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Previous results showed that MDA-MB-468 cells, with 1.9 x 10^6 EGF-R per cell, did not rapidly eliminate activated EGF-R. The variant cell lines S1 and S11 possessing 1.6 x 10^4 and 6.6 x 10^4 EGF-R per cell respectively, did rapidly eliminate activated EGF-R. S1 and S11 lines transduced with EGF-R express EGF-R levels intermediate to the S variants and the 468 cells and eliminate their activated EGF-R at an intermediate rate. We show that phosphatase avctivity is not limiting following receptor amplification and that all phosphotyrosine can be removed within 30 seconds. Our data suggests that PY is prolonged following receptor amplification because the amplified cell lines cannot rapidly segregate ligand and receptor during trafficking. Further, we show that receptor amplification does not increase ligand capture efficiency or increase expression of autocrine ligands. Because basal PY increases following EGF-R amplification increases in a linear fashion this data further supports our model. Lastly, basal phosphotyrosine increases following EGF-R amplification appear to be ligand dependent whereas increases in EGF-R basal PY due to Her2 amplification appear to be ligand independent.

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Glenn Schaden 6/30/99

PI - Signature Date

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Modification of Statement of Work

As this project has progressed, it has naturally diverged from the originally proposed Statement of Work. New results, which will be described in this report, have suggested new directions with potentially very important implications. In addition, I expect to start writing my thesis this year and will complete my Ph.D. training simultaneously with the completion of this grant in June 2000. Because of this, my thesis committee has suggested that I perform specific experiments which will enable me to successfully conclude my ongoing research during my final year.

The original Statement of Work is as follows:

Part I) Determine the percent reduction of EGF-R kinase activity following PMA or EGF induced desensitization for all cell types being tested.

Months 1-12

Part II A) Transfect all cell types with mutant receptors and characterize the total receptor number for each transfectant.

Months 13-24

Part II B) Determine the percent reduction in tyrosine kinase activity following desensitization with either PMA or EGF for all transfected cell types.

Months 25-36

Part II C) Determine the mitogenic response of all transfected cell types.

Months 37-48

Part I was only partially completed. Figure 3 in the End of Year (EOY) report for 1997 (Appendix A1 in this report) shows that 468 cells and the S1 variants were tested for desensitization at T654 by stimulation with PMA. This result showed that phosphorylation of T654 did not appear to be involved in the negative regulation of 468 or its variants. A similar analysis was performed on S11 (data not shown) with the same results. EGF induced desensitization by phosphorylation at S1046/1047 was never performed. By the time we reached

the point at which we would perform this series of experiments, other data presented in the EOY 1997 report, including downregulation and dephosphorylation data, suggested that any contribution S1046/1047 phosphorylation had in the negative regulation of EGF-Rs in our system would be minor. In addition, in the absence of a negative control, mutant receptor A1046/1047 expression in all cell types, the data would be difficult to analyze. Since we had several good experimental leads at that time, we chose to ignore this EGF-R regulatory process in our study.

Part II A was modified based on the results given above for part I. Because desensitization by phosphorylation at T654 did not appear to be a significant regulatory process in these cells and, as already explained, S1046/1047 phosphorylation would probably only have a minor effect on negative regulation, these mutants were never transfected into our cells. However, WT EGF-R were transfected into the variant cell lines and clones from those transfections were analyzed for total receptor number. This data was shown in Figure 1 of the 1998 EOY and is included in this report as Appendix A2.

Part II B and Part II C are obviously no longer on track because of the large number of modifications made in the early phases of work. In addition, for the reasons stated in the other parts, following these leads is no longer either expedient or desirable. The Statement of Work has therefore been modified to reflect actual and future work.

The new Statement of Work is as follows:

Part III) Described in the body of this (EOY 1999) report.

Months 25-36

Part IV A) Show that EGF-R ligand half lives are prolonged in the amplified cell lines.

Months 37-40

Part IV B) Show that basal phosphotyrosine increases following EGF-R amplification are associated with an increase in receptor signaling complexes formed.

Months 41-44

Part IV C) Show that EGF-R basal phosphotyrosine increases in a ligand dependent manner

following EGF-R amplification but in a ligand independent manner following Her2 amplification.

Months 45-48

Introduction

The epidermal growth factor receptor (EGF-R) is a transmembrane tyrosine kinase. Binding to any of the receptors six ligands (EGF, Transforming Growth Factor (TGF) alpha, Heparin Binding EGF (HB-EGF), Amphiregulin (AR), Epiregulin, or Beta-cellulin) activates the receptors intrinsic tyrosine kinase and promotes tyrosine phosphorylation of its carboxy terminal tail (19, 29, 32, 42, 44, 45, 51). This phosphorylation initiates signal transduction through canonical pathways which include Ras, Shc, and PLC gamma (31, 33, 43). Although most of these transduction pathways are thought to be initiated on the cell surface, several points of new evidence suggests that signaling may also occur within the cell (1, 4, 13, 14, 26, 30, 36, 46, 52, 56). Following receptor activation, the EGF-R is rapidly internalized and trafficked to the lysosome, a process termed downregulation, or recycled to the surface (25, 48, 55).

