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13. ABSTRACT (Maximum 200 Words)
Loss is epithelial architecture is thought to be an early event in carcinoma. The mechanism by which oncogenes disrupt epithelial architecture is poorly understood. Previous results from our lab have shown that ErbB2, an oncogene correlated to poor clinical prognosis in breast cancer, can disrupt epithelial cell polarity. My research is aimed at investigating how activation of ErbB2 disrupts epithelial cell polarity.

Activation of ErbB2 is known to induce phophorylation of five tyrosines in its cytoplasmic tail. Using ErbB2 autophosphorylation site mutants, I investigated whether a particular tyrosine residue mediates the ErbB2-induced changes in epithelial cell polarity. In my first series of studies, where one of the five tyrosines was mutated to phenylalanine, I have identified that loss of Tyr 1144 inactivates the ability of ErbB2 to disrupt cell polarity suggesting that Tyr1144 is required for ErbB2-induced changes in polarity. I am in the process of completing my analyses to investigate whether Tyr 1144 or other tyrosine is sufficient to mediate ErbB2-induced changes in polarity and identify the tyrosine residues that mediate the ErbB2-induced changes in the Par protein complex.

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Introduction

The aim of this grant is to investigate the mechanism by which ErbB2, an oncogene commonly overexpressed in breast cancer, regulates the Par complex, one of the three known complexes important in establishing cell polarity [1]. In order to address this question two approaches were taken (1) investigate the contribution of specific autophosphorylation sites to ErbB2-induced changes in epithelial cell polarity and, (2) investigate the role played by basal-lateral localization of ErbB2 in polarized epithelial cells.

Our lab uses a unique ErbB chimeric receptor, in which both the extracellular and transmembrane domains have been replaced with corresponding domains from the p75 low affinity NGFR receptor and dimerization is mediated by a small molecule ligand that binds to the FKBP domain which is fused to C-terminal region of chimeric ErbB2 [2]. In contrast to constitutively active versions of ErbB2, the inducible system offers us the advantage to activate ErbB2 in polarized proliferation-arrested epithelial cells and to investigate the effect of ErbB2 activation on cell polarity [3].

Previous studies have identified five tyrosines in ErbB2's carboxy terminal tail that are autophosphorylated upon ErbB2 dimerization. Other labs have observed that ErbB2 receptor molecules with at least one active autophosphorylated tyrosines (Y) mutants Tyr 1144 (YB), Tyr 1201 (YC), Tyr 1226/1227 (YD) and Tyr 1253 (YE) can promote transformation of fibroblasts [4]. I have generated two classes of autophosphorylation site mutants, a) a series where all but one tyrosine are active and b) a series where only one tyrosine is active.

Previous studies have identified that a 12 amino acid region in the intracellular juxtamembrane region of ErbB2 that is required for basolateral localization of ErbB2 [5]. To investigate the importance of basolateral localization of ErbB2, I have generated mutant versions of ErbB2 that will fail to localize to the basolateral region of polarized epithelial cells and have investigated the ability of mislocalized ErbB2 to disrupt polarity.

Collectively these studies have led to conclusion that Tyr 1144 is required for ErbB2 induced disruption of epithelial cell polarity and that disruption of basolateral localization of ErbB2 not only inactivates its ability to disrupt polarity but also inactives its ability to transform fibroblasts. In order to investigate the importance of these observations in transformation of mammary epithelial acini-like structures, I am using MCF-10A, a human breast epithelial cell line that known to form 3D acini-like structures in culture [6]. In order to investigate the mechanism by which ErbB2 induces disruption of polarity, I am investigating which autophosphorylation tyrosine residue mediates ErbB2-induced changes in Par protein complex.

Body

Using the chimeric receptor as "wild type" ErbB2 I have created two distinct series of mutations in order to investigate the role of individual autophosphorylated tyrosines and ErbB2 localization in its ability to disrupt cell polarity.

