and GDP
HA-rab5(Q79L)	Glu 79 to Leu	Inhbits GTPase; GTP bound
HA-rab5(S34N)	Ser 34 to Asn	GDP ~100X > than GTP

B.



C.



% GTP Bound 28% 66% 3%

Figure 2

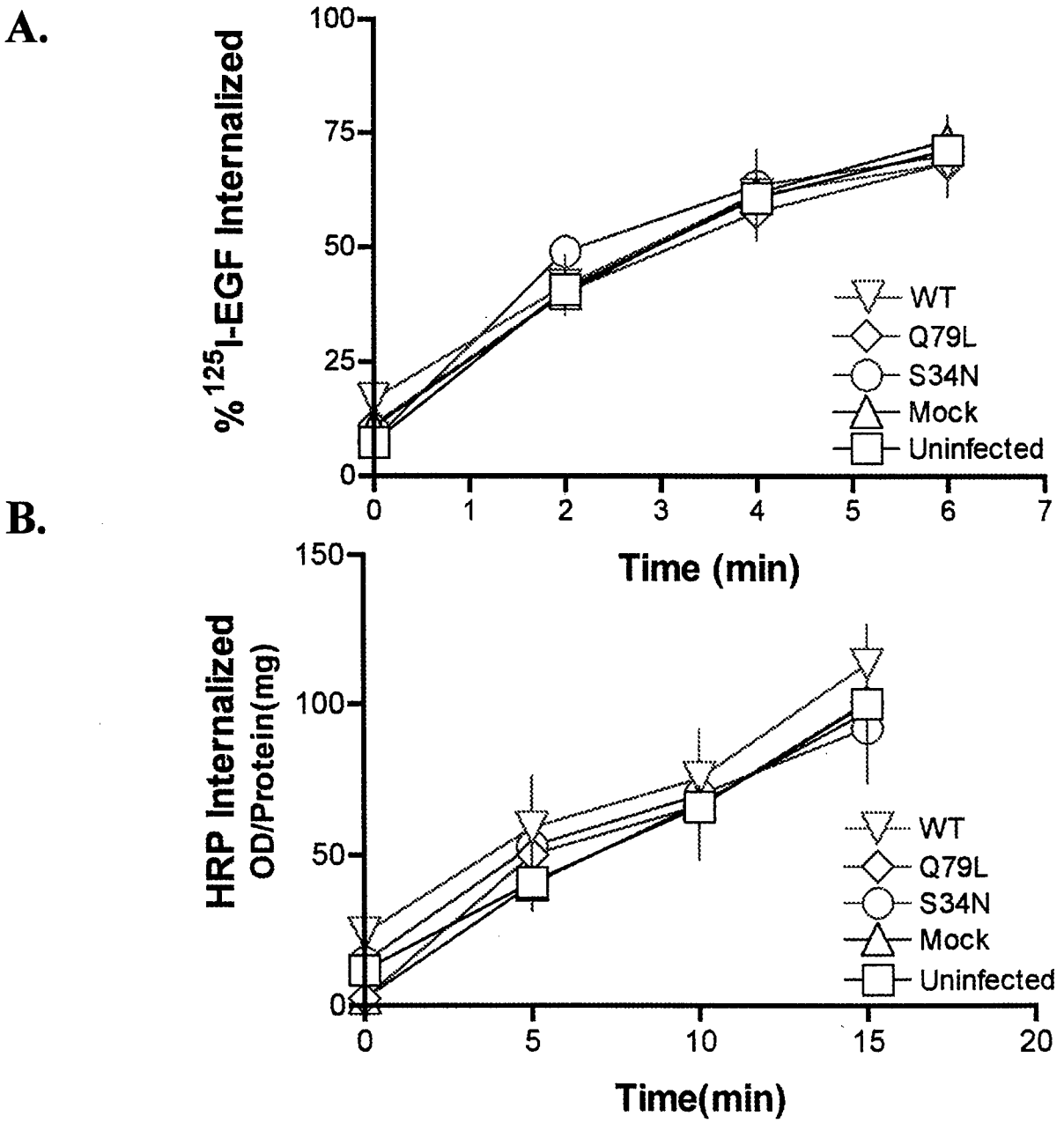

A.
% 125I-EGF Internalized
100
75
50
25
0
0
1
2
3
4
5
6
7
Time (min)
WT
Q79L
S34N
Mock
Uninfected
B.
HRP Internalized
OD/Protein(mg)
150
100
50
0
0
5
10
15
20
Time(min)
WT
Q79L
S34N
Mock
Uninfected