Several points of evidence suggest that the EGF-R is involved in the carcinogenic process. Cells over-expressing EGF-R or its ligands are transformed *in vitro* (12, 17). In addition, both constitutively active and non-internalizing EGF-R mutants are transforming *in vitro* (7, 21, 24, 53). Increased receptor expression in human cell lines correlates with increased tumorigenicity of those cells in nude mice (6, 34, 41). Animal studies have shown that TGF alpha over-expression induces epithelial hyperplasia and carcinoma of the breast (40, 47). In humans, a constitutively activated EGF-R mutant is frequently associated with malignant glioblastoma (21, 54). Lastly, over-expression of the EGF-R in breast cancer is also correlated with poor outcome (23, 37, 39). Together, these points indicate a role for the EGF-R and more significantly, amplification of the EGF-R in the carcinogenic process.

The processes that negatively regulate EGF-R activity can be divided into both covalent and spatial regulatory mechanisms (29, 48, 55). Covalent regulation of the EGF-R includes receptor desensitization and receptor dephosphorylation (29). In desensitization, the EGF-R is phosphorylated on specific threonine and/or serine residues (8-11, 18, 28). This phosphorylation decreases the receptors affinity for ligand and decreases its ability to phosphorylate exogenous substrates (8-11, 18, 28). Receptor dephosphorylation by tyrosine phosphatases eliminates SH2 binding domains and inactivates the receptor (22, 27, 35, 49, 50). Spatial regulatory processes include receptor internalization and downregulation (48, 55). Following receptor activation, the receptor is rapidly removed from the cell surface by a coated pit mediated internalization (48, 55). This prevents the receptor from signaling through pathways which require plasma

membrane localization does not attenuate intracellular receptor signaling . Receptor downregulation targets EGF-Rs for degradation in the lysosomes removing all activated receptor mass and completely abrogating further signaling (25, 48, 55).

Several *in vitro* model systems have been devised to examine the role of EGF-R amplification in carcinogenesis (2, 5, 15, 16, 20). Most take advantage of the fact that, if cell lines have amplified EGF-R levels, application of exogenous EGF is toxic to those cells (5, 15, 16, 20). The threshold model was proposed to explain this phenomenon (15). This model suggests that cells have an optimum level of EGF-R activation for growth promotion. Any increase in receptor actiavtion beyond this optimum level is toxic to the cells (15). By culturing these receptor amplified cell lines with EGF, variant cell lines can be selected which have, in most cases, lost the parental receptor amplification (5, 15, 16). These variant lines, in contrast to their receptor amplified parental lines, are frequently growth stimulated by exogenous EGF (5, 15, 16).

We chose to use the model system based on the MDA-MB-468 (468) cell line developed by Filmus *et al* in order to examine the effect of receptor amplification on cell growth (15, 16). The 468 cell line expresses approximately $1.9x10^6$ EGF-R per cell due to chromosomal amplification. Filmus *et al* isolated variant cell lines from the parental 468 cell line by culturing those cells in EGF. These variant cell lines have lost the parental amplification and two variants, S1 and S11 (S variants), express $1.6x10^4$ and $6.6x10^4$ EGF-R per cell, respectively. In addition, the variant cell lines form smaller, less aggressive tumors in nude mice than do the 468 cell lines (15, 16). This model system suggests that the absolute level of EGF-R per cell could directly correlate with tumor growth rate. Other, similar systems had previously suggested the same thing.

In the initial proposal for this project, we sought to address the following hypothesis: Amplification of the EGF-R in the absence of concomitant increases in the receptors negative regulatory apparatus dysregulates receptor kinase activity and leads to uncontrolled receptor signaling. Early studies used the addition of exogenous ligand to probe for limiting negative regulatory components. These results have allowed us to form a model which explains how receptor amplification increases receptor signaling. In addition, we have begun to explore the similarities and differences between EGF-R amplification and Her2 amplification in breast cancer.

Body of Report

Previous results had shown that EGF-R activation was prolonged following receptor amplification. Desensitization of the EGF-R at T654 did not appear to be a factor in this process. Receptor internalization was greatly attenuated following receptor amplification. This attenuation was in the fraction of receptors internalized not the total number of receptors internalized. Receptor downregulation was also impaired following EGF-R amplification. Phosphatase activity appeared to contribute to attenuation of receptor PY in the unamplified cell lines but not in the amplified cell lines. Lastly, PY/EGFR declined sharply over time in the unamplified but not the amplified cell lines. This indicated that removal of PY was limited by downregulation in amplified cell lines but not in the unamplified cell lines.

Figure 4 in EOY 1997 demonstrated that tyrosine phosphatases were actively eliminating activated receptors by dephosphorylation in the S variants but not in the parental 468 receptor amplified cell line. Unfortunately, that experiment was performed by treating the cells with orthovanadate to block phosphatase activity. Orthovanadate is know to cause several phosphatase independent effects and is therefore not the ideal reagent for performing this analysis. Since then, a new method has been devised to compare phosphatase activity using the compound PD 153035 so we wished to reanalyze our data from 1997 using this method (3). PD 153035 is a specific inhibitor of the EGF-R tyrosine kinase (3). The degree of EGF-R tyrosine phosphorylation can be described by the following equation: L + R <---> LR <---> LR*. L is the ligand, R is the receptor, LR is the inactive ligand/receptor complex and LR* is the tyrosine phosphorylated ligand/receptor complex. At any point in time, the degree of receptor activation is reflected by the difference of receptor activation by ligand and the degree of receptor inactivation by tyrosine phosphatases. Treatment with PD 153035 blocks the transition of LR to LR* by inhibiting the receptors kinase activity. Therefore, no new activated complexes are formed and the degree of phosphorylation at any point in time reflects the decay of LR* back to LR and L + R by phosphatase activity. In simplest terms, following treatment with PD 153035, the speed at which PY disappears is a reflection of the rate of EGF-R dephosphorylation and therefore the effectiveness of its tyrosine phosphatases (3).