1. Investigate the contribution of specific autophosphorylation sites

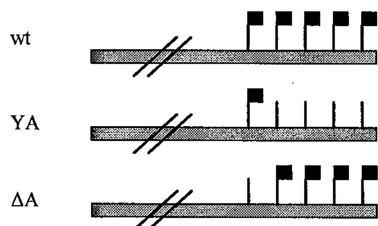

Figure 1: Examples of Y and Δ mutants. In Y mutants all but one of the autophosphorylation sites is mutated to phenylalanine, whereas in delta mutants 4 autophosphorylation sites remain intact and only one is mutated to phenylalanine.

The first series of mutations, herein referred to as the "Delta" mutations, have only one of the five tyrosines mutated to phenylalanine (Figure 1). This Delta series is used to determine which, if any, of the sites are required for ErbB2's ability to disrupt polarity.

1a. Delta mutations

All 5 delta mutations - ΔA, ΔB, ΔC, ΔD and ΔE – have been made and expressed in epithelial cells. In addition two control mutations have been created, 1) all five tyrosines are mutated to phenylalanine (phosphotyrosine deficient – PYD) and, 2) a kinase-null mutant (kd). In order to be able to directly compare the effect of activating different mutant versions of ErbB2, I selected clonal populations of epithelial cells that have comparable levels of expression of chimeric ErbB2. The clones were further screened for their ability to undergo ligand–induced tyrosine phosphorylation (Figure 2).

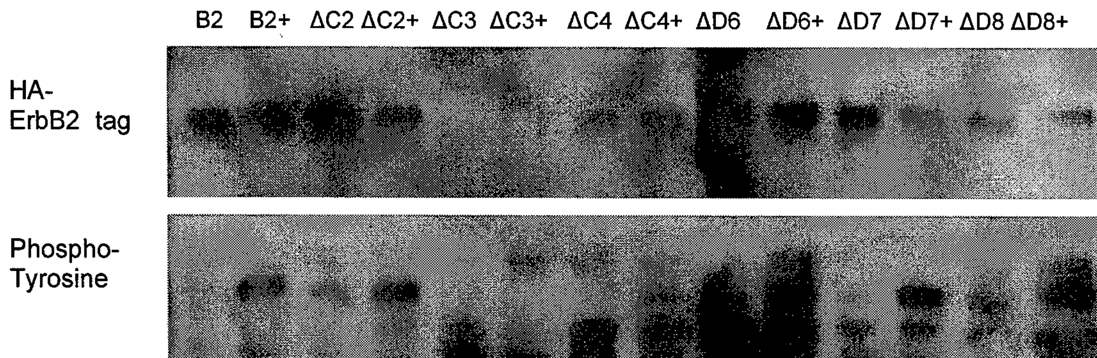

Figure 2. An example of selection of clones expressing the autophosphorylation site muations. After clones were isolated they were tested first for expression of the receptor by doing a western blot for the chimeric receptor tag, HA. They were then tested for inducible activation of the receptor by stimulating the cells with dimerizer for 20 minutes before lysis. An increase in phosphotyrosine signal of the receptor indicates that the receptor is active. A wild type control of ErbB2 (B2) shows the level of receptor expression that is known to cause a phenotype in cells express the wild type receptor. All clones chosen for testing express the receptor at this level or a level slightly higher. In this case, ΔC2 ΔD6 and ΔD8, were chosen for further testing in polarity assay.

Our lab has previously shown that stimulation of epithelial cells expressing the wild type chimera results in (a) activation of the receptor and (b) disruption of polarity as visualized by changes in ZO-1, a tight junction marker. These changes occur only upon addition of the synthetic ligand and therefore can be induced polarized epithelia. Activation of ErbB2 that lacks Tyr 1201 (delta C) or Tyr 1226/7 (delta D) induced disruption of ZO-1 localization, however, activation of ErbB2 mutants that lack Tyr 1028 or Tyr 1144 did not induce disruption of ZO-1 localization suggesting that Tyrosines 1201 and 1226/7 are not required for ErbB2-induced disruption of cell polarity and tyrosines 1028 and 1144 are critical mediators of ErbB2-induced changes in cell polarity. (Figure 3). Experiments are being done to determine the effect of stimulating ΔE. Dankort et al have shown previously that the autophosphorylated tyrosine sites play redundant roles in the transformation of rat fibroblasts, suggesting that the requirement for Tyr 1144 may be an epithelial specific effect. I am conducting experiments to confirm that ΔB and ΔC constructs are capable of transforming fibroblasts.

