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ORIGINAL ARTICLE

Cisplatin induces PKB/Akt activation and p38^{MAPK} phosphorylation of the EGF receptor

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Cisplatin is an effective DNA-damaging antitumor agent employed for the treatment of various human cancers. In this study, we report that Cisplatin activates PKB/Akt in several cancer cell lines and that this activation is mediated by EGFR, Src and PI3-kinase. Inhibition of PI3-kinase activity decreases the survival of the cells exposed to Cisplatin, suggesting that Cisplatin-induced PKB/Akt activation may lead to Cisplatin resistance. While investigating the EGFR-dependent PKB/Akt activation in MDA-MB-468 cells, we found that the EGFR receptor undergoes a gel mobility shift upon Cisplatin treatment, which is mediated by p38^{MAPK}. An EGFR, in which threonine 669 was mutated to alanine (A669), is phosphorylated by p38^{MAPK} to a much lesser extent, suggesting that threonine 669 is a p38 phosphorylation site. We found that Cisplatin induces EGFR internalization, which is mediated by p38^{MAPK}-dependent phosphorylation of the receptor on threonine 669. Our results identify the EGFR as a new substrate of p38 and identify threonine 669 as a new phosphorylation site that regulates EGFR internalization. Together, these results suggest that Cisplatin has side effects, which may alter the signaling pattern of cancer cells and modulate the desired effects of Cisplatin treatment.
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Introduction

PKB/Akt is a cytoplasmic serine and threonine kinase that positively regulates metabolism, cell cycle progression and cell survival (reviewed by Kandel and Hay, 1999; and Franke *et al*., 2003). PKB/Akt also promotes anchorage-independent survival, cell migration and angiogenesis (Kandel and Hay, 1999). PKB/Akt was shown to be downstream of PI3-kinase, a phospholipid kinase activated by a variety of growth factors, cytokines, G-protein-coupled receptors and adhesion molecules. Any signaling event that increases PI3-kinase activity leads to the activation of PKB/Akt through its phosphorylation on threonine 308 (T308) and serine 473 (S473) (Franke *et al*., 1995; Kennedy *et al*., 1997).

Cisplatin is an effective DNA-damaging antitumor agent utilized for the treatment of various human cancers (Siddik, 2003). Cisplatin treatment often leads to the acquisition of chemoresistance (Kartalou and Essigmann, 2001). A number of mechanisms have already been found and further understanding of the cellular responses to Cisplatin is important to optimize its use in the clinic. Recent studies have documented that Cisplatin triggers the activation of the Jun-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) cascades in tumor cells or transformed cell lines (Pandey *et al*., 1996; Benhar *et al*., 2001; Deschesnes *et al*., 2001). p38 kinase activates a variety of downstream substrates and is believed to play an important role in coordinating a variety of cellular events, such as cell growth and cell death (Dent *et al*., 2003). We have previously reported that Cisplatin activates EGFR and p38^{MAPK} (Benhar *et al*., 2001, 2002). In addition, Src tyrosine kinase was also shown to be activated by Cisplatin and to be involved in EGFR activation upon Cisplatin treatment (Benhar *et al*., 2002). This study extends these previous findings and here we show that Cisplatin activates PKB/Akt, as measured by T308 phosphorylation, and that this activation is dependent on EGFR and Src in MDA-MB-468 cells. We also show that inhibition of PI3-kinase activity decreases the survival of the cells exposed to Cisplatin, suggesting that Cisplatin-induced PKB/Akt activation may lead to Cisplatin resistance. Cisplatin treatment activates also the p38 MAPK, which we found to phosphorylate the EGFR at threonine 669. We observed that Cisplatin induces EGFR internalization through the phosphorylation of threonine 669 by p38 MAPK. The implications of these effects of Cisplatin are discussed.

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Results

PKB/Akt is activated by Cisplatin in various cell lines

In order to examine if Cisplatin-induced PKB/Akt activation is a general effect, we examined its ability to activate PKB/Akt in several cell lines, by measuring PKB/Akt T308 phosphorylation. We treated the Rat1, Panc-1, Ovcar-3, MCF-7 and MDA-MB-468 cell lines with Cisplatin and examined the level of PKB/Akt phosphorylation (Figure 1a and b). In Rat1 fibroblasts and in Panc-1 pancreatic cancer cells, slight phosphorylation of PKB/Akt was detected after 4 h of Cisplatin exposure and increased with time of Cisplatin exposure (Figure 1b). In the ovarian cancer cells, Ovcar-3, PKB/Akt was phosphorylated 4 h after Cisplatin exposure and the phosphorylation decreased with longer exposure times. In MCF-7 breast cancer cells, PKB/Akt phosphorylation was high 8 h after Cisplatin exposure and decreased by 16 h after exposure. We conclude that the activation of PKB/Akt by Cisplatin is widespread among cancer cell lines, is dose and time dependent. Cisplatin, like EGF, induces PKB/Akt phosphorylation at threonine 308 and serine 473 and leads its activation inferred by the analysis of the phosphorylation state of its endogenous substrate GSK3 (Figure 1c). Activation of PKB/Akt by Cisplatin, as measured by T308 phosphorylation, is blocked by the PI3-kinase inhibitor LY294002, strongly suggesting that Cisplatin-dependent PKB/Akt activation is due to the upstream activation of PI3-kinase (Figure 1c).

Inhibition of PI3-kinase by LY294002 sensitizes the cells to Cisplatin

PKB/Akt was shown to inhibit cell death caused by a wide variety of apoptotic stimuli (Kandel and Hay, 1999). In order to assess if the activation of PKB/Akt by Cisplatin induces cell survival upon Cisplatin treatment, we compared cell survival after Cisplatin treatment to the survival of cells treated with Cisplatin together with the PI3-kinase inhibitor LY294002. In both the MDA-MB-468 and Panc-1 cell lines, LY294002 markedly decreased cell survival owing to Cisplatin treatment by up to 50%, where the effects were most noticeable at 24–48 h after application of the agents (Figure 2). Thus, activation of PI3-kinase and most probably PKB/Akt by Cisplatin reduces its cytotoxicity, enhancing the resistance of cells to Cisplatin treatment.