Figure 1 shows that phosphatases do not become limiting following receptor amplification. Contrary to what was expected, receptor amplification increases the rate of

receptor dephosphorylation. We saw no increase in receptor dephosphorylation rate when endocytosis was blocked (data not shown) suggesting that the rate of dephosphorylation on the surface was equivalent to the rate of dephosphorylation inside the cell. Therefore, the 468 cells were not dephosphorylating their receptors faster because their rate of internalization was attenuated. Other researchers have shown that SH2 binding proteins compete with tyrosine phosphatases for access to PY residues (38). Following EGF-R amplification, the number of EGF-R binding SH2 proteins available per receptor is expected to fall. Therefore, fewer EGF-R PY residues would be protected by SH2 containing proteins from phosphatase activity following receptor amplification. If true, this would increase the apparent rate of dephosphorylation in this assay following receptor amplification and suggest that substrate availability, not phosphatase activity, is rate limiting in these cells. Regardless of the mechanism, almost all tyrosine can be removed from the EGF-R within 30 seconds, and all PY is removed within 15 minutes (data not shown) regardless of receptor number per cell. We therefore propose that tyrosine phosphatases are not limiting following EGF-R amplification.

By decreasing the rate of internalization, receptors associated with amplified cell lines remain in contact with extra-cellular ligand. By decreasing the efficiency of downregulation, the receptors may remain in contact with ligand during trafficking to the lysosomes. We postulate that receptor activation is prolonged following amplification because the amplified cell lines cannot rapidly segregate EGF-R from ligand while the unamplified cell lines can. This establishes a loop where the receptor is activated by ligand, inactivated by phosphatases and reactivated by continual stimulation with ligand. Therefore, receptor signaling in the amplified lines can only be attenuated by downregulation. In contrast, the unamplified lines rapidly segregate ligand from receptor during trafficking and are able to inactivate the receptor by dephosphorylation and thus show a rapid attenuation of signaling.

If ligand is degraded simultaneously with receptor in the amplified cell lines as our hypothesis predicts, and because receptor downregulation is attenuated following amplification, we predict that ligand half life should also be decreased in the amplified cell lines compared to the unamplified cell lines. Preliminary results, shown in Figure 2 indicates that this is indeed the case. Figure 2 shows that the half life of EGF in the 468 cells is significantly slower than the half life of ligand in either S1 or S11XR6 cells. This data supports our hypothesis proposed above. We are currently repeating this result with all cell types and at different ligand concentrations to

determine whether or not this effect can be extrapolated to physiological ligand levels. In addition, we are using other ligands which disassociate easier from the EGF-R to determine if they exhibit this same decrease in half life following receptor amplification. These experiments will be performed as part IV A of the revised Statement of Work (above).

We wanted to determine if the amplified lines were making more ligand than the unamplified lines and/or if the amplified lines were capturing ligand more effectively than the unamplified cell lines. Figure 3 (this report) shows an ELISA analysis of ligand levels for each cell type plus or minus the blocking antibody 225. Because a collaborator of ours, Dr. Peter Dempsey, performs these ELISAs for us, I do not have the assay analytical details at this time. Prior to treatment with 225, the amount of TGF alpha in the media (TGFa, "pg/ml/10E6 cells" column) is identical regardless of receptor number per cell. This suggests that the receptors are not capturing more ligand following receptor amplification. If they were, the ligand concentration in the media would decrease as receptor number per cell increased. This same data also suggests that the amplified cell lines are not making more ligand. However, amplification could result in higher ligand expression levels and increased capture efficiency. If so, ligand concentration released to the media may appear artificially similar in both amplified and unamplified cell lines. To rule out this possibility we treated the cells with the EGF-R antagonistic antibody 225. If the receptors are all prevented from binding ligand, the amount of ligand in the media reflects ligand synthesized while eliminating the influence of capture efficiency. Our results following treatment with 225 suggest that ligand synthesis and release is not increased following receptor amplification. Whether or not the the slight elevation in amplified cell line numerical values are significantly different requires more experimental duplicates so that we can determine the experimental error. We have already begun performing these experiments. The results for AR ("AR, pg/ml/10E6 cells" column) seem to suggest the same thing as the TGF alpha results (above). However, the AR is at the limit of detection for the assay and requires more replicates to make any firm statements. Combined, these results suggest that amplification of the EGF-R does not increase capture efficiency and that it does not lead to an increase in autocrine ligand expression. Therefore, these two hypotheses as a general mechanism allowing amplification to increase signaling capacity seem unlikely, at least in our system.

All of our results to this point have used the addition of exogenous ligand as an integral part of our assays. We wanted to know if receptor PY in our cells increased following receptor amplification in the absence of exogenous ligand. This "basal" PY level should recapitulate the physiological situation more closely than the addition of exogenous ligand does. Figure 4 shows that any increase in receptor level is associated with aconcomittant increase in receptor associated basal PY. The linear function clearly shows that any increase in receptor number is associated with a proportional increase in basal PY. A linear increase in basal PY following receptor number increases would be expected if amplification increased PY by homodimerization, by prolonging receptor ligand interactions, or by stochastic receptor activation.

