Heterodimerization of the epidermal-growth-factor (EGF) receptor and ErbB2 and the affinity of EGF binding are regulated by different mechanisms

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When clathrin-dependent endocytosis is inhibited in HeLa cells by overexpression of a K44A (Lys^{44} → Ala) mutant of the GTPase dynamin, high-affinity binding of epidermal growth factor (EGF) to the EGF receptor (EGFR) is disrupted [Ringerike, Stang, Johannessen, Sandnes, Levy and Madshus (1998) J. Biol. Chem. **273**, 16639–16642]. We now report that the effect of [K44A]dynamin on EGF binding was counteracted by incubation with the non-specific kinase inhibitor staurosporine (SSP), implying that a protein kinase is responsible for disrupted high-affinity binding of EGF upon overexpression of [K44A]dynamin. The effect of [K44A]dynamin on EGF binding was not due to altered phosphorylation of the EGFR, suggesting that the activated kinase is responsible for phosphorylation of a substrate other than EGFR. The number of EGFR molecules was increased in cells overexpressing [K44A]dynamin, while the number of proto-oncoprotein ErbB2 molecules was unaltered. EGF-induced receptor dimerization was not influenced by overexpression of [K44A]dynamin. ErbB2–EGFR heterodimer formation was found to be ligand-independent, and the number of heterodimers was not altered by overexpression of [K44A]dynamin. Neither SSP nor the phorbol ester PMA, which disrupts high-affinity EGF–EGFR interaction, had any effect on the EGFR homo- or hetero-dimerization. Furthermore, the EGF-induced tyrosine phosphorylation of ErbB2 was not affected by overexpression of [K44A]dynamin, implying that EGFR–ErbB2 dimers were fully functional. Our results strongly suggest that high-affinity binding of EGF and EGFR–ErbB2 heterodimerization are regulated by different mechanisms.

Key words: affinity, endocytosis, ErbB2, kinase activity, dimerization.

INTRODUCTION

The epidermal-growth-factor receptor (EGFR) belongs to a family of receptors with intrinsic tyrosine kinase activity [1,2]. Binding of epidermal growth factor (EGF) to EGFR leads to receptor dimerization and activation of the tyrosine kinase [3–6]. This results in receptor auto- or trans-phosphorylation on several tyrosine residues in the cytoplasmic tail and in tyrosine phosphorylation of several substrate proteins [7,8].

The EGFR exists in different states with respect to affinity for EGF [9], and the mechanisms involved in EGFR trans-modulation are still enigmatic. Involvement of threonine and/or serine phosphorylation of the EGFR has been suggested. This is based on the fact that activation of protein kinase C (PKC)-dependent signalling pathways by the phorbol ester PMA leads to a loss of high-affinity EGF binding as well as inhibition of receptor tyrosine kinase activity [10,11]. The loss of high-affinity EGF binding correlates with PKC-mediated phosphorylation on Thr^{654} in the EGFR [12–14]. Additionally, it has been demonstrated that sites of phosphorylation other that Thr^{654} in EGFR may negatively regulate the kinase activity of the EGFR, as well as the affinity of the EGF–EGFR interaction [14]. The EGFR can also be phosphorylated on threonine and/or serine residues in a PKC-independent manner [13,15–22], and these phosphorylations may negatively regulate the affinity of the EGF–EGFR interaction [13,15]. Furthermore, the non-specific serine, threonine, and tyrosine kinase inhibitor staurosporine (SSP) has been shown to increase the number of high-affinity EGFRs in A431 cells, and this increase correlated with a decrease in the phosphoserine and phosphothreonine content of the EGFR [23]. There is therefore compelling evidence that serine and threonine kinases are involved in modulation of EGFR function.

High-affinity EGFRs were demonstrated to be cytoskeleton-associated, and the EGFR binds specifically to actin [24–27]. However, removal of the actin-binding site did not affect the affinity of the EGFR for EGF, and the possibility of affinity-modulating proteins has been suggested [28,29].

EGFR can form homodimers or heterodimers with other members of the EGFR family, namely ErbB2, ErbB3 and ErbB4 [30–34]. Dimeric forms of the EGFR were demonstrated to bind EGF with high affinity [35]. In murine fibroblasts transfected with cDNA encoding ErbB2 and EGFR, exposure to EGF resulted in formation of ErbB2 and EGFR heterodimers and unusually high affinity for EGF [36]. ErbB2 overexpression has been reported to decrease the rate of EGF dissociation from EGFR and to delay the EGFR inactivation [32,37]. This implied that association of ErbB2 and EGFR modifies the function of the EGFR. However, it was recently reported that overexpression of ErbB2 did not detectably alter EGFR affinity at 0 °C, nor potentiate the signalling through the EGFR [38]. On the basis of these contradictory results, the authors raised the possibility that the effect of dimerization of EGFR and ErbB2 on EGF–EGFR interaction may depend on cell-specific factors [38].