Activation of PKB/Akt by Cisplatin in MDA-MB-468 is dependent on EGFR receptor, PI3-kinase and Src

The EGFR inhibitor, tyrphostin AG1478 and the Src inhibitor PP1, block the phosphorylation of PKB/Akt by Cisplatin in a dose-dependent manner, suggesting the involvement of Src and EGFR in the activation of PKB/Akt by Cisplatin (Figure 3).

EGFR undergoes gel mobility shift upon Cisplatin treatment of MDA-MB-468 cells

While investigating the involvement of the EGFR in the cellular response to Cisplatin in MDA-MB-468 breast cancer cells, we observed that the receptor undergoes a gel mobility shift upon Cisplatin treatment, in a time and dose-dependent manner (Figures 1c and 4a–c). As p38 ^{MAPK} is activated upon Cisplatin treatment (Benhar *et al*., 2001), we examined whether there is a correlation between p38 activation and the gel mobility shift of the EGFR. Figures 4a–b shows a similar time and dose response for the activation of p38 and EGFR gel shift. The gel mobility shift of the EGFR was also observed when the receptor was immunoprecipitated with monoclonal antibody 108, from cells previously treated with Cisplatin. The shift was induced by Cisplatin treatment and was not detected in EGFR immunoprecipitates from untreated cells or cells treated with EGF (Figure 4c).

The gel mobility shift of the EGFR is owing to p38-dependent phosphorylation

Application of alkaline phosphatase to the EGFR immunoprecipitated from Cisplatin-treated cells abrogated the gel mobility shift, indicating that the shift was owing to phosphorylation (Figure 4d).

Besides p38, Cisplatin activates also extracellular signal-protein kinase and JNK MAPK kinases (Fan and Chambers, 2001). In order to study which of these kinases may be involved in the phosphorylation of the receptor, we examined the effects of the p38 inhibitor SB202190, the MEK inhibitor U0126 (Calbiochem, Darmstadt, Germany) and the JNK inhibitor SP600125 (Calbiochem) on the Cisplatin-dependent gel mobility shift status of the EGFR. Figure 5a shows that only SB202190 inhibited the Cisplatin-induced gel shift of the receptor.

To examine the possible activation of p38 by other anticancer agents and their effects on the EGFR, we treated MDA-MB-468 cells with several anticancer agents in parallel to Cisplatin and analysed the gel mobility shift and tyrosine phosphorylation state of the EGFR, in parallel to the activation of p38. Only Cisplatin and Epirubicin, which strongly activate $p38^{MAPK}$, led to the gel shift of the EGFR (Figure 5b), further supporting the notion that $p38^{MAPK}$ is involved in EGFR phosphorylation on serine and/or threonine.

p38 phosphorylates the EGFR on threonine 669

To determine if $p38^{MAPK}$ can directly phosphorylate the EGFR, we assayed the kinase activity of $p38^{MAPK}$ using immunoprecipitated EGFR as the substrate and a recombinant constitutively active form of p38, $p38^{F327S}$ (Bell *et al*., 2001). As can be seen in Figure 6, $p38^{MAPK/F327S}$ phosphorylated the EGFR in a dose-dependent manner. In order to saturate the tyrosine phosphorylation sites on the EGFR, non-radioactive ATP was added, before the addition of $p38^{F327S}$ and [γ-^{32}P]ATP ('Materials and methods'). We believe that the EGFR was therefore strongly autophosphorylated by the non-radioactive ATP, and this is the reason that no gel shift was observed, although we cannot completely exclude the possibility that another phosphorylation event, not