We assume that the increase in basal PY level seen following EGF-R amplification (Figure 4) is associated with increased growth promoting signal transduction. If so, increases in receptor basal PY per cell should also increase the number of signaling complexes associated with the EGF-R in that cell. We are currently performing co-immunoprecipitation studies to address this question and preliminary data (not shown) suggest that this is indeed the case. We have proposed to address this question more completely in part IV B of the newly proposed Statement of Work (above).

If basal PY increased in a ligand independent manner following receptor amplification, for example by homodimerization or stochastic receptor activation, then blocking receptors from being activated by ligand with antagonistic antibodies should have no effect on basal PY. Figure 5 shows that this is not what is observed experimentally. Batimistat is a matrix metalloprotease inhibitor which prevents release of EGF-R ligands, including TGF alpha. EGF-R ligands are released from the plasma membrane by proteolytic cleavage. This cleavage event is required to enable those ligands to bind and activate the EGF-R. Therefore, Batimistat treatment prevents ligand/receptor interactions and blocks autocrine signaling. Treatment with 225 almost completely blocks basal in S1 and S11 cells but its ability to block basal PY decreases with increasing receptor concentration per cell. As expected, Batimistat blocks basal PY similar to what is seen for 225 treatment: complete blockage in S1 and S11 and decreased blocking ability at higher receptor numbers per cell. However, the combination of 225 and Batimistat simultaneously works as good as either treatment alone in S1, S11, S1XR13 and S11XR6 but better than either treatment alone in the highly amplified 468 line. This data shows that receptor

amplification, at least in the 468 cell line, results in little, if any, receptor activation by ligand independent mechanisms. In addition, blocking autocrine stimulation of the EGF-R by inhibiting ligand cleavage and ligand binding simultaneously results in a cumulative inhibition exceeding either treatment alone. This result may have direct clinical applications as both 225 and Batimistat are currently in, or are finishing, clinical trials for breast cancer treatment.

Amplification of the Her2, an EGF-R family member, is also associated with poor outcome in breast cancer. Her2 does not appear to have its own ligand and is activated by heterodimerization with other EGF-R family members. The 468 cells used in the above studies do not have detectable Her2 expression. MTSV is a breast cell line which expresses a low level of Her2. Transfection was used to artifically amplify Her2 in in the parental cell line MTSV and the CE2 cell line, which expresses high levels of Her2, was generated. Her2 amplification, in CE2, increases EGF-R basal PY above that seen in MTSV. For this reason, we examined the effect of antagonistic antibody 225 and Batimistat treatment in these cells in an effort to determine if this increased EGF-R activation was ligand dependent or ligand independent. Figure 6 shows that amplification of Her2 appears to increase the phosphorylation of EGF-R in a ligand independent fashion. Although the lanes had equal protein concentrations loaded, there is much less EGF-R per lane in the CE2 gel as compared to the MTSV gel lanes (data not shown). Because of this, the EGF-R associated basal PY is much higher in the CE2 cells as compared to the MTSV cells. PD 153035 treatment eliminates all basal PY suggesting that the basal PY bands are indeed phosphorylated EGF-R. MTSV cells basal PY is inhibited only slightly, if at all, with 225 or Batimistat treatment alone. However, it is significantly decreased following treatment with both simultaneously. The CE2 cell basal PY, in contrast, is not inhibited by any of the treatments. This data suggests that, in contrast to EGF-R amplification, Her2 amplification activates EGF-R PY in a ligand independent manner. In all probability, this activation is due to ligand independent heterodimerization between the EGF-R and Her2. Amplification of Her2 would make this heterodimerization more likely by increasing the chance of random collisions and could therefore increase the EGF-R basal PY. If the primary oncogenic effect of Her2 amplification was EGF-R signaling, this result has immediate clinical consequences. This data suggests that following Her2 amplification in breast cancer, neither 225 or Batimistat would be effective treatment modalities. Likewise, breast cancers with amplified EGF-R are less likely to be treatable by 225 or Batimistat if they express Her2. Further studies regarding basal activation

following EGF-R amplification and Her2 amplification and if that activation is ligand dependent or independent will be further addressed in part IV C of the new Statement of Work (above).

The data generated in this project has suggested a new model for prolonged receptor activation following EGF-R amplification. In addition, we have shown that EGF-R amplification appears to increase signaling potential in a ligand dependant manner while amplification of the Her2 receptor appears to increase signaling potential in a ligand independent manner. These findings have direct clinical relevance. First, maximal decreases in EGF-R signaling following its amplification require blockage of the autocrine loop by more than one mechanism. Second, Her2 expression will decrease the efficacy of any treatment which inhibits EGF-R autocrine activity.

Conclusions

1) EGF induced receptor activation, as measured by PY, is prolonged when the EGF-R is amplified and the decrease in speed of attenuation correlates with receptor level.

2) EGF induced receptor downregulation is attenuated when the EGF-R is amplified and this attenuation is correlated with receptor level.

3) Tyrosine phosphatase activity does not become limiting following EGF-R amplification and all receptors can be completely dephosphorylated within 30 seconds.

4) Phosphotyrosine is prolonged following receptor amplification because ligand cannot be efficiently uncoupled from the EGF-R during trafficking.

5) Receptor amplification does not increase cellular capture efficiency or ligand expression.

6) Receptor amplification increases the cells dependency on their autocrine loops.

7) Basal PY increases linearly following EGF-R amplification.