Dynamin is a GTPase participating in the pinching off of clathrin-coated pits, and overexpression of a mutant form

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; SSP, staurosporine; PKC, protein kinase C; PKA, cAMP-dependent protein kinase A; FBS, fetal-bovine serum; TBS, Tris-buffered saline [10 mM Tris/HCl (pH 7.6)/137 mM NaCl]; ECL® (Amersham), enhanced chemiluminescence; ECF, enhanced chemifluorescence; BS^3, bis(sulphosuccinimidyl) suberate; Cl_3Ac, trichloroacetic acid; PTA, phosphotungstic acid; SAB, sodium acetate buffer containing 0.5 M NaCl; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; PI 3-kinase, phosphoinositol 3-kinase; K44A, Lys^{44} → Ala; AP, alkaline phosphatase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase.

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of dynamin, [K44A]dynamin (where Lys^{44} has mutated to Ala) inhibits endocytosis from clathrin-coated pits [39,40]. We recently demonstrated that overexpression of [K44A]dynamin in HeLa cells disrupted high-affinity binding of EGF to the EGFR [41], in addition to strongly inhibiting clathrin-dependent endocytosis of the EGFR [41–43]. Our initial aim in the present study was to investigate possible mechanisms whereby [K44A]dynamin disrupted high-affinity EGF–EGFR interaction. We found that the non-specific kinase inhibitor SSP restored the level of high-affinity EGF–EGFR interaction in cells overexpressing [K44A]-dynamin, implying activation of a kinase as being responsible for the desensitization of the EGFR. However, we observed no changes in EGFR phosphorylation upon overexpression of [K44A]dynamin that could explain the disrupting effect of [K44A]dynamin on the EGF–EGFR interaction. SSP did not affect the inhibition of clathrin-dependent endocytosis induced by overexpression of [K44A]dynamin, indicating that the mechanism responsible for desensitization of the EGFR is different from the mechanism responsible for inhibition of endocytosis.

In cells overexpressing [K44A]dynamin, the amount of EGFR was increased, while the amount of ErbB2 was unaltered. The absolute number of heterodimers of EGFR and ErbB2 was unaltered, and the heterodimerization was found not be affected by addition of ligand. Nor was the heterodimerization affected by incubation with SSP or PMA, reagents that clearly affected affinity of EGF binding. This strongly suggests that EGFR–ErbB2 heterodimerization is not in itself responsible for the high-affinity binding of EGF in HeLa cells and further suggests that ligand binding and heterodimerization is regulated by different mechanisms.

EXPERIMENTAL

Materials

Human recombinant EGF was from Bachem Feinchemikalien AG, Budendorf, Switzerland. ^{125}I-EGF and [^{32}P]phosphate were from Amersham Pharmacia Biotech. The Src kinase inhibitor PD 180970-0000 was kindly provided by Parke-Davis (Ann Arbor, MI, U.S.A.). All other chemicals were from Sigma, unless otherwise noted.

Antibodies

Antibodies used were sheep anti-EGFR (Gibco BRL Life Technologies, Paisley, Renfrewshire, Scotland, U.K.), mouse anti-phosphotyrosine (pTyr) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.), mouse anti-(activated EGFR) (pY1173) (Upstate Biotechnology, Lake Placid, NY, U.S.A.), mouse anti-ErbB2 (NeoMarkers Inc., Union City, CA, U.S.A.), mouse anti-activated ErbB2 (pY1248) (Upstate Biotechnology), rabbit anti-ErbB2 (NeoMarkers), mouse anti-ErbB3 (NeoMarkers) and mouse anti-ErbB4 (NeoMarkers). Alkaline phosphatase (AP)-conjugated donkey anti-sheep IgG, AP-conjugated goat anti-rabbit and AP-conjugated donkey anti-mouse IgG, peroxidase-conjugated donkey anti-sheep IgG and peroxidase-conjugated donkey anti-mouse IgG were all from Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.