Figure 3A

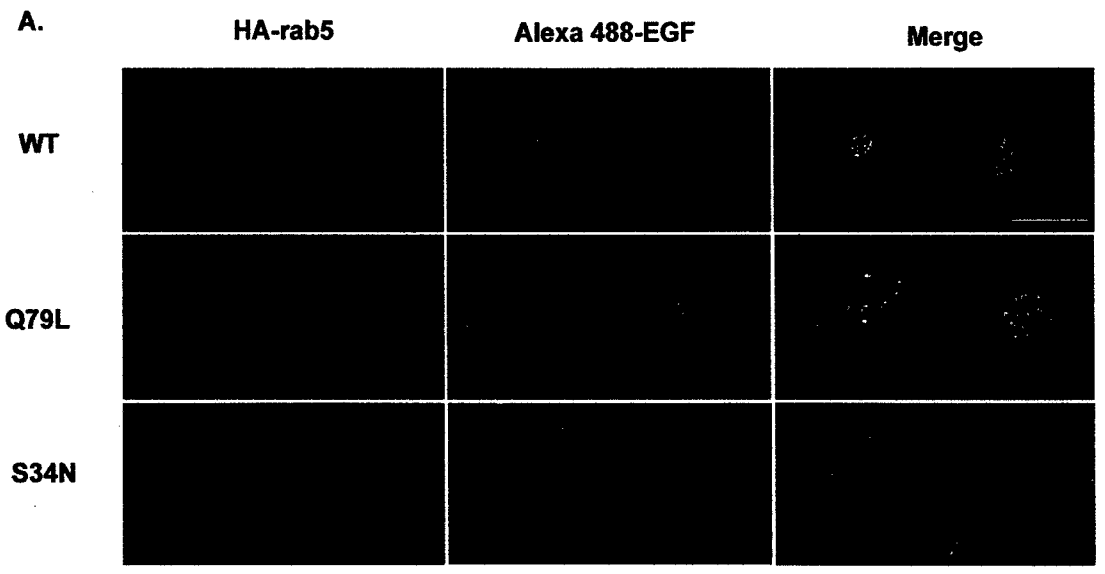
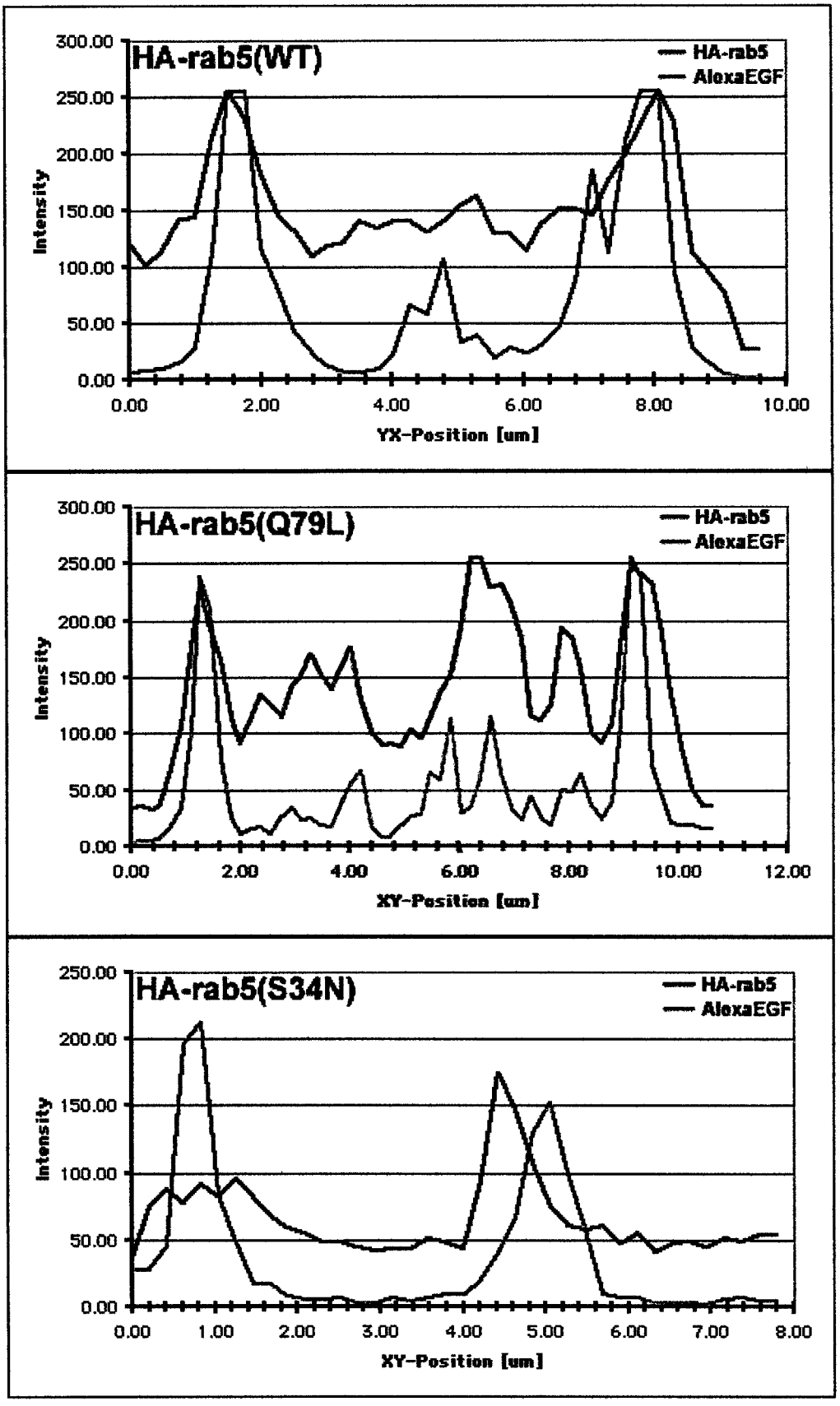