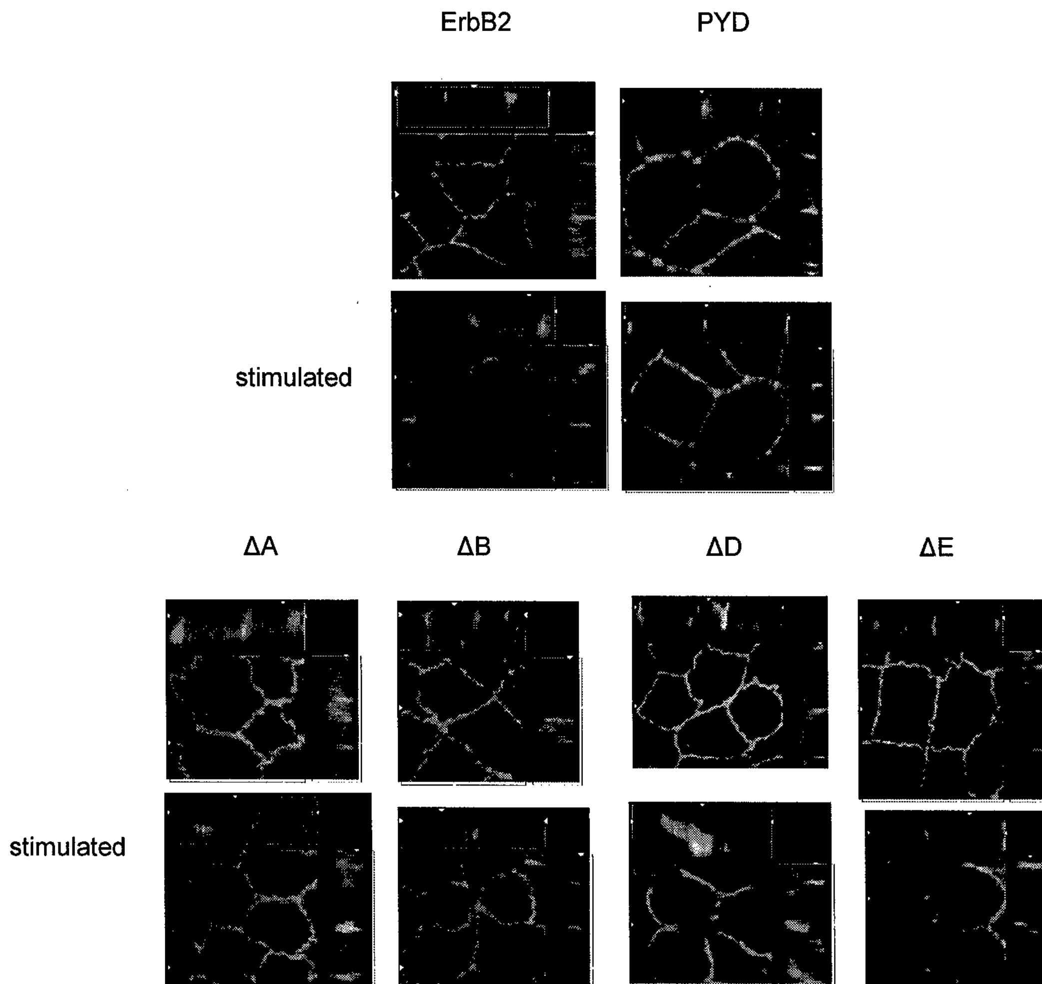

Figure 3. Immunoflouresence of clones expressing delta mutations both before and after stimulation of the receptor. In each case, an xy view in the plane of the tight junctions is flanked by a yz view and topped by an xz view of the field. ErbB2 is a wild type clone known to cause disruption of polarity. PYD (the phosphotyrosine deficient mutation) is included as a negative control. The rest are representative clones of the various mutations. Nuclei are stained blue with DAPI, the receptor tag is visualized in green, ZO-1, the marker of tight junctions, is stained red.