Cell culture

HeLa cells stably transfected with a plasmid encoding [K44A]-dynamin, where the promoter is negatively controlled by tetracycline (Tet-Off system) [39], was provided by Dr. Sandra L. Schmid, The Scripps Research Institute, La Jolla, CA, U.S.A. Cells were grown in Costar 3275 flasks (Costar, Badhoevedorp, The Netherlands). The medium used was Dulbecco's Modified Eagles Medium (DMEM) containing 3.7 g/l sodium bicarbonate (BioWhittaker, Walkersville, MD, U.S.A.) containing 400 μg/ml geneticin (Gibco BRL), 200 ng/ml puromycin, 2 mM L-glutamine (BioWhittaker), 1 × penicillin/streptomycin/fungizone mixture (BioWhittaker) and 1 μg/ml tetracycline supplemented with 10 % (v/v) fetal-bovine serum (FBS) (BioWhittaker). Cells were seeded at a density of 15000 cells/cm^2, and the cells were grown for 24 h at 37 °C in the presence (non-induced) or absence (induced overexpression of [K44A]dynamin) of 1 μg/ml tetracycline. After 24 h, cells were serum-starved by incubation for another 24 h in the same medium with only 0.5 % FBS. When cells were treated with ligand, EGF (10 nM) was added to the serum-starved cells on ice in minimal essential medium (MEM) (Gibco BRL) without bicarbonate and with 0.1 % (w/v) BSA. After 15 min the cells were washed three times in ice-cold PBS (137 mM NaCl/2.7 mM KCl/1 mM Na_2HPO_4/2 mM NaH_2PO_4) to remove unbound ligand. The cells were then chased in MEM without bicarbonate and with 0.1 % (w/v) BSA at 37 °C for the indicated time periods. In some experiments cells were pretreated with various chemical compounds: 100 nM SSP (dissolved in DMSO) for 30 min; 10 μM bisindolylmaleimide I (GF109203X, Calbiochem–Novabiochem International, La Jolla, CA, U.S.A.) (dissolved in DMSO) for 90 min; 10 μM H89 (Calbiochem–Novabiochem International) (dissolved in DMSO) for 2 h; 10 μM trifluoperazine (dissolved in doubly distilled water) for 15 min; 500 nM wortmannin (dissolved in DMSO) for 10 min; 0.1 μg/ml PMA (dissolved in DMSO) for 15 min; 0.5 μM PD 180970-0000 (Parke–Davis) (dissolved in DMSO) for 2 h; 50 μM PD 98059 (Calbiochem–Novabiochem International) (dissolved in DMSO) for 1.5 h; or 20 μM genistein (dissolved in DMSO) for 30 min at 37 °C before addition of EGF in the presence of the same chemical compounds.

Scatchard plot

Cells in 24-well microtitre plates were incubated for 3 h on ice with increasing concentrations of ^{125}I-EGF (0.05–7.5 ng/ml) in 50 μl or in 500 μl when concentrations of ^{125}I-EGF were $\leqslant$ 0.25 ng/ml. The total binding was measured after incubation with ^{125}I-EGF in MEM without bicarbonate and with 0.1 % (w/v) BSA in the absence of unlabelled EGF, while the non-specific binding was measured after incubation with ^{125}I-EGF in the same medium, but in the presence of unlabelled EGF (200 nM). The medium was collected and counted for radioactivity in a γ-radiation counter. The cells were washed three times with ice-cold PBS to remove unbound ligand before they were hydrolysed in 1 M NaOH and counted in a γ-radiation counter. The specific binding (total binding minus non-specific binding) was analysed by non-linear curve-fitting to equations describing one $[Y = B_{max} \cdot X/(K_d + X)]$ or two $[Y = B_{max_H} \cdot X/(K_{d_H} + X) + B_{max_L} - X/(K_{d_L} + X)]$ binding sites using Prism 2.01 (GraphPad Software). B_{max} is the total number of specific binding sites (receptors) per cell, K_d the equilibrium dissociation constant, X the concentration of free ^{125}I-EGF in the medium and Y the amount of specific ^{125}I-EGF binding per cell. The subsubscripted H and L in B_{max_H}, B_{max_L}, K_{d_H} and K_{d_L} indicates that these are the B_{max} and K_d values for high- and low-affinity EGFR respectively. For the two-site model, the data were weighted by $1/Y^2$. The observed data were plotted as described by Scatchard [44], using Prism 2.01.