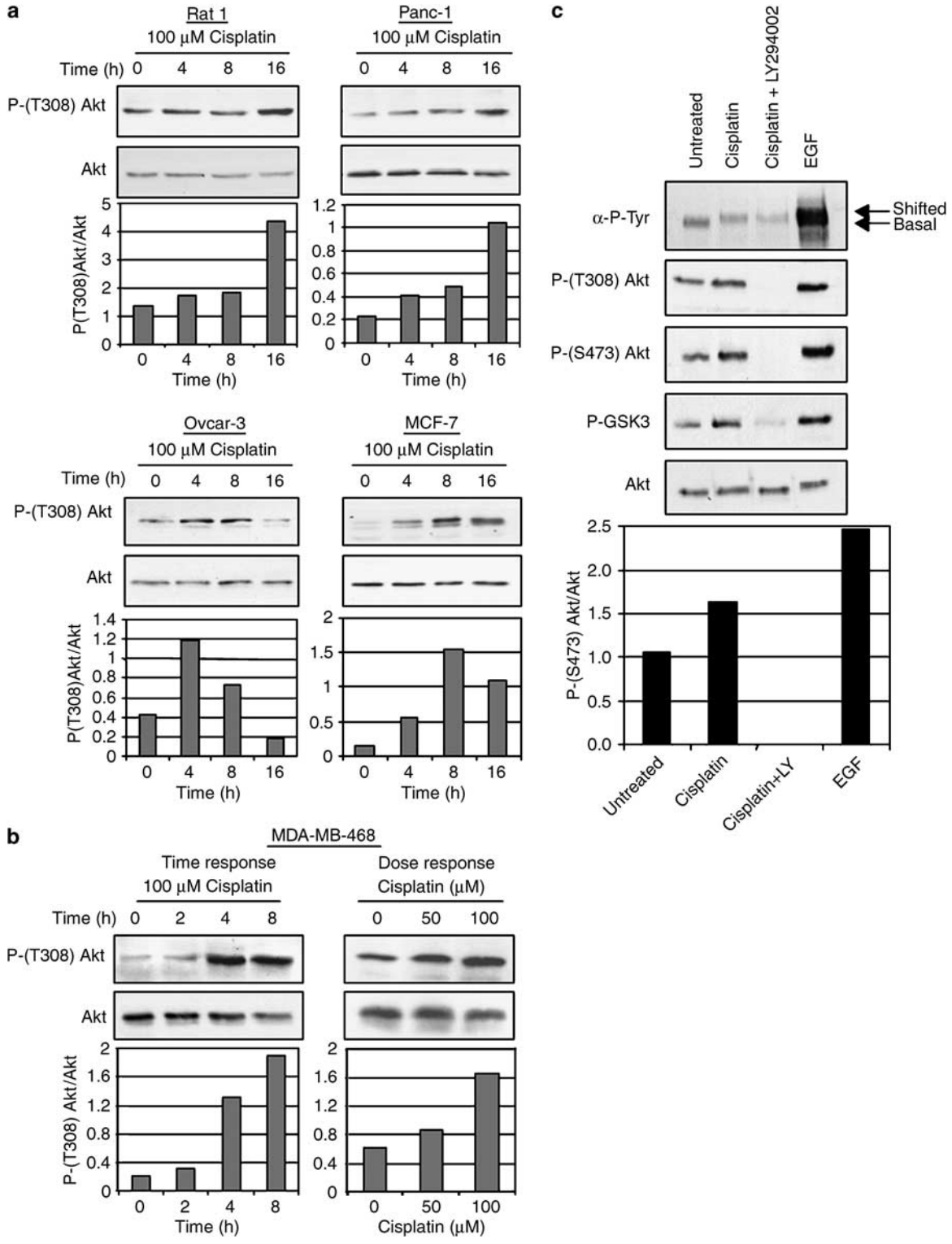

Figure 1 PKB/Akt is activated, as measured by T308 phosphorylation, by Cisplatin in various cell lines. Cells were starved for 12 h, followed by treatment with 100 μM Cisplatin for the time indicated or with the indicated Cisplatin concentration for 8 h. The cells were lysed and lysates analysed by immunoblot with α-P (T308) Akt. Following stripping, the blots were reacted with α-Akt antibodies. The graphs show the amount of P (T308) Akt normalized to the amount of Akt. (**a**) Time response to Cisplatin of Rat1, Panc-1, Ovcar-3 and MCF-7 cells. (**b**) Dose and time response to Cisplatin of MDA-MB-468 cells. (**c**) MDA-MB-468 cells were starved for 12 h and then treated with Cisplatin (100 μM, 8 h), Cisplatin (100 μM, 8 h) + LY294002 (20 μM) or with EGF (100 nM, 10 min). Lysates were analysed by immunoblot with the indicated antibodies. The figures are representative for at least three experiments.

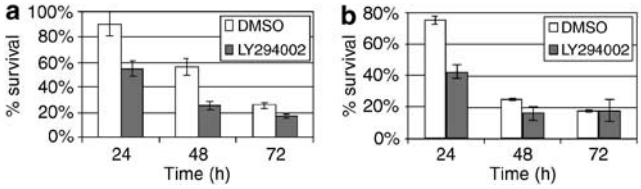

Figure 2 Inhibition of PI3-kinase by LY294002 sensitizes the Panc-1 and MDA-MB-468 cells to Cisplatin-induced cell death. Survival of Panc-1 (**a**) and MDA-MB-468 cells (**b**) following treatment with 50 μM (**a**) and 20 μM (**b**) Cisplatin in the presence of LY294002 (20 μM) or vehicle (DMSO). The percentage of surviving cells was determined by the automated microculture methylene blue assay. Results represent data of six independent wells; values are means $\pm$ s.d.

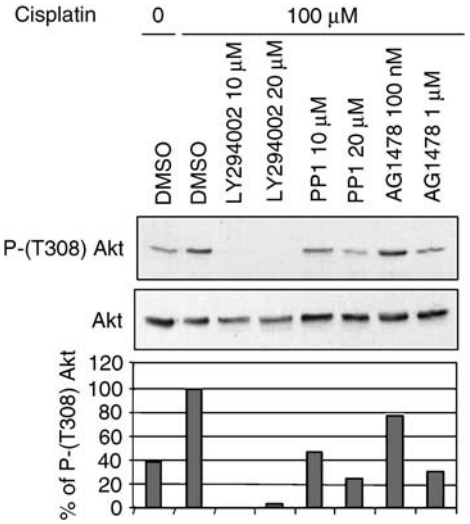

Figure 3 Inhibition of Cisplatin-induced PKB/Akt T308 phosphorylation with PI3-kinase, Src and EGF receptor inhibitors. MDA-MB-468 cells were starved for 12 h. LY294002, PP1 or AG1478 were added and half an hour later 100 μM Cisplatin was also added for 8 h (0: no Cisplatin; 100 μM: with Cisplatin). Lysates were analysed by immunoblot with α-P (T308) Akt. Following stripping, the blot was reacted with α-Akt antibodies. The phosphorylation level of each sample was normalized to Akt levels. The values are shown as percentage of the maximal activity level in the absence of inhibitor (designated 100%). The figure is from a representative result of three experiments.

mediated by $p38^{MAPK}$, is necessary for the gel shift of the EGFR to occur. Using the Scansite program (Obenauer *et al*., 2003), we found five putative consensus sites for p38 phosphorylation within the EGFR: T669, S487, S967, T823 and T1008. It was previously reported that a peptide corresponding to the T669 consensus site was employed as a $p38^{MAPK}$ substrate in $p38^{MAPK}$ kinase assays (Young *et al*., 1997). We therefore examined if $p38^{MAPK}$ can phosphorylate mutant EGFR in which T669 is mutated to alanine (EGFR (A669)). Figure 6b shows that EGFR (A669) is phosphorylated by $p38^{MAPK/F327S}$ to a much lower extent than the wild-type receptor, indicating that this site is indeed a substrate for $p38^{MAPK}$.

Phosphorylation of threonine 669 leads to Cisplatin-induced internalization of the EGFR

Phosphorylation of the EGFR on T654 by PKC was reported to regulate the internalization and turnover of the EGFR (Lin *et al*., 1986). In order to assess if there is an effect of p38 on the internalization of the EGFR, we treated MDA-MB-468 cells with Cisplatin or with Cisplatin together with SB202190 and stained the cells with EGFR antibody.

In the control cells, the EGFR is localized to the cell surface, whereas treatment with Cisplatin leads to the internalization of the EGFR. Addition of the p38 inhibitor SB202190 together with Cisplatin inhibits the internalization of the EGFR. This finding indicates that p38 activation by Cisplatin leads to the internalization of the EGFR.

In order to see whether Cisplatin-induced internalization of the EGFR is dependent on the activation of the receptor, we treated the cells with the EGFR kinase inhibitor AG1478. Figure 7 shows that there is no effect of AG1478 on the internalization of the EGFR in MDA-MB-468 cells indicating that the kinase activity of the EGFR is not involved in Cisplatin induced internalization.