1b. Y mutations

The second series of mutations, herein referred to as the "Y" mutations, have all but one of the five tyrosines mutated to phenylalanine (Figure 1). The Y series is used to determine which, if any, of the autophosphorylation tyrosines are sufficient for ErbB2's ability to disrupt polarity. Tyr 1201 (YC), Tyr 1226/7 (YD), and Tyr (1253) YE have all been in tested in clonal populations with equal levels of expression, chosen in the same manner and expressing the same level as the delta mutations. None of the sites, tested thus far, have shown the ability to induce disruption of polarity in a polarized, proliferation arrested monolater (Figure 4). I am currently testing YB (Tyr 1144) for the ability to disrupt polarity. It will be interesting to determine if this site is sufficient, since lack of this tyrosine (ΔB) prevents ErbB2 mediated disruption of polarity.

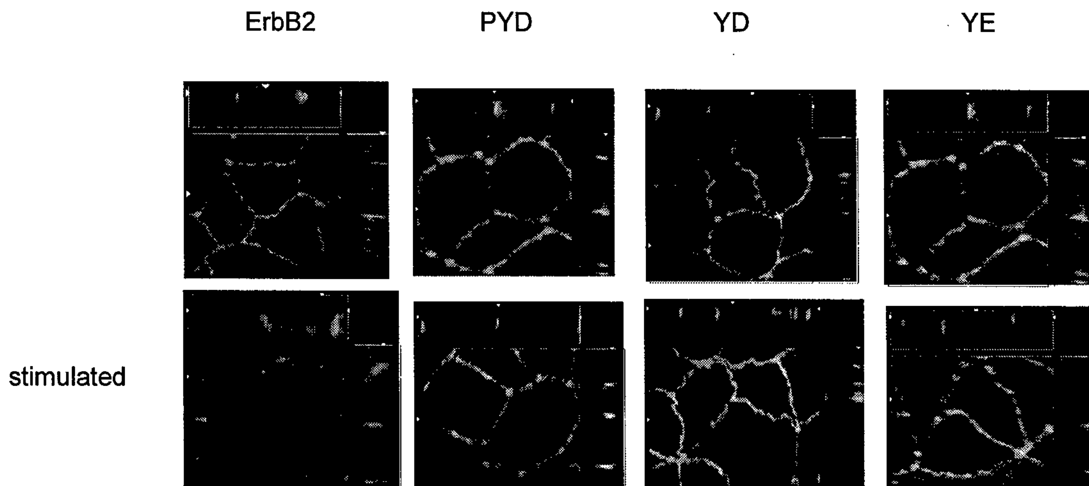

Figure 4. Immunoflouresence of clones expressing Y mutations both before and after stimulation of the receptor. In each case, an xy view of the field is flanked by a yz view and topped by an xz view of the field. ErbB2 is a wild type clone known to cause disruption of polarity. PYD (the phosphotyrosine deficient mutation) is included as a negative control. The rest are representative clones of the various mutations. Nuclei are stained blue with DAPI, the receptor tag is visualized in green, ZO-1, the marker of tight junctions, is stained red.

2. Investigate the role played by basal-lateral localization of ErbB2 in polarized epithelial cells

Previous studies have shown that mutation of 12 amino acids in the juxtamembrane region of the intracellular domain of ErbB2 cause the protein to localize to the apical, instead of the basal-lateral membrane. Mutation of only the first 8 amino acids results in a mixed apical/basal-lateral membrane localization [5]. I have used these mutations in the chimeric ErbB2 receptor in order to study the importance of ErbB2 localization to its ability to disrupt cell polarity.