Western-blot analysis

After incubation as indicated in the legends to the Figures, cells were lysed in SDS/PAGE sample buffer [10 mM Tris/HCl

(pH 6.8)/5 mM EDTA/50 mM NaF/30 mM sodium pyrophosphate/2% (w/v) SDS (Bio-Rad)/4% (v/v) β-mercaptoethanol/1 mM Na_3VO_4/1 mM PMSF] on ice for 10 min. Then 4% (v/v) glycerol and 0.005% (w/v) Bromophenol Blue were added to the lysates. The lysates were incubated at 95 °C for 10 min and subjected to SDS/PAGE [45], and the proteins were electrotransferred to PVDF membranes (Hybond-P; Amersham Pharmacia Biotech) [46]. The membranes were incubated with primary antibodies (dissolved in Tris-buffered saline [TBS; 10 mM Tris/HCl (pH 7.6)/137 mM NaCl] with 0.1% (v/v) Tween 20, containing 1% fat-free dry-milk) overnight at 4 °C before incubation with AP-conjugated or peroxidase-conjugated secondary antibody (dissolved in TBS with 0.1% (v/v) Tween 20, containing 1% fat-free dry-milk) for 2 h at room temperature. The immunobinding was detected by enhanced chemifluorescence (ECF) or enhanced chemiluminescence (ECL®, Amersham), following the manufacturer's instructions. ECF was measured and quantified using a phosphorofluoroimager (STORM840 Gel and Blot Imaging System; Molecular Dynamics).

Immunoprecipitation

Cells were lysed in immunoprecipitation buffer [PBS containing 10 mM EDTA, 1% (v/v) Triton X-100, 10 mM NaF, 200 units/ml aprotinin (Fluka Chemie AG, Buchs, Switzerland], 1 mM PMSF, 1 mM *N*-ethylmaleimide and 1 mM Na_3VO_4) on ice for 20 min before the lysates were centrifuged at 20000 ***g*** for 15 min at 4 °C. Antibody bound to Protein G-coupled or Protein A-coupled Sepharose beads (Amersham Pharmacia Biotech) was then added to the supernatant fraction and immunoprecipitation was performed at 4 °C for 60 min. The immunoprecipitates were washed three times with immunoprecipitation buffer and once with PBS diluted 1:10, before addition of SDS/PAGE sample buffer (as described above under 'Western-blot analysis'). The immunoprecipitated proteins were subjected to SDS/PAGE and Western-blotting as described above.

Cross-linking of the EGFR

Cells in 10-cm-diameter Petri dishes were incubated as described in the legends to the Figures and then washed three times in ice-cold PBS before incubation on ice for 20 min in PBS containing 3 mM of the non-permeable cross-linking reagent bis(sulphosuccinimidyl) suberate (BS^3) (Pierce). In all experiments, a freshly prepared solution of BS^3 was used. To stop the cross-linking reaction, glycine (250 mM final concn.) was added, and the cells were further incubated for 5 min on ice. The cells were washed three times in ice-cold PBS before they were scraped loose and transferred to test tubes. Cells from two Petri dishes were collected in one test tube. The cells were centrifuged at 410 ***g***, before removal of the supernatant and lysis of the cells on ice in 50 μl SDS/PAGE sample buffer containing 250 mM glycine. The lysates were homogenized by using Qiashredder (Qiagen GmbH, Hilden, Germany), before being subjected to SDS/PAGE using 3–15% gradient gels (Bio-Rad Laboratories) and Western blotting, using an antibody to the EGFR.

^{125}I-EGF–cell interaction experiments

^{125}I-EGF (100 nM) in MEM without bicarbonate and with 0.1% (w/v) BSA was added to cells in 24-well microtitre plates, and the cells were incubated on ice for 15 min. The cells were washed three times with ice-cold PBS before chase in MEM without bicarbonate and with 0.1% (w/v) BSA at 37 °C for the indicated time periods. The medium was collected, and ^{125}I-EGF was precipitated using 5% trichloroacetic acid (Cl_3Ac) containing 1% phosphotungstic acid (PTA) (Cl_3Ac-PTA) as described in [47]. Both the Cl_3Ac-precipitable ^{125}I-EGF (^{125}I-EGF in the medium) and the Cl_3Ac-soluble radioactivity (degraded ^{125}I-EGF) was measured in a γ-radiation counter. Cells were washed three times in ice-cold PBS, treated with 0.2 M sodium acetate buffer, pH 4.5 or 7.4, containing 0.5 M NaCl (SAB pH 4.5/SAB pH 7.4) on ice for 10 min, and washed once with the same buffer. ^{125}I-EGF was precipitated from the cells with Cl_3Ac-PTA and washed three times with Cl_3Ac-PTA before the precipitate was dissolved in 1 M NaOH and counted for radioactivity in a γ-radiation counter. Radioactivity measured from cells treated with SAB pH 4.5 represents internalized ^{125}I-EGF, while radioactivity measured from cells treated with SAB pH 7.4 represents both internalized and surface-localized ^{125}I-EGF.