8) Basal PY increases following EGF-R amplification are ligand dependent and blocking receptor/ligand interactions in more than one way gives cumulative inhibition of basal receptor activation.

9) Amplification of Her2 increases the basal PY of EGF-R by a ligand independent mechanism.

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Whole Cell Extract
Anti-PY Western

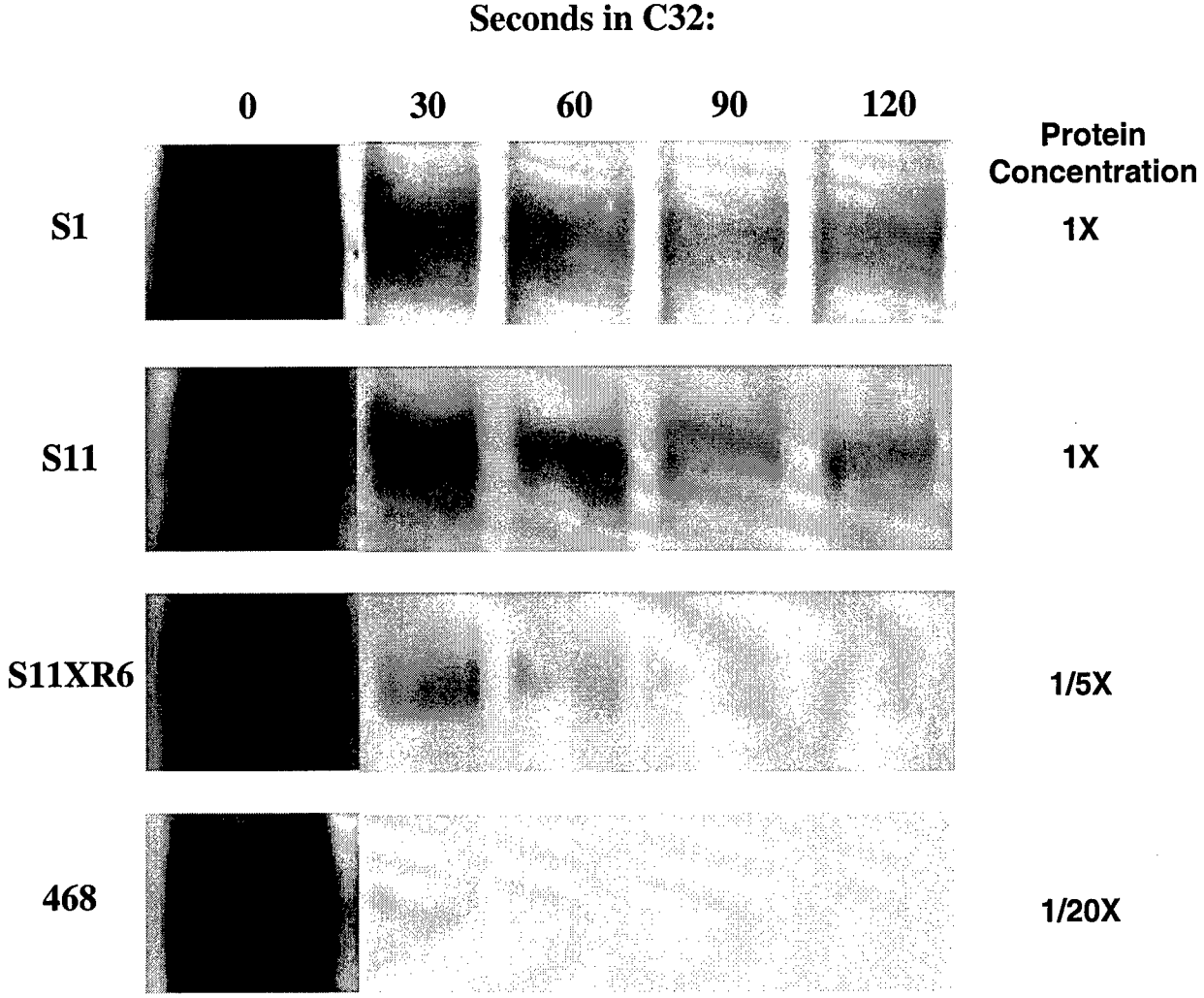

Figure 1: *Receptor amplification accelerates apparent rate of EGF-R dephosphorylation.* Equal numbers of S1, S11, S11XR6 or 468 cells were treated for 10 minutes with 100 ng/ml EGF. The cell media was then changed to media containing 4 uM PD 153035. At 0, 15, 30, or 60 seconds post PD 153035 treatment, cells were lysed in RIPA and the EGF-R was resolved by SDS-PAGE. PY was visualized by Western blotting with anti-phosphotyrosine antibodies. S1 and S11 lanes had 1X protein concentration, the S11XR6 lane had 1/5X protein concentration and 468 lanes had 1/20X protein concentration.