Figure 3B


HA-rab5(WT)
HA-rab5
AlexaEGF
Intensity
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.00
2.00
4.00
6.00
8.00
10.00
YX-Position [um]
HA-rab5(Q79L)
HA-rab5
AlexaEGF
Intensity
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
XY-Position [um]
HA-rab5(S34N)
HA-rab5
AlexaEGF
Intensity
250.00
200.00
150.00
100.00
50.00
0.00
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1.00
2.00
3.00
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7.00
8.00
XY-Position [um]

Figure 6A

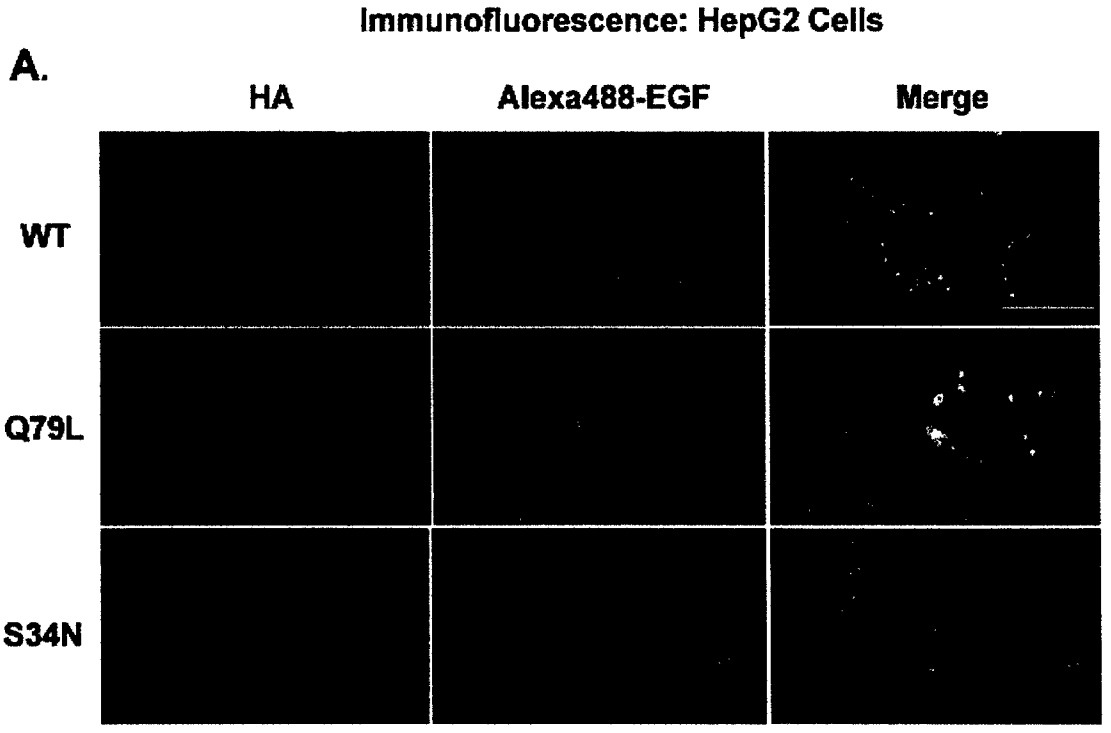