^{32}P-labelling of EGFR

Cells were seeded 48 h prior to the experiment. In the last 24 h before the experiment, the cells were incubated in the absence or presence of tetracycline in phosphate-free MEM containing 3.7 g/l sodium bicarbonate, 400 μg/ml geneticin, 200 ng/ml puromycin, 2 mM L-glutamine and 1 × penicillin/streptomycin/fungizone mixture, supplemented with 0.5% (v/v) FBS. The medium was changed to phosphate-free MEM without bicarbonate 30 min before the experiment. [^{32}P]Phosphate (1 mCi/ml) was then added to cells in phosphate-free MEM without bicarbonate, and the cells were incubated for 3 h at 37 °C. The cells were washed three times in ice-cold PBS and lysed on ice for 10 min in PBS containing 1% (w/v) SDS, 1 mM PMSF, 200 units/ml aprotinin, 10 μg/ml leupeptin and 1 mM Na_3VO_4. The lysates were homogenized with Qiashredder, and the EGFR was immunoprecipitated with anti-EGFR antibody coupled to Protein G–Sepharose in SDS-immunoprecipitation buffer [1% (v/v) Triton X-100, 0.5% (w/v) SDS, 0.25% (w/v) sodium deoxycholate, 0.5% (w/v) BSA, 1 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 20 mM NaF, 10 μg/ml leupeptin and 200 units/ml aprotinin dissolved in PBS]. The immunoprecipitated proteins were subjected to SDS/PAGE and electrotransferred to PVDF membranes, as described above.

When measuring the ^{32}P content of the EGFR, the radioactivity of the immunoprecipitated EGFR was measured before the membranes were incubated with an antibody to EGFR to quantify the amount of immunoprecipitated EGFR by ECF. Both radioactivity and ECF were quantified using the STORM840 Gel and Blot Imaging System.

When performing phosphoamino acid analysis, phosphorylated EGFR electrotransferred to a PVDF membrane was excised from the membrane and hydrolysed for 120 min at 110 °C in 6 M HCl. After freeze-drying, phosphoamino acids were separated in the first dimension by electrophoresis in acetic acid/formic acid/water (78:25:897, by vol.) at pH 1.9 on thin-layer cellulose-coated sheets (Merck, Darmstadt, Germany) and in the second dimension by chromatography in isobutyric acid/0.5 M NH_4OH (5:3, v/v). Radiolabelled phosphoamino acids were located by autoradiography and identified by co-migration of phosphamino acid standards revealed by ninhydrin [48].

RESULTS

SSP counteracts the disrupting effect of [K44A]dynamin on high-affinity EGF–EGFR interaction

We have previously demonstrated that high-affinity EGF–EGFR interaction is disrupted by overexpression of [K44A]dynamin

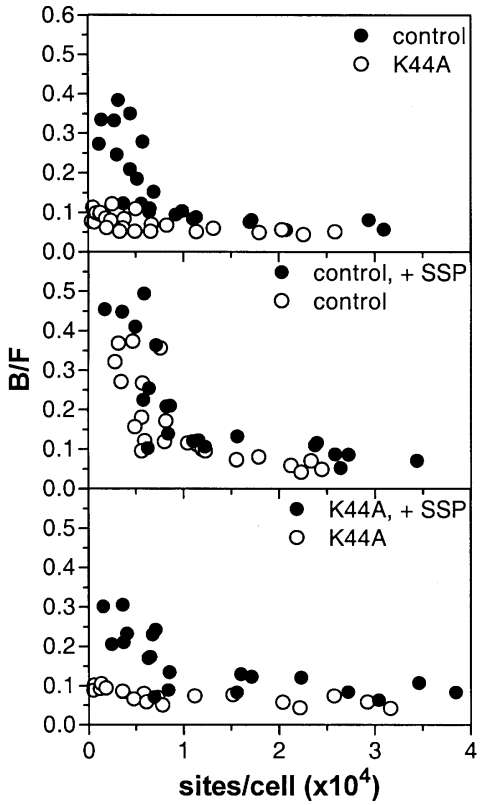

Figure 1 Effect of SSP on the level of high-affinity EGFRs in cells with or without overexpression of [K44A]dynamin