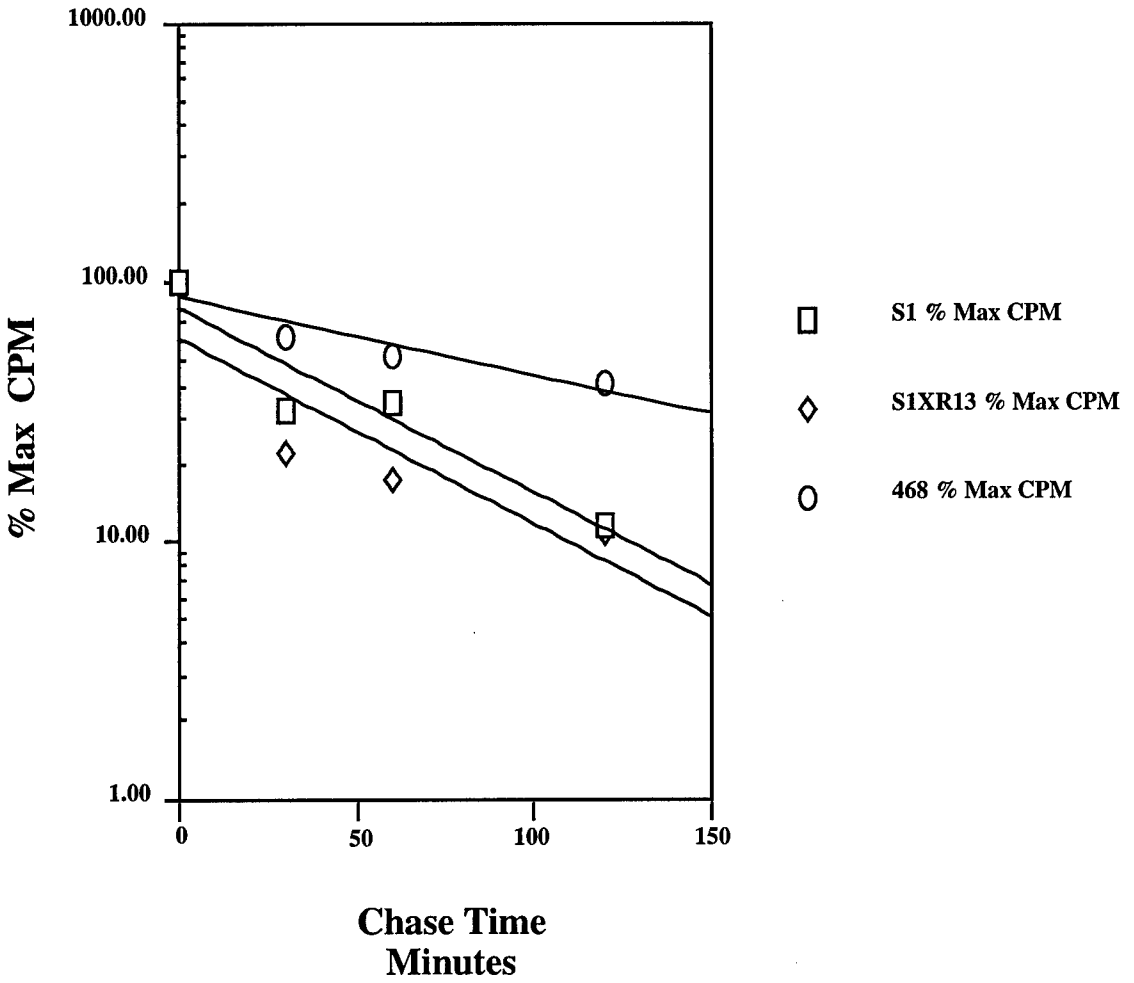

Figure 2: *Ligand half life decreases as EGF-R is amplified.* Equal numbers of cells were treated with 100 ng/ml I125 labeled EGF for 10 minutes at 37C. The media was then aspirated and the radio-labeled EGF was chased with plain media for 0, 30, 60, or 120 minutes at 37 C. At each timepoint, cells were solubleized in 2% SDS and the total radioactivity associated with the cells was determined by gamma counting.

Cell Type	Cell Number	% Growth Inhibition by 225	TGFalpha pg/ml	TGFa (pg/ml/10E6 cells)	AR pg/ml	AR (pg/ml/10E6 cells)
S1	4.13E+06		14	3	46	11
S1 + 225	3.72E+06	90	109	29	58	16
S11	5.24E+06		19	4	77	15
S11 + 225	4.19E+06	80	134	32	42	10
S1XR13	4.69E+06		17	4	78	17
S1XR13 + 225	2.10E+06	45	122	58	65	31
S11XR6	4.98E+06		0	0	0	0
S11XR6 + 225	2.84E+06	57	83	29	0	0
MDA-468	2.51E+06		12	5	116	46
MDA-468 +225	1.43E+06	57	79	55	42	29

Figure 3: *EGF-R amplification does not increase ligand capture efficiency or increase ligand expression.* Equal numbers of cells were treated with or without 20 ug/ml 225 for 24 hours at 37 C. Media was removed, frozen at -20 and then analyzed by ELISA for TGF alpha and AR concentration. Cell numbers for each plate were determined by Coulter counting at the time of media aspiration.