Immunofluorescence: HepG2 Cells
A.
HA
Alexa488-EGF
Merge
WT
Q79L
S34N

Figure 6B

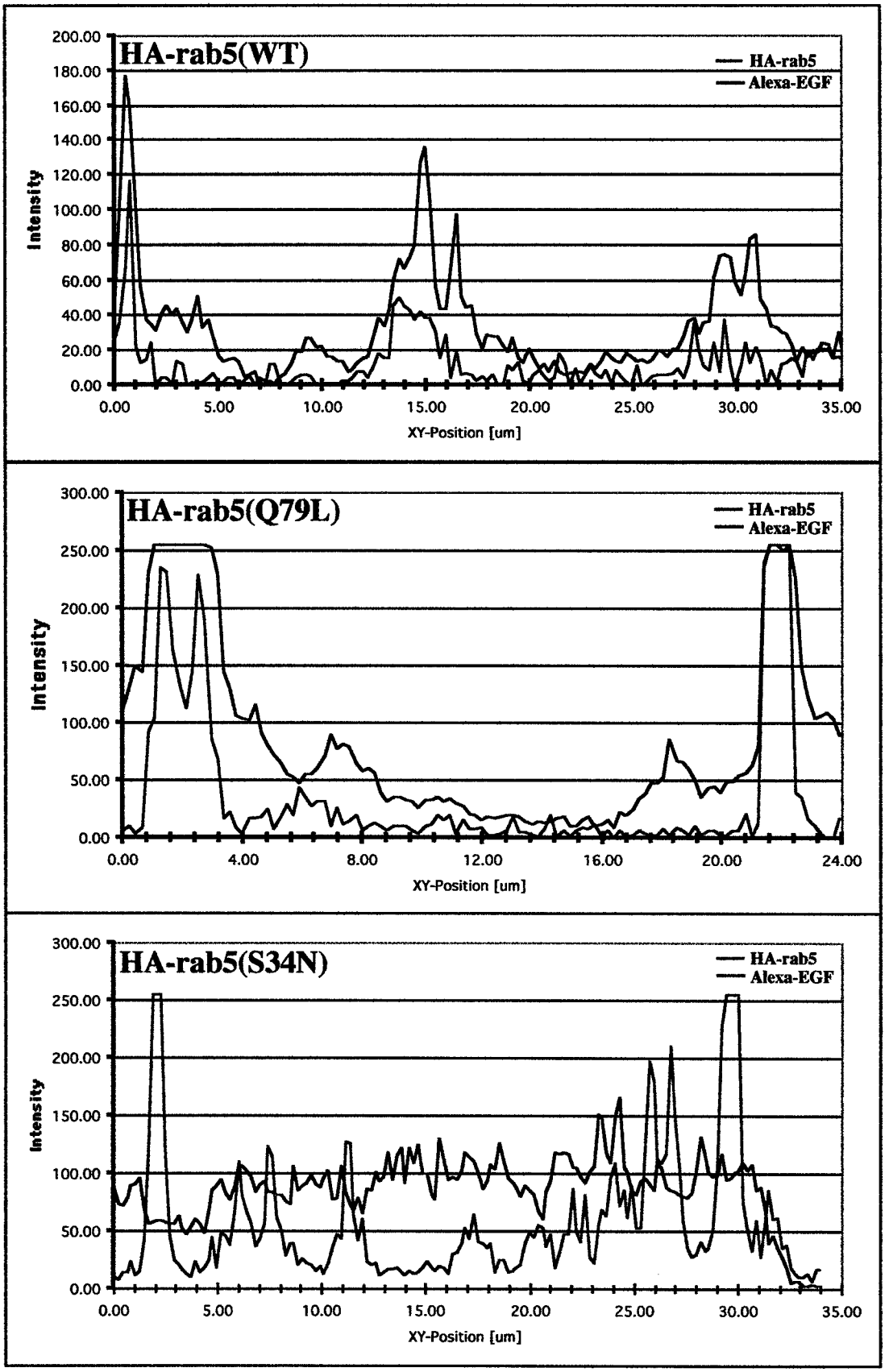

HA-rab5(WT)
HA-rab5
Alexa-EGF
Intensity
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
XY-Position [um]
HA-rab5(Q79L)
HA-rab5
Alexa-EGF
Intensity
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.00
4.00
8.00
12.00
16.00
20.00
24.00
XY-Position [um]
HA-rab5(S34N)
HA-rab5
Alexa-EGF
Intensity
300.00
250.00
200.00
150.00
100.00
50.00
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
XY-Position [um]