After transferring these mutations to the chimeric receptor I tested for the ability of the receptor to autophosphorylate in the presence of the synthetic ligand. The chimeric ErbB2-12A receptors were deficent in autophosphorylation both in epithelial cells and in HEK293 cells. In contrast, ErbB2-8A is autophosphorylated in response to synthetic ligand in both these systems (Figure 5). Without being able to activate 12A we could not study the effects of localization on receptor function, therefore the rest of the studies were conducted solely with the ErbB2-8A mutation.

After confirming the apical/basal-lateral localization of ErbB2-8A (Figure 6), cells were FACS sorted into separate populations (low, medium, high) and ligand-induced autophosphorylation was again confirmed (Figure 5). Low expressing ErbB2-8A and wild type populations were both tested for the ability to disrupt epithelial cell polarity. While wild type was able to disrupt the tight junctions, visualized with the marker ZO-1, ErbB2-8A was not (Figure 7). Neither an increase in the ErbB2-8A receptor expression level nor a longer stimulation time was able to cause ErbB2-8A induced disruption of polarity (data not shown). Therefore the ErbB2-8A mutation is incapable of disrupting epithelial cell polarity.

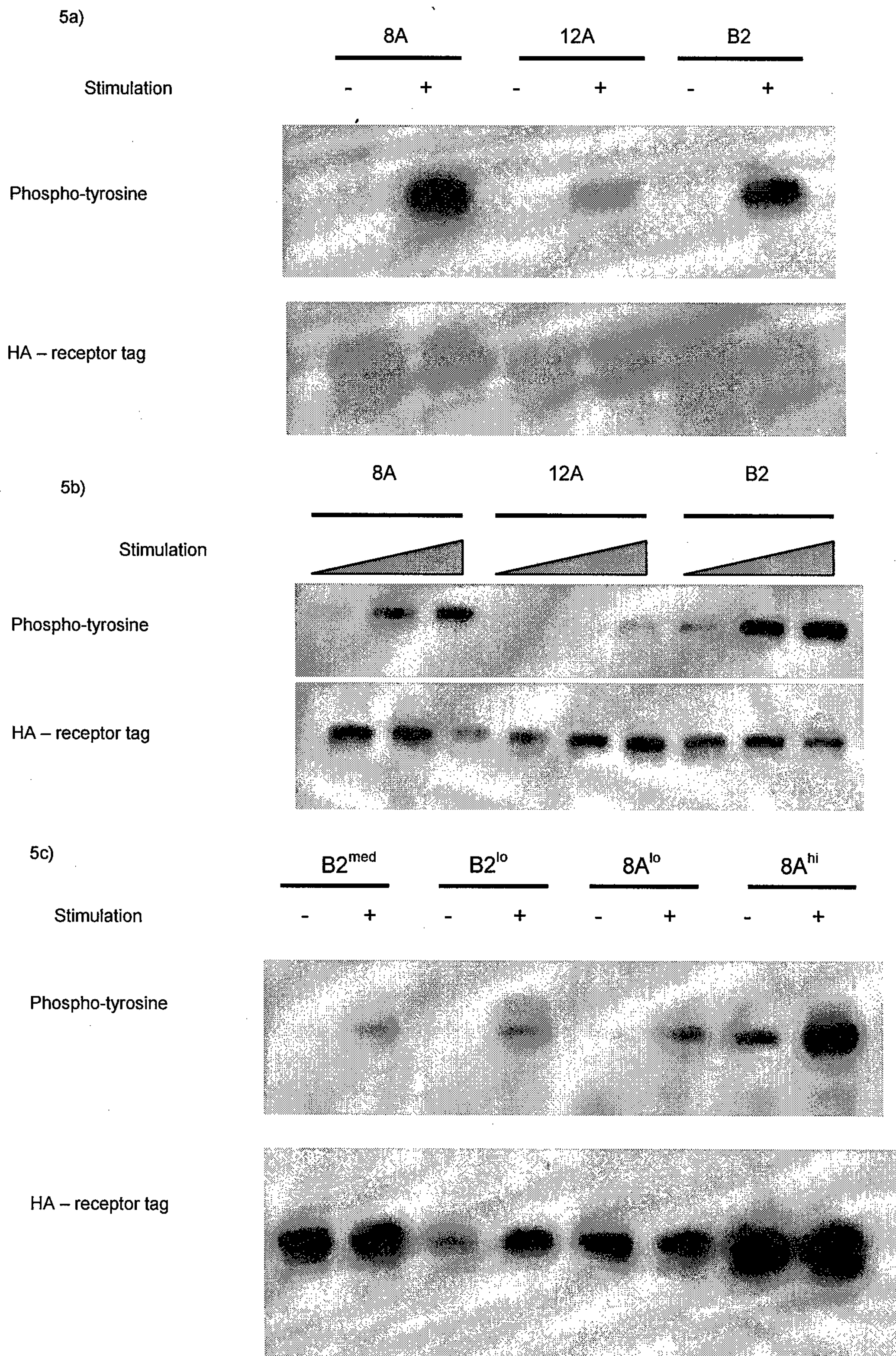