To test if the phosphorylation of threonine 669 by p38 is involved in the internalization of the EGFR we compared the localization of a wild type and a mutant EGFR in which T669 is mutated to alanine (EGFR (A669)) in Cho-K1 cells that do not express endogenous EGFR. In order to better visualize and quantify the localization of the EGFR we first fixed the cells and stain the extracellular domain of the receptor (Cy3-extracellular staining) in order to see the receptors that are localized in the plasma membrane. Then we permeabilized the cells and stained again the receptor (Cy5-permeabilized cells) in order to see also the internalized receptor. In this way, we could quantitate the intensity ratio between the receptor localized in the plasma membrane (Cy3) to the overall receptor (Cy5) in about 10 cells of each treatment. Moreover, to better visusalize the localization of the receptor, a deconvoluted image of the permeabilized Cy-5 stained receptor is shown (Figure 8a).

Figure 8b shows that the wild type receptor is internalized upon Cisplatin treatment. Treatment with the p38 inhibitor SB202190 inhibits the internalization of the receptor in these cells similarly to MDA-MB-468 cells. Interestingly, the mutant EGFR (A669) is mainly refractive to Cisplatin-induced internalization (the small decrease in the Cy3/Cy5 ratio in is not statistically significant), indicating that phosphorylation threonine 669 is essential for Cisplatin induced internalization of the EGFR.

Discussion

Treatment of cancer by Cisplatin often results in the development of resistance. A number of mechanisms have been found such as accelerated DNA repair

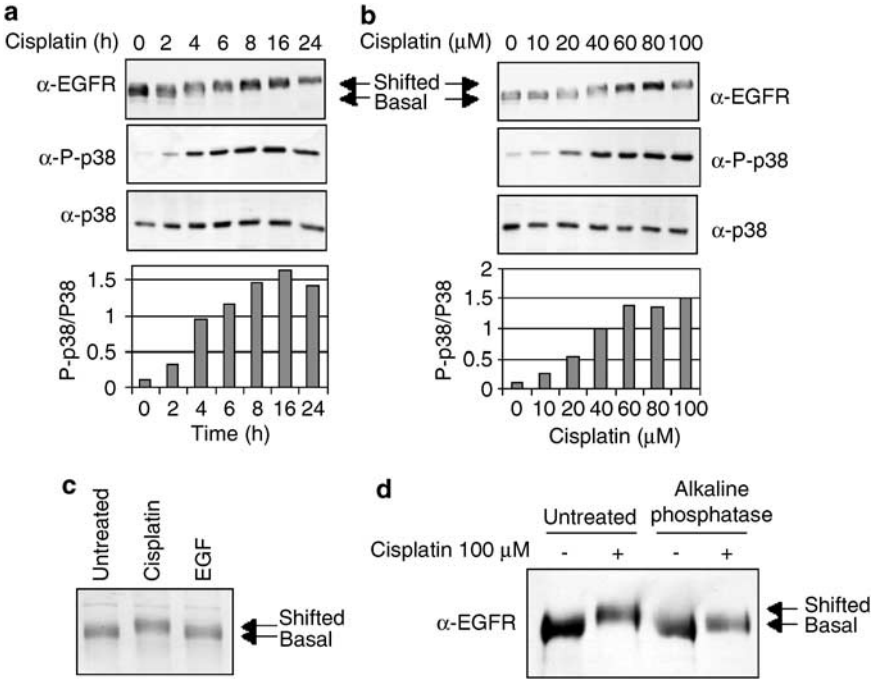

Figure 4 EGF-undergoes a phosphorylation-dependent gel mobility shift upon Cisplatin treatment. (**a**–**b**) MDA-MB-468 cells were starved for 12 h. Cells were treated with 100 μM of Cisplatin for the indicated time periods (**a**) or with Cisplatin at the indicated concentrations for 8 h (**b**). Lysates were analysed by immunoblot with the indicated antibodies. Graphs represent the intensity of the phosphorylated p38 (P-p38) normalized to the intensity of the total p38. Figures represent a result from three experiments. (**c**) MDA-MB-468 cells were starved for 12 h. Cells were untreated, treated with 100 μM of Cisplatin for 8 h or with 10 nM EGF for 5 min. Cells were then lysed and EGFR receptor was immunoprecipitated. Immunocomplexes were separated in 8% SDS–PAGE and the gel was stained with Coomassie. (**d**) MDA-MB-468 cells were starved for 12 h and then untreated (−) or treated (+) with 100 μM Cisplatin for 8 h. Cells were then lysed and EGF receptor was immunoprecipitated. Each immunoprecipitate was divided into two tubes: one was treated with alkaline phosphatase, the other one was left untreated. Immunoblot was performed with α-EGFR antibody. The arrows indicate the two forms of the EGF receptor.

(Siddik, 2003). Here we report that Cisplatin activates PKB/Akt in several cancer cell lines by its phosphorylation on T308 (Figure 1). Inhibition of the Cisplatin-induced activation of PKB/Akt by the PI3-kinase inhibitor LY294002 results in a 50% decrease in the survival of MDA-MB-468 and Panc-1 cells after Cisplatin treatment (Figure 2), whereas LY294002 alone does not influence cell death (data not shown). These results clearly show that one of the possible mechanisms of resistance to Cisplatin is mediated by PKB/Akt. This finding is in accordance with the finding that Cisplatin as well as other DNA-damaging agents synergize in promoting apoptosis in PC3 and LNCaP prostate cancer cell lines with PKB/Akt inhibitors (Litman *et al*., submitted). Previous studies, using several cell lines, suggested a role for PKB/Akt in the response to chemotherapeutic drugs (West *et al*., 2002). Cisplatin was shown to activate PKB/Akt and phosphorylate BAD in ovarian cancer cell lines, and PKB/Akt activation was found to be important for cell survival upon Cisplatin treatment (Hayakawa *et al*., 2000). A similar finding was shown with renal tubular epithelial cells, where PKB/Akt was found to inhibit the activation of caspases (Kaushal *et al*., 2001). Moreover, a recent study showed that chemosensitivity induced by adenoviral *E1a* gene expression is owing to the inhibition of PKB/Akt activation upon Cisplatin treatment (Viniegra *et al*., 2002). Our study shows that PKB/Akt is activated by Cisplatin, as measured by T308 phosphorylation, in ovarian, pancreatic and breast cancer cell lines, as well as in Rat1 fibroblasts, indicating that PKB/Akt activation by Cisplatin is a widespread phenomenon (Figure 1).