Figure 5

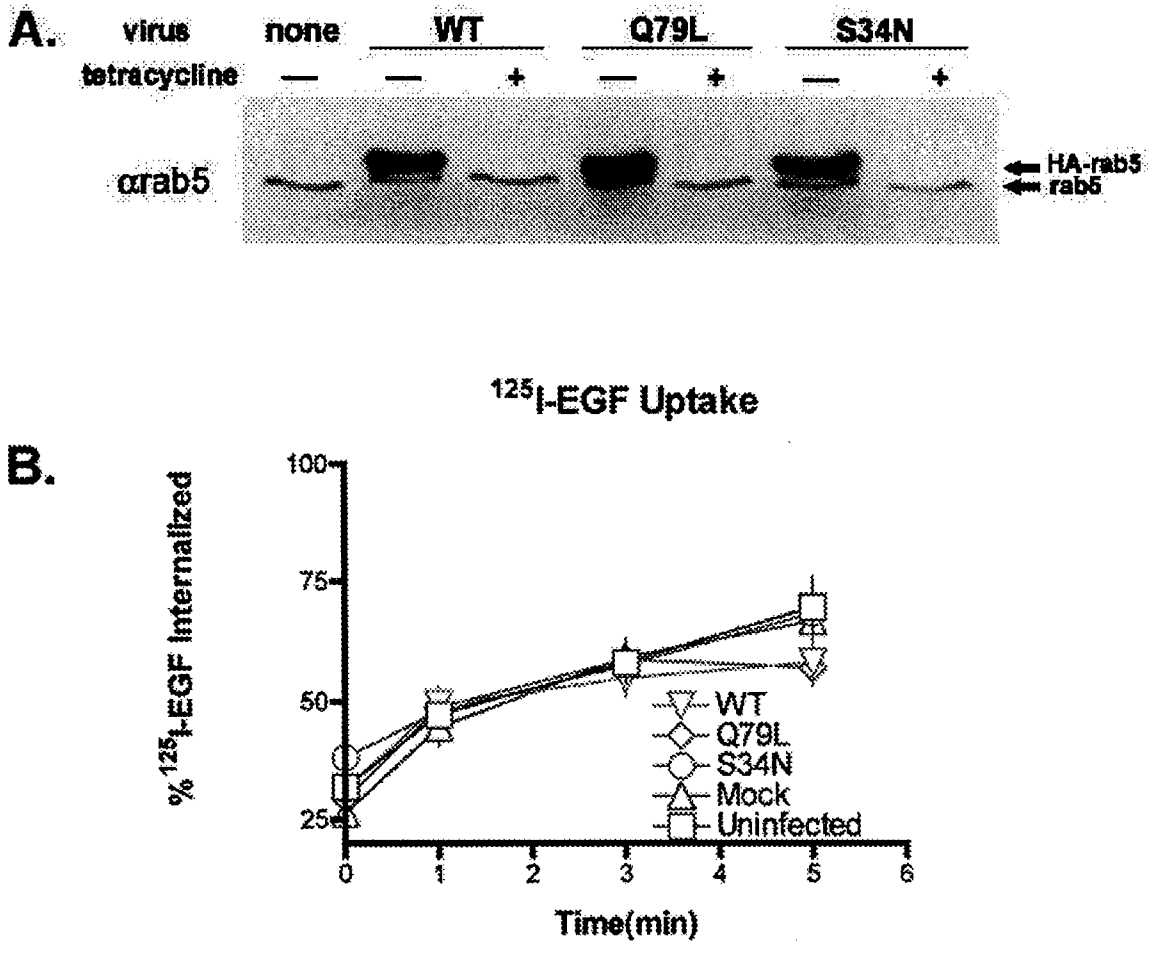

A.
virus
none
WT
Q79L
S34N
tetracycline
—
—
+
—
+
—
+
αrab5
HA-rab5
rab5
B.
125I-EGF Uptake
% 125I-EGF Internalized
100
75
50
25
0
1
2
3
4
5
6
Time(min)
WT
Q79L
S34N
Mock
Uninfected