Figure 5. Mislocalization mutant ErbB2-8A autophosphorylates upon stimulation, but ErbB2-12A is deficient in autophosphorulation. Mislocalization mutations were tested first in epithelial cells (a) and then in fibroblasts at increasing concentrations of dimerizer (b) for the ability to autophosphorylate. FACS sorted populations of epithelial cells expressing wild type or 8A ErbB2 were also tested for phosphorylation (c). Cells were stimulated for 20 min before lysis. Whole cell lysates were then run out and blotted for phosphotyrosine before being stripped and reprobed for HA, the receptor tag.

I further tested this mutation for the ability to transform rat fibroblasts, which lack the apical-basal polarity of an epithelial cell. Whereas stimulated wild type ErbB2 could induce foci in a focus forming assay, ErbB2-8A could not. Therefore, the ErbB2-8A mutation, although capable of autophosphorylating, has lost its ability to transform fibroblast and to disrupt polarity in epithelial cells.

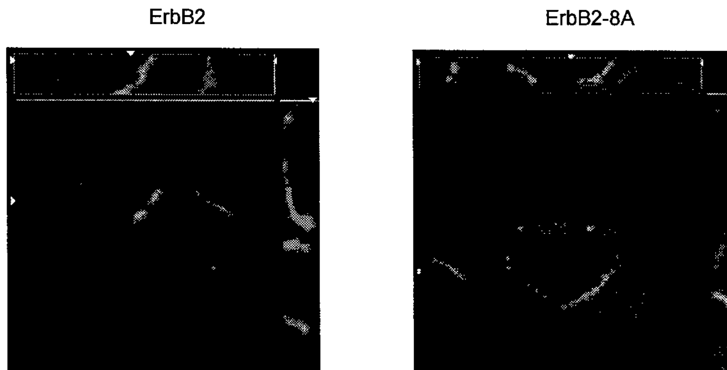

Figure 6. 8A mutations causes ErbB2 to mislocalizes to the apical, as well as lateral membrane. Immunoflouresence of MDCK cells expressing wild type and 8A ErbB2 was performed to visualize the localization of the receptor in epithelial cells. Nuclei are stained in blue with DAPI. The chimeric receptors are recognized via the HA tag and stained green.