By pharmacological means, we showed that the phosphorylation and thus activation of PKB/Akt by Cisplatin is dependent on Src, EGFR and PI3-kinase (Figures 3). These proteins can be placed in one signal-transduction pathway, with EGFR and Src upstream of PI3-kinase. It is interesting that in MDA-MB-468 cells Cisplatin induces EGFR internalization in a p38-dependent manner (Figure 7). Internalized EGFR may be still able to signal to the PI3-kinase-PKB/Akt module as the inhibition of p38-mediated EGFR internalization does not block its Cisplatin-induced activation of PKB/Akt. Indeed, internalization of the EGFR was found to induce tyrosine phosphorylation (and therefore activation; Cuevas *et al*., 2001)) of p85 subunit of PI3-kinase. This study shows that the activation of the prosurvival signal PKB/Akt by Cisplatin may be taken into account and its use may be combined with signaling inhibitors like those inhibiting the PI3-kinase module, Src and EGFR. It remains to be elucidated by what mechanism

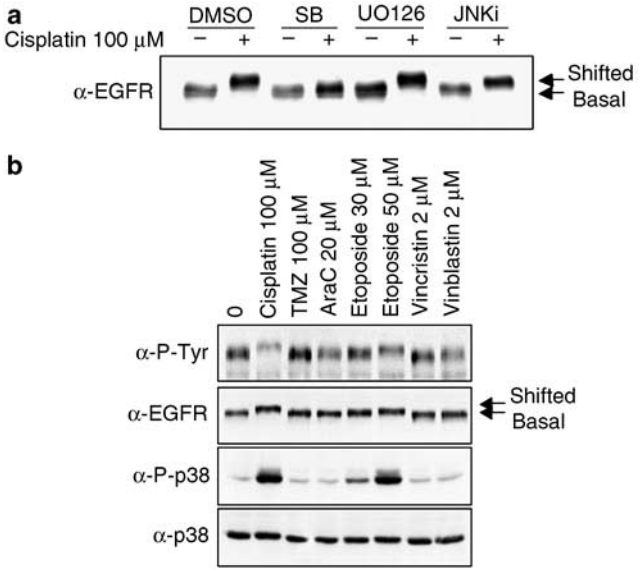

Figure 5 Gel mobility shift of the EGFR is owing to p38-dependent phosphorylation. (**a**) MDA-MB-468 cells were starved for 12 h. Cells were treated with Cisplatin (100 μM) and SB203580, UO126 or JNK inhibitor at a concentration of 20 μM. Lysates were analysed by immunoblot with anti-EGFR antibody. The arrows indicate the two forms of the EGF receptor. (**b**) Only the anticancer drugs that activate p38 cause a gel shift in the EGF receptor. MDA-MB-468 cells were starved for 12 h and then treated with various anticancer drugs in the indicated concentrations for 8 h. Lysates were analysed by immunoblot with the indicated antibodies. TMZ: Temozolamide, AraC: 1-β-D-Arabinofuranosylcytosine. The arrows indicate the two forms of the EGF receptor.

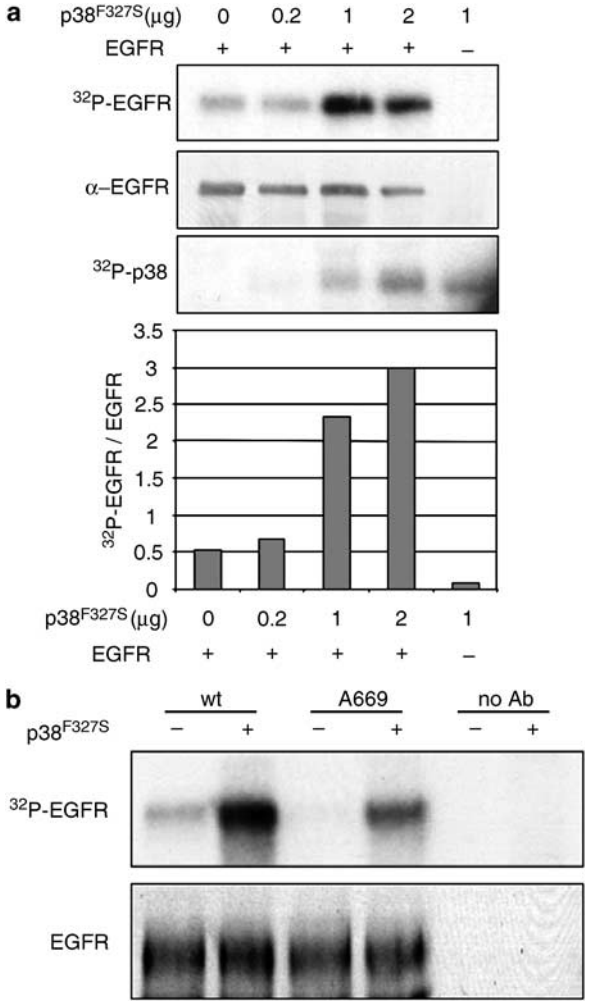

Figure 6 EGFR is directly phosphorylated by recombinant p38. (**a**) EGF was immunoprecipitated from MDA-MB-468 cells. The immunoprecipitated EGFR was then tested for phosphorylation by the indicated amounts of recombinant, constitutively active $p38^{F327S}$, as described in 'Materials and methods'. Immunoprecipitation without EGFR antibody was performed as a negative control. The phosphorylated EGFR as well as phosphorylated p38 (^{32}P-p38) were detected by autoradiography. Autophosphorylated p38 (^{32}P-p38) was also detected. The total EGFR present in the reaction was detected by immunoblot with anti-EGFR antibody. The graph represents the amounts of phosphorylated EGFR (^{32}P-EGFR) normalized to the total EGFR present in the reaction. (**b**) p38 phosphorylates the EGF receptor at threonine 669. CHO cells were transfected with wild-type (wt) or with mutant EGFR (T669 to A669). Twenty-four hours, after transfection the cells were lysed and immunoprecipitation of the EGFR was performed. The immunoprecipitates were subjected to kinase assay with or without recombinant active $p38^{F327S}$ as described in 'Materials and methods'. The phosphorylated EGFR was detected by autoradiography. The total EGFR present in the kinase reaction was detected by immunoblot with anti-EGFR antibody. Immunoprecipitation without α-EGFR (no Ab) was performed as a negative control. Figures represent the result of two experiments.