Figure 4A

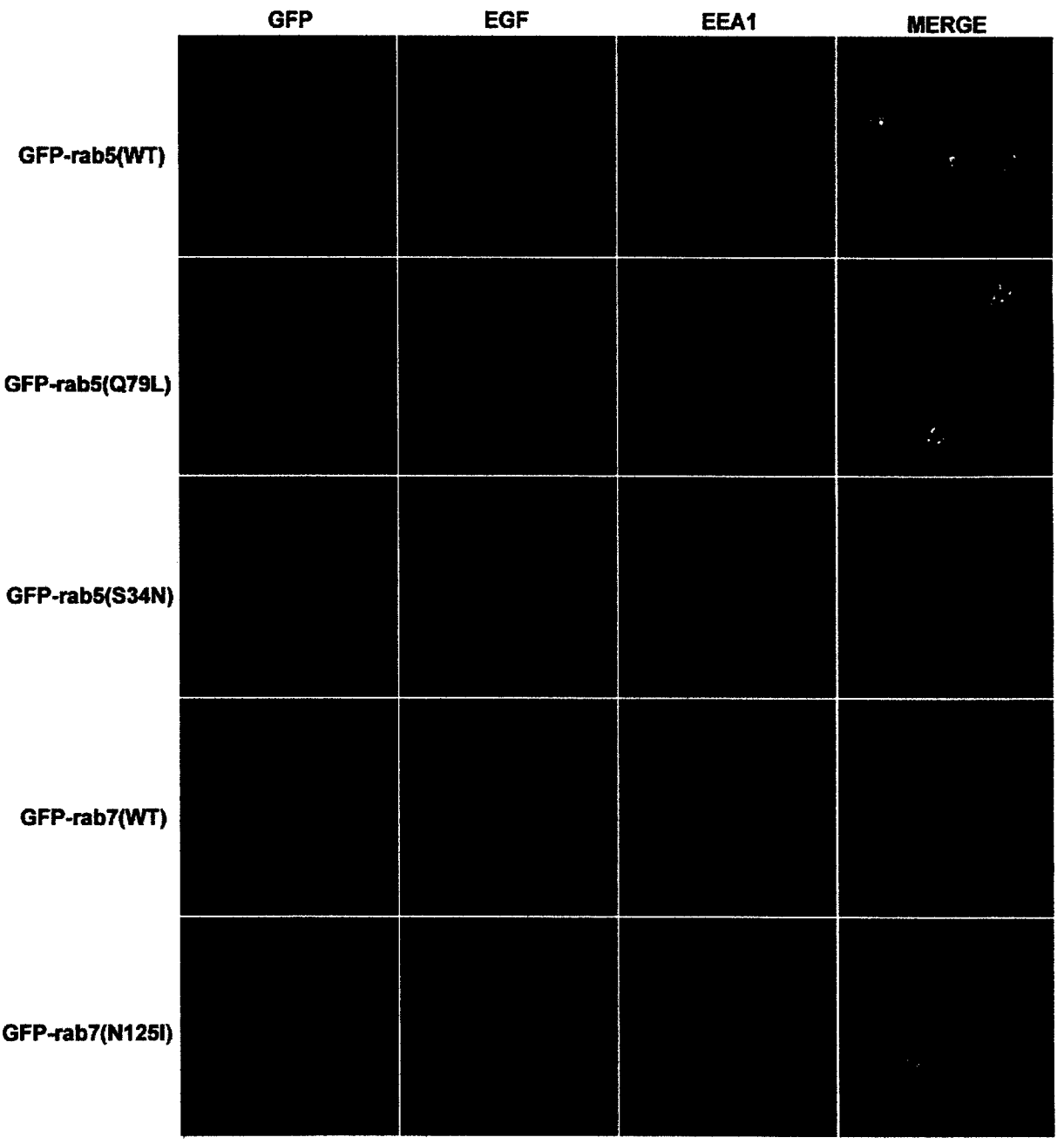

GFP
EGF
EEA1
MERGE
GFP-rab5(WT)
GFP-rab5(Q79L)
GFP-rab5(S34N)
GFP-rab7(WT)
GFP-rab7(N125I)