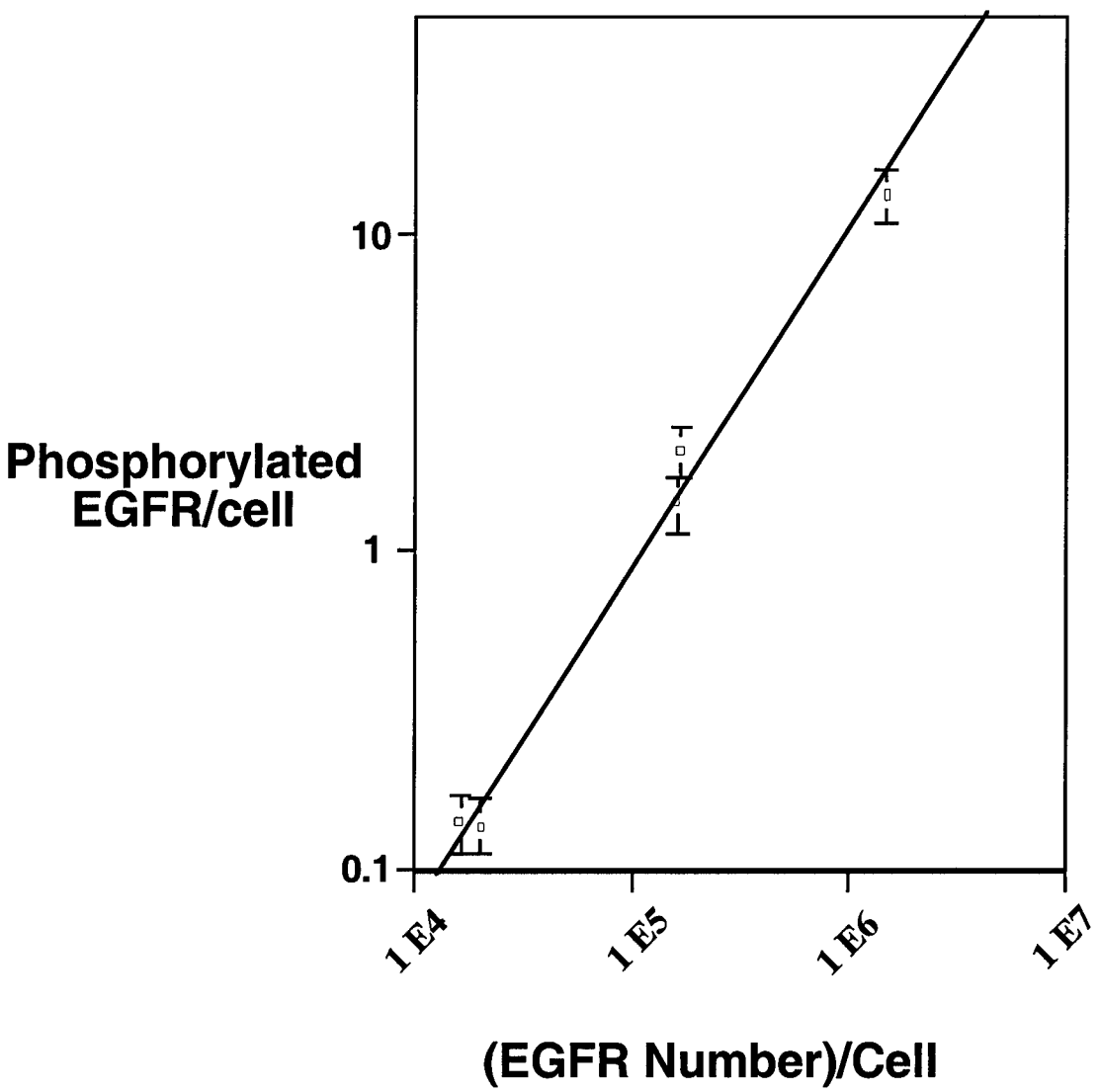

Figure 4: *EGF-R amplification concomittantly amplifies receptor basal PY levels per cell.* Equal numbers of cells per plate for S1, S11, S1XR13, S11XR6 and MDA-MB-468 were lysed in RIPA and the PY associated with the EGF-R was analyzed by ELISA. This basal PY was plotted against the number of EGF-Rs per cell in each cell type.