Cisplatin actually activates EGFR and Src in cancer cells. One possibility is the inhibition of phosphatases by the reactive oxygen species generated by Cisplatin treatment (Benhar *et al*., 2001).

While studying the role of EGFR in the Cisplatin-mediated activation of PKB/Akt, we observed that treatment of MDA-MB-468 breast cancer cells with Cisplatin induces p38 activation, in turn leading to EGFR serine/threonine phosphorylation (Figures 4 and 5). This phosphorylation was accompanied by a gel mobility shift of the EGFR, which was abrogated by the treatment with alkaline phosphatase (Figure 4d) and by the p38 inhibitor SB202190 (Figure 5a). Similar effects were produced by Epirubicin that leads to p38 activation but not by other antitumor agents that do not activate p38 (Figure 5b). Activated p38 directly phosphorylated EGFR in a cell-free assay (Figure 6), but a mutant EGFR T669A was phosphorylated by p38 to a much lesser extent (Figure 6b). Cisplatin-induced EGFR phosphorylation is accompanied by the internalization of the latter and inhibition of p38 activation by Cisplatin inhibits receptor internalization (Figure 7). Interestingly, kinase activity of the receptor is not necessary for its internalization.

By expressing wild-type and A669 mutant EGFR in CHO-K1 cells, we showed that the phosphorylation of threonine 669 by p38 is essential for Cisplatin-induced internalization of the receptor (Figure 8). Taken together, these results show, for the first time, that EGFR internalization can be regulated by p38 phosphorylation of the EGFR at threonine 669.

Phosphorylation of the EGFR at serine and threonine residues has been intensively studied over the years. Threonine 654 was shown to be phosphorylated by

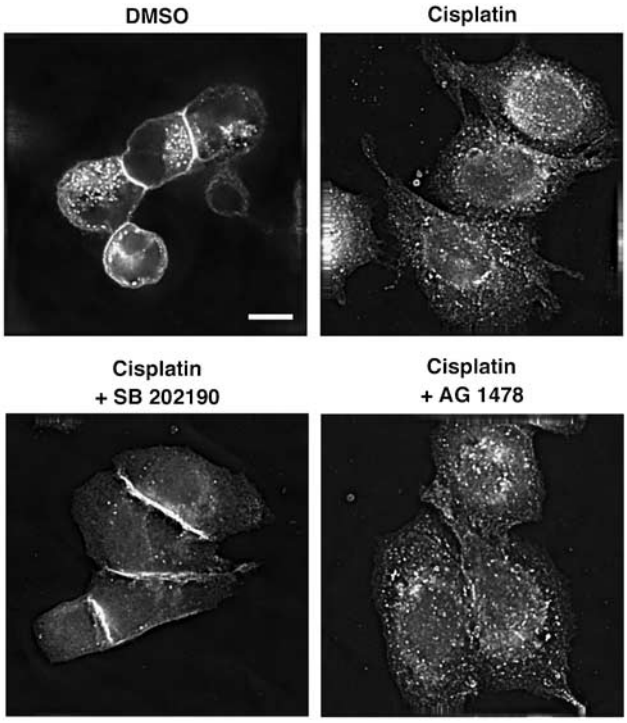

Figure 7 SB202190 inhibits the Cisplatin-dependent internalization of the EGFR. MDA-MB-468 cells were starved for 12 h. Cells were treated with Cisplatin (100 μM) with or without DMSO (Control), SB202190 (10 μM) or AG 1478 (1 μM) as indicated. The cells were fixed, permeabilized and stained with anti-EGFR antibody and Cy3-conjugated anti-mouse antibody. Bar: 10 μm.

protein kinase C, leading to negative regulation of the receptor (Hunter *et al*., 1984). A later study showed that phosphorylation of this site enhances EGFR trafficking to recycling endosomes leading to the sequestration from the cell surface, but inhibiting receptor degradation (Bao *et al*., 2000).

Threonine 669 of the EGFR was first described as the major site of phosphorylation on the EGFR in cells (Heisermann and Gill, 1988). Previous reports show that the EGFR is phosphorylated on T669 after treatment with EGF, phorbol esters or thapsigargin (Takishima *et al*., 1988, 1991; Northwood *et al*., 1991). This effect was shown to be mediated by ERK (Northwood *et al*., 1991; Takishima *et al*., 1991). Several studies have looked for a physiological involvement of T669 phosphorylation in the inhibition of EGF-stimulated receptor kinase activity and in the decrease of EGF high-affinity binding (Countaway *et al*., 1989, 1990; Morrison *et al*., 1993). We have also assessed this issue in our study and no consistent effects were noted. Our study shows for the first time that threonine 669 is necessary for the Cisplatin-dependent internalization of the EGFR. Our finding is in line with a study by Heisermann *et al*. (1990), showing that a mutant EGFR unable to be phosphorylated on serine 671 and T669 the EGF-induced internalization was impaired. It is interesting to note that Bagowski *et al*. (1999), showed that T654 and T669 are phosphorylated by the activation of PKD in Rat1 cells, abrogating EGFR signaling to JNK.