HeLa cells with ('K44A') or without ('control') overexpression of [K44A]dynamin were preincubated for 30 min in the absence (upper panel) or presence (middle and lower panels) of 100 nM SSP at 37 °C before addition of increasing concentrations of ^{125}I-EGF on ice in the absence or presence of SSP. The cells were further incubated on ice for 3 h before the medium was collected and the cells were hydrolysed in 1 M NaOH. The medium and the hydrolysed cells were counted for radioactivity in a γ-radiation counter. One representative experiment out of three is demonstrated, except for the control, where one representative experiment out of six is demonstrated. Scatchard analysis was performed as described in the Experimental section. B/F: B denotes specific binding of ^{125}I-EGF (c.p.m.), whereas F denotes c.p.m./50 μl of medium. B_{max} and K_d are shown in Table 1.

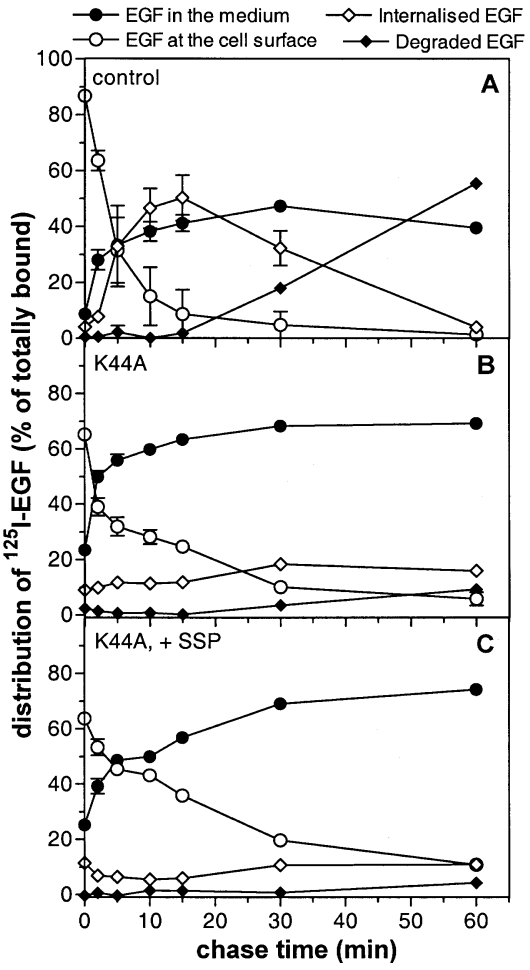

Figure 2 SSP does not counteract the inhibitory effect of [K44A]dynamin on EGF endocytosis

HeLa cells without (**A**) or with (**B**, **C**) overexpression of [K44A]dynamin, were preincubated with (**C**) or without (**A**, **B**) 10 nM SSP for 30 min. Then 10 nM ^{125}I-EGF was added to the cells on ice, and the cells were further incubated for 15 min on ice in the absence or presence of SSP. The cells were washed and chased at 37 °C for the time periods indicated, in the absence or presence of SSP. The medium was collected and analysed for degraded or intact ^{125}I-EGF, and the cells were analysed for internalized or surface-localized ^{125}I-EGF, as described in the Experimental section. One representative experiment with two parallels is demonstrated (minimum and maximum are indicated).

Table 1 B_{max} and K_d of the EGFR

HeLa cells overexpressing [K44A]dynamin (K44A) or HeLa cells not overexpressing K44A dynamin (Control) were preincubated with or without SSP or PMA as described in the Experimental section. The cells were incubated with increasing concentrations of ^{125}I-EGF on ice for 3 h in the absence or presence of SSP or PMA before the medium was collected and the cells were hydrolysed in 1 M NaOH. The medium and the hydrolysed cells were subsequently counted for radioactivity in a γ-radiation counter. Scatchard analysis was performed as described in the Experimental section. The numbers in the B_{max_H} column represent the number of high-affinity EGFR at the cell surface. B_{max_H} and K_{d_H} represent the results for the high-affinity EGFR, while K_{d_L} represents the results for the low-affinity EGFR.