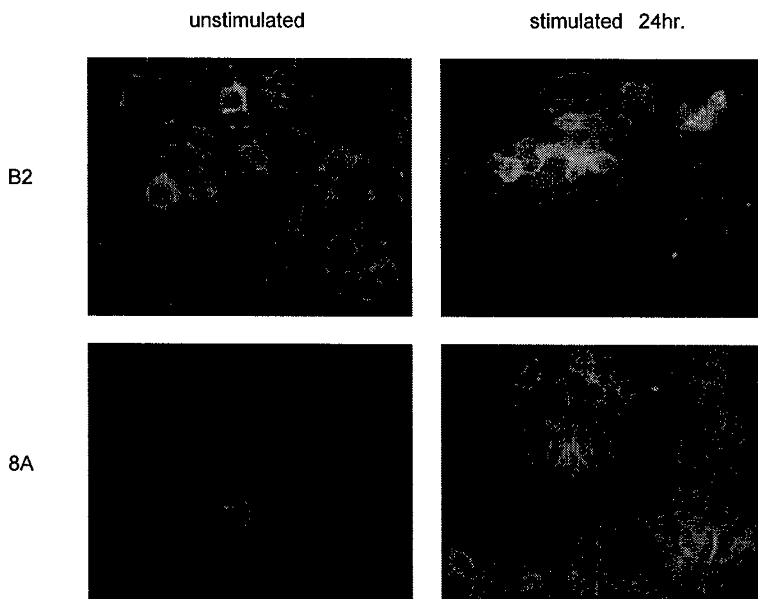

Figure 7. ErbB2-8A mutation does not disrupt epithelial cell polarity. FACS sorted populations of epithelial cells expressing either ErbB2 or ErbB2-8A at similar levels were grown on filters to form polarized monolayers. After polarization and proliferation arrest they were stimulated for 24 hrs. Nuclei are stained blue with DAPI, the receptor tag is visualized in red, and Zo-1, the marker of tight junctions, is stained green.

Key Accomplishments

- determination that ErbB2-8A, although capable of autophosphorylating, cannot disrupt epithelial cell polarity or transform fibroblasts.
- identification of tyrosine 1144 as necessary for ErbB2 mediated disruption of cell polarity.
- determination that tyrosines 1201, 1226/1227are not necessary for disruption of polarity.
- determination that tyrosines 1201, 1226/1227, and 1253 are not sufficient for ErbB2 mediated disruption of cell polarity.

Conclusion

Two approaches were taken to investigate the ability of ErbB2 to regulate cell polarity. The first, mutation of the autophosphorylated tyrosines, has lead to the discovery that tyrosine 1144 is necessary for ErbB2 mediated disruption of polarity in epithelial cells. The second, mislocalization of the receptor, has proved to have interesting possibilities as to the role the juxtamembrane region plays in ErbB2 regulated cell transformation, but is an ineffective tool for studying the role of localization in ErbB2 mediated transformation. Therefore, future studies will focus on the autophosphorylation site mutations.

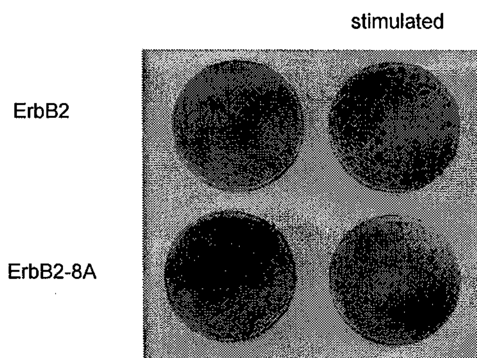

Figure 8. ErbB2, but not ErbB2 8A, transforms rat1 fibroblasts. ErbB2 and ErbB2-8A DNA were transfected into rat1 fibroblasts. Cells were allowed to grow to confluency before stimulant was added. Fourteen days later, plates were fixed and stained with Giemsa.

Once our initial studies are complete and it is determine if tyrosine 1144 is sufficient, as well as necessary, for disruption of polarity mutations involving this site will be used to characterize the mechanism by which ErbB2 regulates the Par complex. In the interest of time and well-established polarity techniques, these initial screens have been done in Madin Darby Canine Kidney cells. Now that a specific site has been targeted as a regulator of polarity a smaller set of ErbB2 mutations will be expressed in MCF-10A cells to determine the biochemical interactions mediated by the individual tyrosines, as well as the role each tyrosine plays in ErbB2 mediated transformation of mammary epithelial cells.

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