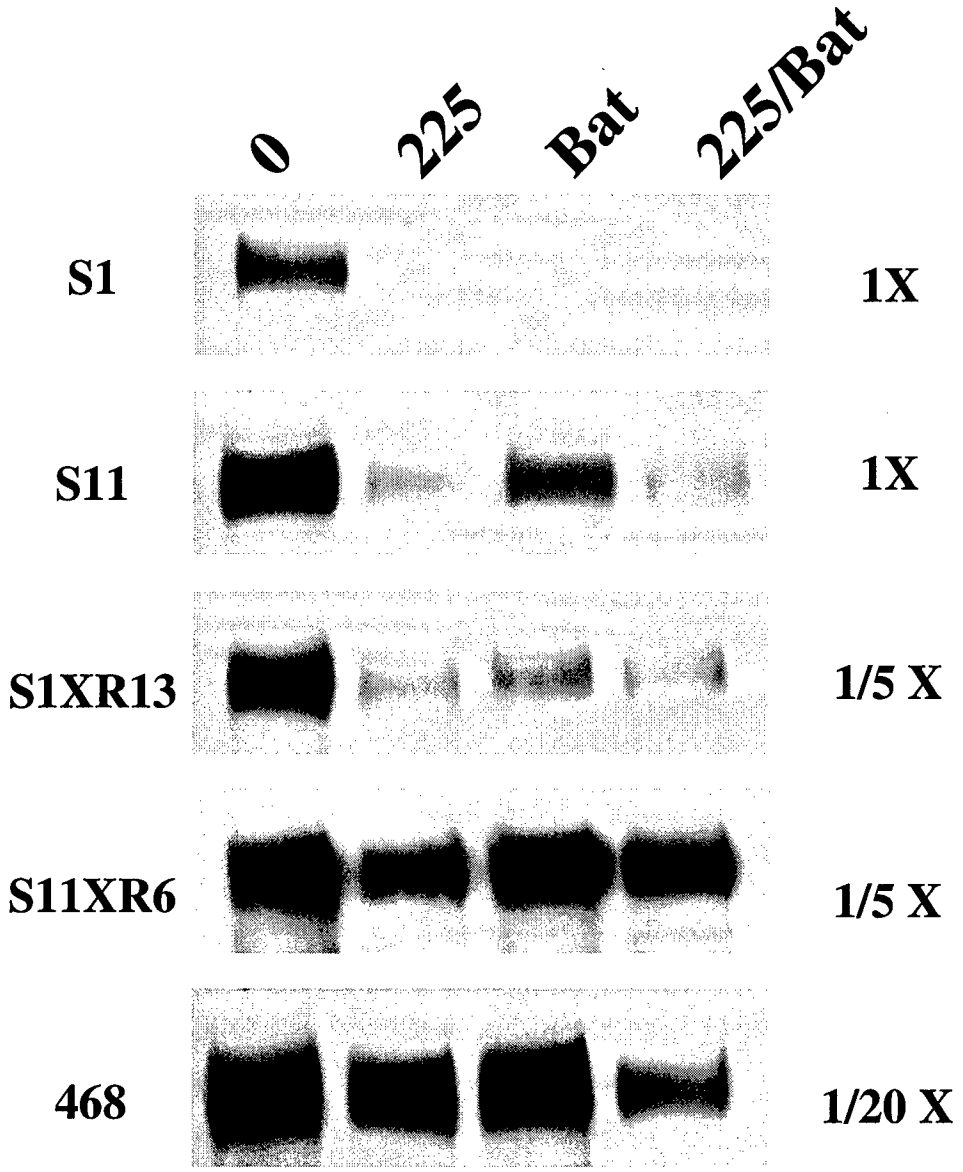

Figure 5: *Increase in basal PY following receptor amplification is ligand dependent.* Equal numbers of S1, S11, S1XR13, S11XR6 and MDA-MB-468 cells were treated for 24 hours with 20 ug/ml EGF-R antagonistic antibody 225, 10 ug/ml Batimistat or 20 ug/ml 225 and 10 ug/ml Batimistat simultaneously. All cells were lysed in RIPA, resolved by SDS-PAGE and the PY associated with the receptor was visualized by anti-phosphotyrosine antibody Western blotting. Protein levels were loaded in each well were adjusted so that S1 and S11 have 1X protein concentration, S1XR13 and S11XR6 are loaded at 1/5X concentration and 468 cells are loaded at 1/20X protein concentration.

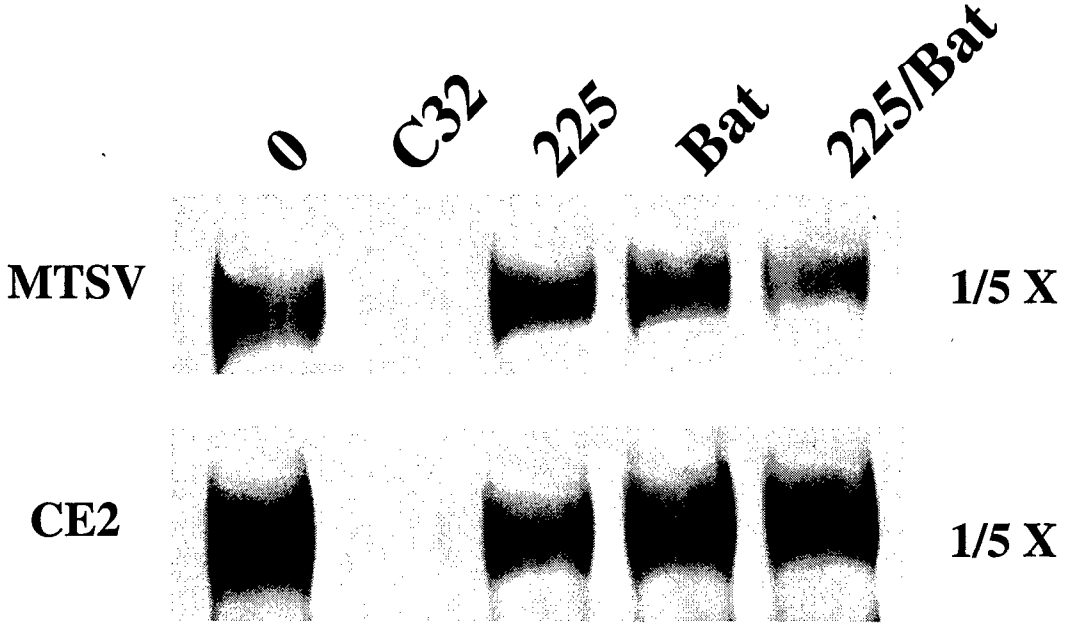

Figure 6: *Her2 amplification increases EGF-R basal PY in a ligand independent manner.* Equal numbers of MTSV and CE2 cells were treated with nothing, 20 ug/ml antagonistic antibody 225, 10 ug/ml Batimistat or 20 ug/ml 225 and 10 ug/ml Batimistat simultaneously. A replica plate of "no treatment" was treated with 4 uM PD 153035 for two minutes before lysis. The cells were then lysed with RIPA and the EGF-R was immunoprecipitated with anti-EGF-R antibodies and resolved by SDS-PAGE. PY associated with the EGF-R was visualized by Western Blotting with anti-phosphotyrosine antibodies.