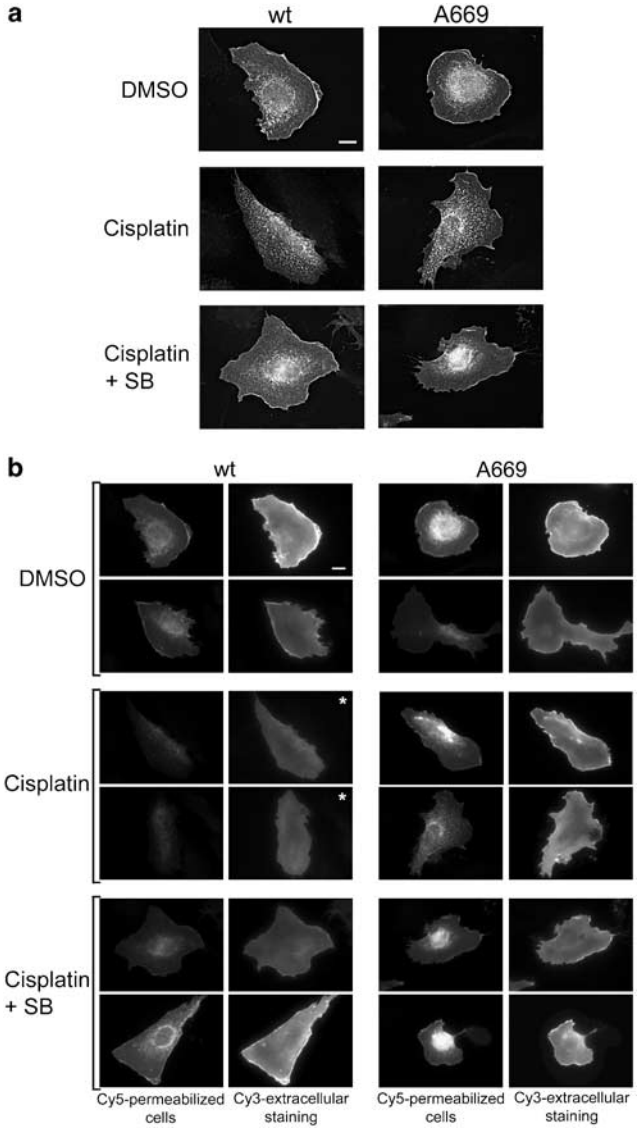

Figure 8 Phosphorylation of threonine 669 is essential for Cisplatin-induced internalization of the EGFR. CHO-K1 cells were transfected with wt or A669 EGFR. One day after transfection, the cells were treated with Cisplatin (100 μM) with or without DMSO (Control) or SB202190 (10 μM) as indicated, fixed and then stained with anti-EGFR antibody and Cy3-conjugated anti-mouse antibody (Cy3 extracellular staining). Immediately afterwards, the cells were permeabilized with 0.1% Triton X-100 for 5 min and then stained again with anti-EGFR antibody and Cy5-conjugated antimouse antibody (Cy5-permeabilized cells). (**a**) Deconvoluted images of one representative cell for each treatment after permeabilization (image intensities were adjusted in order to visualize the receptor localization). Bar: 10 μm. (**b**) Representative images of two cells of each treatment before and after permeabilization (original image intensities). *Exposure for these images was twice as the others in order to better visualize the EGFR staining. Bar: 10 μm. (**c**) The ratio of Cy3 to Cy5 intensity of eight to 10 cells of each treatment. $*P<0.125$, $**P<0.0001$. Figure represents two experiments.

It is possible that signaling of EGFR to JNK is dependent on receptor internalization. PKD was not shown to directly phosphorylate T669, allowing for the possibility that p38 acts downstream of PKD.

Alternatively, T669 might be a substrate for several protein kinases.

In this work, we analyse two different molecular events that follow Cisplatin treatment. First, the activation of PKB/Akt by the drug, leading most probably to enhanced survival, and second, the phosphorylation of the EGFR by p38, leading to its internalization. Internalized EGFR can form signaling complexes in endosomes, which may trigger qualitative different signals than receptors localized at the plasma membrane (Vieira *et al*., 1996). Internalization-defective EGFR was shown to increase cell proliferation and lead to lower activation of the ERK pathway together with lower tyrosine phosphorylation of the p85 subunit of PI3-kinase (Vieira *et al*., 1996). Benhar *et al*. (2002) showed that AG1478 decreases the survival of Cisplatin-treated cells, although this drug does not inhibits the EGFR internalization, it inhibits the signaling of the receptor. Together, we can propose that Cisplatin-induced internalization of the receptor may switch the signaling pathways from proliferation into survival. In summary, both of the effects described in this paper may shed light on the mechanism of chemoresistance to Cisplatin and other p38-activating agents.

Materials and methods

Reagents and antibodies

All the tissue culture reagents were purchased from Biological Industries Bet-Haemek Ltd, Kibbutz Beit Haemek, Israel; LY294002, SB202190, U0126, SP600125, Microcystin LR and Epirubicin were from Calbiochem; AEBSF and aprotinin were from Roche (Mannheim, Germany); Temozolamide was a generous gift from A Burgess (Ludwig Institute for Cancer Research, Melbourne, Australia); Cisplatin (Abiplatin), Etoposide and Vincristin were from ABIC (Nethanya, Israel). PP1 and AG1478 were synthesized as described previously (Osherov and Levitzki, 1994; Schindler *et al*., 1999). All other chemicals were purchased from Sigma Aldrich, St Louis, MO, USA.

Antibodies were obtained as follows: antiphospho-tyrosine (PY20), Akt 1/2 and anti-p38 from Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-phospho-p38, phospho-Akt (Ser473 and Thr308) and phospho-GSK3 α/β from Cell Signaling, Danvers, MA, USA; anti-EGFR C111.6 was a generous gift from Y Yarden (The Weizamnn Institute of Science, Rehovot, Israel) and anti-EGFR M108 was a generous gift from J Schlessinger (Yale University School of Medicine, New Haven, CJ, USA). Secondary antibodies were all from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell culture

The MDA-MB-468 cell line was a generous gift from A Ullrich (Max Planck Institute, Martinsreid, Germany). Rat1 fibroblasts were a gift from Professor M Oren (Weizmann Institute, Rehovot, Israel). Ovcar-3, an ovarian cancer cell line, and Panc-1, a pancreatic cancer cell line were a gift from Professor JQ Chen (Fox Chase Cancer Center, Philadelphia, PA USA). All these cell lines were grown in Dulbecco's modified Eagle's medium (DMEM). CHO (DG44) cells were grown on MEMα supplemented with 30 μM thymidine and nucleotides. CHO-K1 cells were grown on F12 (HAM). All the media were supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were grown in a humidified atmosphere of 6% CO_2 at 37°C. Unless otherwise stated, cells were seeded in 60 mm dishes with 4 ml growth medium and were treated on the third day (after 12 h starvation where stated) as indicated. Inhibitors were added an hour before Cisplatin treatment. The concentration of DMSO in the controls was equal to the concentration of DMSO in inhibitor containing media and never exceeded 0.1%.