Treatment of cells transfected with [K44A]dynamin	B_{max_H}*	K_{d_H} (pM)	K_{d_L} (pM)
Control	6204 ± 2341	14.0 ± 7.0	820.6 ± 565.2
K44A	0		1676.0 ± 167.4
K44A + SSP	5215 ± 1715	26.5 ± 10.6	2102.0 ± 845.0
Control + SSP	7174 ± 2729	8.4 ± 5.6	829.0 ± 511.9
Control + PMA	0		1323.0 ± 389.9

* The values represent pooled results (means ± S.E.M., $n = 3$, except for the control, where $n = 6$).

when assayed 48 h after start of expressing the transgene. Furthermore, autophosphorylation of the EGFR was reduced upon overexpression of [K44A]dynamin in HeLa cells [41]. A Scatchard plot demonstrating EGFR binding characteristics in HeLa cells with or without overexpression of [K44A]dynamin is shown in Figure 1. This is a Tet-Off system, and, when tetracycline is present in the growth medium, there is no expression of [K44A]dynamin. Control cells therefore represent HeLa cells stably transfected with a plasmid encoding [K44A]dynamin, grown in the presence of tetracycline. [K44A]dynamin is overexpressed when tetracycline is removed from the growth medium. Consistent with previous findings [41], the high-affinity class of receptors in control cells (cells not overexpressing [K44A]-dynamin) (6204 ± 2341 receptors) has an apparent K_d of 14.0 ± 7.0 pM, and the low-affinity class of EGFR has an apparent K_d of 820 ± 565.2 pM. In cells overexpressing [K44A]-