Figure 4B

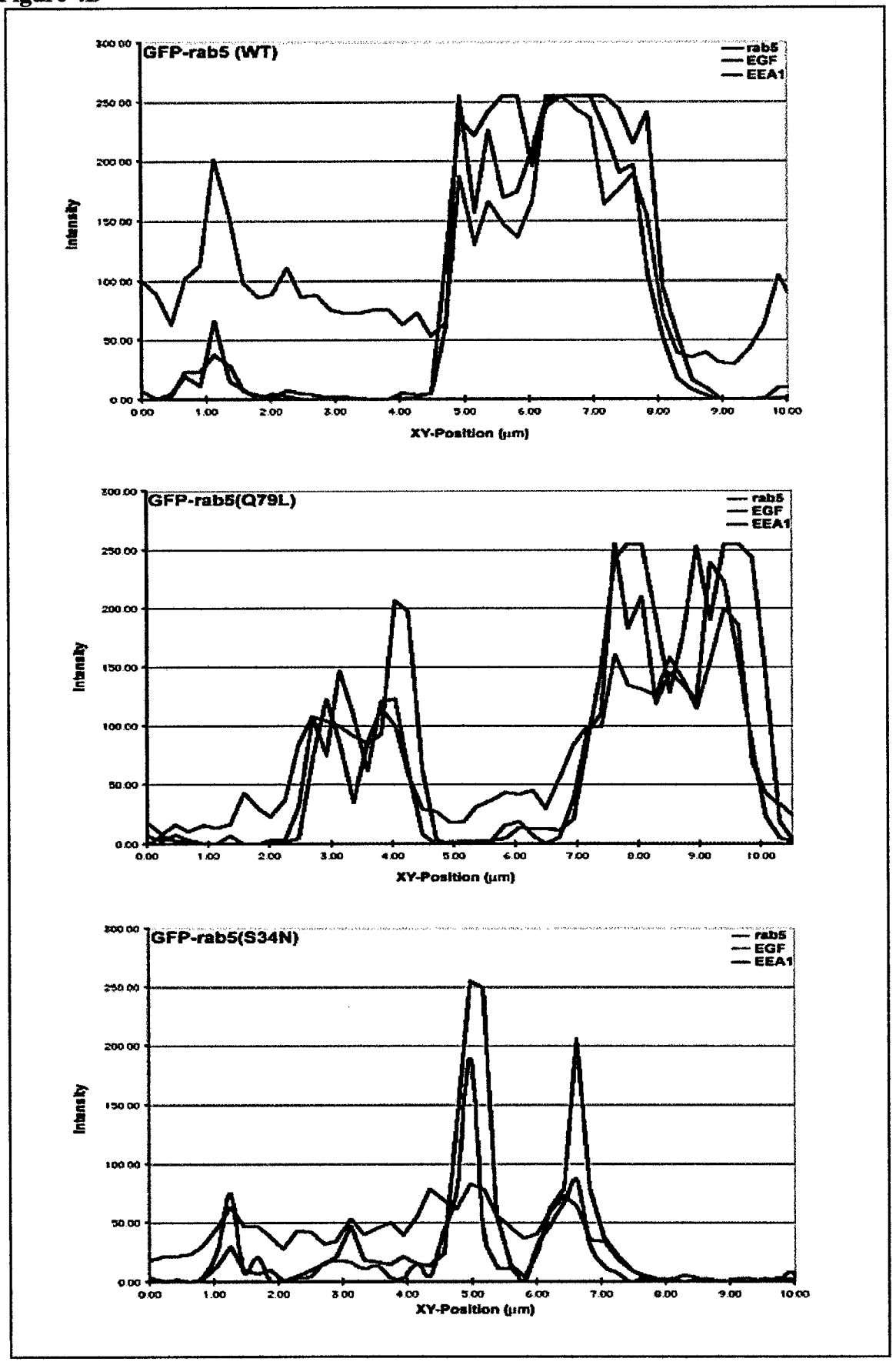
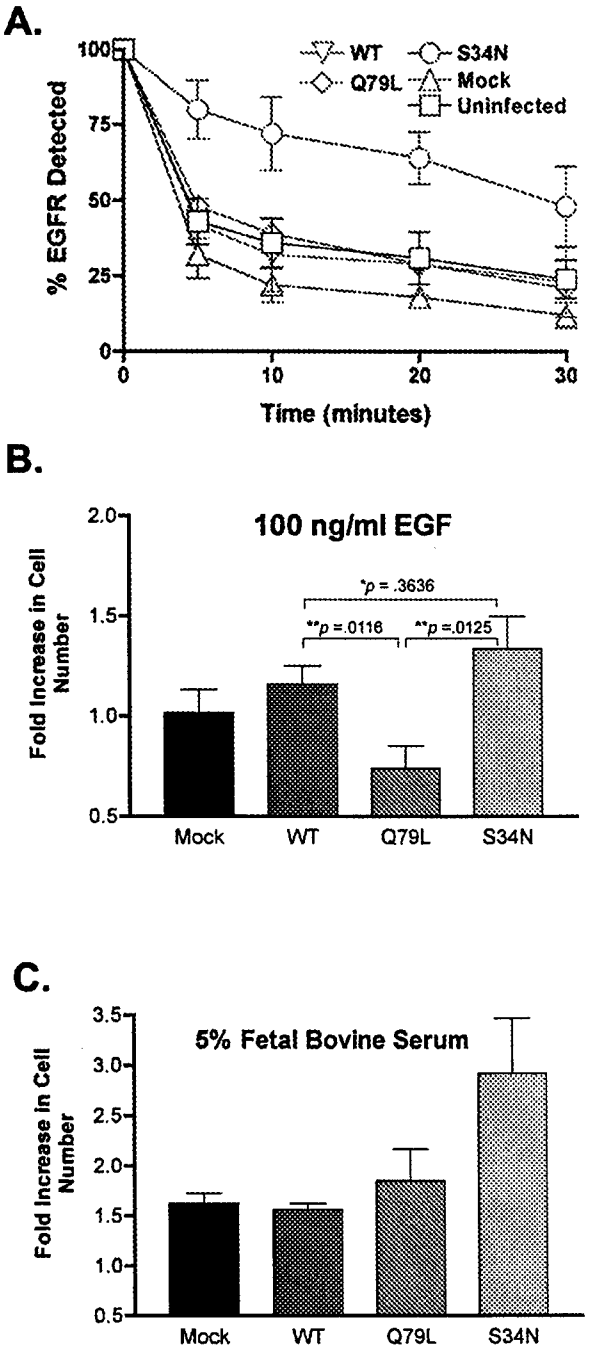

GFP-rab5 (WT)
rab5
EGF
EEA1
Intensity
XY-Position (µm)
GFP-rab5(Q79L)
rab5
EGF
EEA1
Intensity
XY-Position (µm)
GFP-rab5(S34N)
rab5
EGF
EEA1
Intensity
XY-Position (µm)

Figure 7 - Dinneen and Ceresa



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 27 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed list of technical documents. Request the limited distribution statement assigned to the documents listed be changed to "Approved for public release; distribution unlimited." These documents should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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