Cell survival assay

Cells were seeded in 96-microculture-well plates. Eight thousand and 6000 cells per well for MDA-MB-468 and Panc-1, were used respectively. The day after, cells were treated as indicated. The fraction of surviving cells was measured after the indicated times using the automated microculture methylene blue assay as described in (Benhar *et al*.,2001).

Plasmids

pXER encoding the wild-type EGFR and pXER (Ala^{669}) encoding the mutant EGFR T669A were a generous gift from R Davis (Countaway *et al*., 1989).

Cell lysis, immunoprecipitation, immunoblotting and Coomassie staining

Denaturated cell lysates (for immunoblot) were prepared by scraping the cells in the presence of 2 × Laemmli sample buffer (Laemmli, 1970) and boiling the samples for 10 min.

Lysates for immunoprecipitation of the EGFR were prepared by scraping the cells at 4°C with lysis buffer containing 50 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) pH 7.5, 1 mM ethylenediaminetetraaceticacid (EDTA), 1 mM ethyleneglycoltetraacetate (EGTA), 1% Triton X-100, 150 mM NaCl, 1 mM sodium orthovanadate, 20 mM β-glycerolphosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 μM Microcystin LR, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 313 μg/ml benzamidine and 0.2 mM AEBSF. Lysates were centrifuged at 19 000 *g* for 10 min and supernatants were subjected to immunoprecipitation.

EGFR was immunoprecipitated from 250 μg protein of MDA-MB-468 lysates or from 1 mg protein of EGFR-transfected CHO lysates. The lysates were first pre-cleared with 20 μl of protein A sepharose beads for 2–4 h at 4°C. In parallel, 20 μl of protein A beads were coupled to monoclonal antibody 108, by incubating them with 10 μl of (6 μg/μl total protein NH_4SO_4 precipitated) mAb108 antibody. After coupling, the beads were washed of excess antibody four times with PBS. Following pre-clearing, the lysates were incubated for 18 h with the coupled beads at 4°C. Immunocomplexes were washed four times with washing buffer containing 50 mM HEPES (pH 7.5), 1% Triton X-100, 150 mM KCl. 2 × Laemmli sample buffer (Laemmli, 1970) was added to the beads and the samples were boiled for 3–10 min before SDS–PAGE separation.

For immunoblotting, aliquots of cell extracts containing the same amount of protein were resolved by sodium dodecyl sulphate (SDS–PAGE) and electroblotted onto nitrocellulose membranes (Sartorius, Goettingen, Germany). After incubation of the membranes with the appropriate antibodies, specific bands were visualized using enhanced chemiluminescence. For densitometric analysis, several exposures were performed and only sub-saturation exposures were analysed. Densitometry of immunoblots was performed with NIH image 1.61 software.

Coomassie staining of gels was carried out with GelCode Blue stain reagent (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions.

Phosphatase assay of the EGFR
After immunoprecipitation of the EGFR, the immunocomplex-coupled beads were divided into two tubes. One tube was left untreated. In the other tube, the immunocomplex-coupled beads were suspended in 36 μl phosphatase buffer (supplied by manufacturer as a 10 $\times$ solution, Roche, Germany) containing 5 μl of shrimp alkaline phosphatase (1 U/μl, Roche, Germany) and incubated for 1 h at 30°C with shaking. The reaction was stopped by adding 12 μl of 4 $\times$ Laemmli sample buffer. The samples were boiled for 3 min, loaded onto an SDS–PAGE and subjected to immunoblot.

p38 kinase assay
EGFR immunoprecipitates were washed twice with washing buffer (described above) and twice with kinase buffer containing 25 mM HEPES (pH) 7.5, 20 mM $MgCl_2$, 20 mM β-glycerolphosphate, 5 mM para-nitrophenolphosphate, 0.1 mM sodium orthovanadate and 1 mM dithiothreitol (DTT). Immunoprecipitates were then resuspended in 30 μl kinase buffer containing 40 μM ATP and incubated for 40 min at 30°C with shaking, for EGFR autophosphorylation. Then, the beads were washed once and resuspended in 30 μl kinase buffer containing 20 μM ATP, 10 μCi [γ-^{32}P] ATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) and recombinant $p38^{F327S}$ (a kind gift from D Engelberg) (Bell *et al*., 2001) as indicated. The p38 kinase reaction was performed for 30 min at 30°C with shaking. The reaction was stopped by adding 10 μl of 4 $\times$ Laemmli sample buffer. The samples were boiled for 3 min, loaded onto an SDS–PAGE, electroblotted onto nitrocellulose membrane and subjected to autoradiography. Following autoradiography, the membranes were subjected to immunoblotting.

Immunostaining
MDA-MB-468 cells were plated on coverslips in DMEM containing 10% FCS and maintained at 37°C with 5% CO_2. Twenty-four hours after seeding, the medium was replaced with DMEM without serum for 12 h. After treatment, fixation was performed at room temperature, as follows. Cells were washed once with PBS, fixed and permeabilized in 3% paraformaldehyde, 0.5% Triton X-100, for 2 min, followed by 30 min incubation in 3% paraformaldehyde. The fixed cells were incubated with anti-EGFR antibody (111.6) for 40 min at room temperature, then washed three times with PBS and incubated with Cy3-conjugated anti-mouse antibody for another 40 min After staining, coverslips were mounted in Elvanol.

CHO-K1 cells were seeded in six-well plates, transfected with Lipofectamine Plus (Invitrogen, Life Technologies, Carlsbad, CA, USA) and then transferred to coverslips, precoated with fibronectin (10 μg/ml; Sigma) for 1 h. After treatment, the cells were fixed in 3% paraformaldehyde, stained with anti-EGFR as described for MDA-MB-468 cells and then permeabilized with 0.1% Triton X-100, for 5 min and stained again with anti-EGFR and Cy-5-conjugated secondary antibody.

Fluorescent images were recorded with DeltaVision system (Applied Precision Inc., Issaquah, WA, USA) on an Axioverted microscope (Zeiss, Oberkochen, Germany). A series of *z*-sections were taken at 2 μm intervals. The images were deconvoluted and an image from the central part of the cell was chosen. The images of the extracellular Cy3 were taken in the membrane plane only. Image intensity was measures using the Priism software package (www.msg.ucsf.edu/IVE).

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