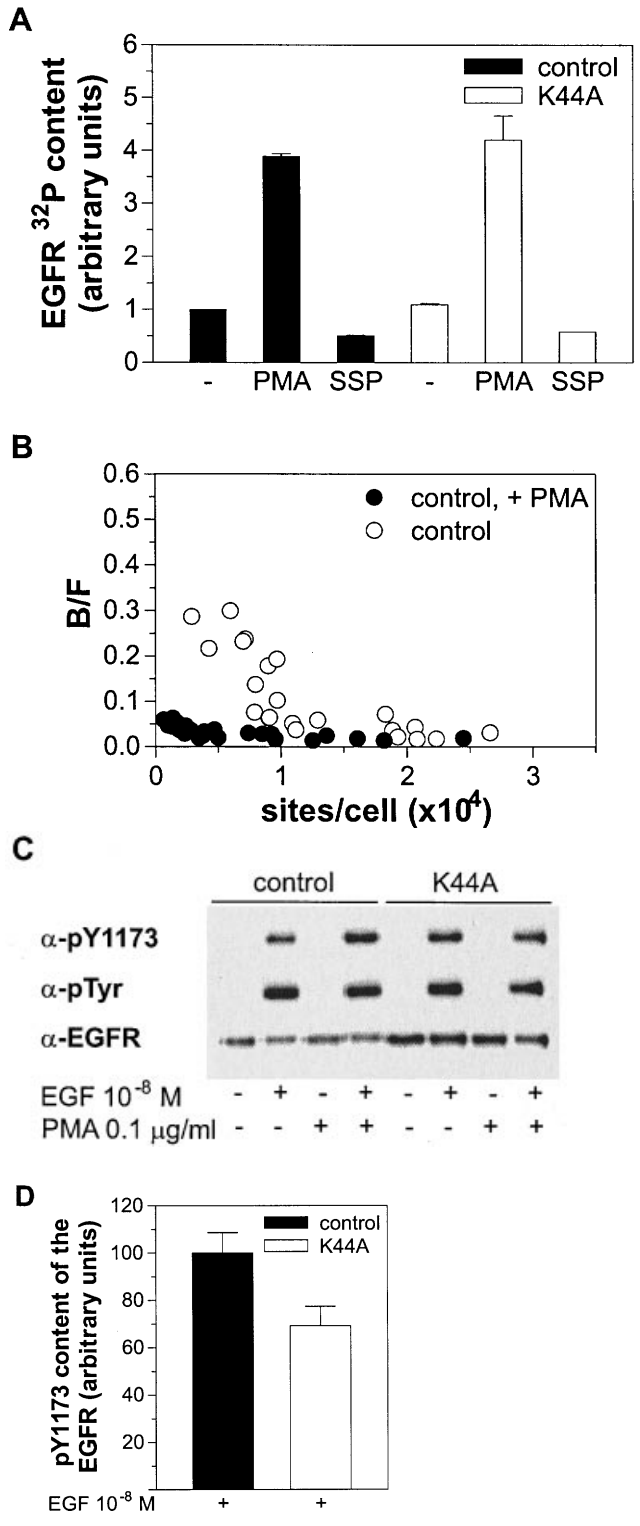

Figure 3 Effect of [K44A]dynamin on the phosphorylation of the EGFR

(**A**) HeLa cells with ('K44A') or without ('control') overexpression of [K44A]dynamin in 24-well plates were incubated in phosphate-free medium for 24 h (during the serum starvation) as described in the Experimental section. Cells were labelled for 3 h at 37 °C with 1 mCi/ml [^{32}P]phosphate in phosphate-free medium. SSP (100 nM) was added to the cells 30 min before lysis of the cells, and 0.1 μg/ml PMA was added to the cells 15 min before lysis. EGFR was immunoprecipitated, and the immunoprecipitated proteins were subjected to SDS/PAGE and subsequently electrotransferred to a PVDF membrane as described in the Experimental section. The radioactivity of the immunoprecipitated EGFR was measured using the STORM840 instrument. The membrane was then blotted with an antibody to EGFR, and the intensitiy of the EGFR band was measured using the STORM840 instrument as described in the Experimental section. The radioactivity of immunoprecipitated EGFR was normalized to the intensity of the EGFR band. Three independent experiments were performed, and one representative experiment with two parallels is demonstrated (minimum and maximum are indicated). (**B**) HeLa cells without overexpression of [K44A]dynamin (control) were preincubated with 0.1 μg/ml PMA for 15 min at 37 °C before addition of increasing concentrations of ^{125}I-EGF on ice. The cells were further incubated on ice for 3 h in the presence of PMA before Scatchard analysis was performed as described in the Experimental section. One representative experiment out of three is shown. B/F: B denotes specific binding of ^{125}I-EGF (c.p.m.), whereas F denotes c.p.m./50 μl of medium. B_{max} and K_d values are given in Table 1. (**C**) HeLa cells were preincubated with or without 0.1 μg/ml PMA for 15 min at 37 °C before incubation with or without 10 nM EGF on ice for 15 min. Then the cells were lysed and subjected to Western blotting using an antibody to phosphotyrosine (pTyr), an antibody to activated EGFR (pY1173) or an antibody to the EGFR, as described in the Experimental section. (**D**) The intensity of the immunobinding in (**C**) was measured as described in the Experimental section. The intensity of the pY1173 band was normalized to the intensity of the EGFR band. The data presented in (**D**) show the means for two independent experiments with the mean and the maximum indicated.

dynamin, the Scatchard plot is linear, indicating only one receptor class. These receptors have an apparent K_d of 1676 ± 167.4 pM (Figure 1 and Table 1), showing that they bind EGF with low affinity.

In order to investigate whether overexpression of [K44A]-dynamin reduced high-affinity EGF binding by activating a kinase, we preincubated HeLa cells overexpressing [K44A]-dynamin with the non-specific kinase inhibitor SSP. Then the binding of ^{125}I-EGF to cells on ice was measured and analysed by Scatchard plotting. As demonstrated in Figure 1, lower panel, SSP (100 nM) clearly restored high-affinity EGF-binding in cells overexpressing [K44A]dynamin, suggesting that overexpression of the K44A mutant of dynamin affected the affinity of the EGF–EGFR interaction by activating an SSP-inhibitable kinase. In SSP-treated cells overexpressing [K44A]dynamin, the high-affinity class of receptors (5215 ± 1715 receptors) had an apparent K_d of 26.5 ± 10.6 pM, and the low-affinity class of receptors had an apparent K_d of 2102 ± 845 pM (Table 1). Treatment of the HeLa cells not overexpressing [K44A]dynamin (middle panel of Figure 1) with 100 nM SSP had no significant effect on the number of high-affinity EGFRs (Figure 1 and Table 1), neither had treatment with concentrations of SSP as high as 3 μM (results not shown).

SSP does not counteract the inhibitory effect of [K44A]dynamin on endocytosis of the EGFR

It was necessary to investigate whether SSP could possibly counteract the effect of [K44A]dynamin on the number of high-affinity EGFRs by counteracting the block in clathrin-dependent endocytosis. Trafficking of ligand-bound EGFR was therefore investigated in HeLa cells not overexpressing [K44A]dynamin, in HeLa cells overexpressing [K44A]dynamin and in SSP-treated HeLa cells overexpressing [K44A]dynamin. The cells were incubated with ^{125}I-EGF on ice before washing away unbound ligand and chasing the cells at 37 °C. At different time points, the fate of the initially bound ^{125}I-EGF was analysed as described in the Experimental section. Medium was collected to determine the amount of ^{125}I-EGF dissociated from the EGFR, as well as degraded EGF, and the cells were analysed before and after a low-pH wash to estimate intracellularly localized versus plasma-membrane-localized ^{125}I-EGF. In cells not overexpressing [K44A]dynamin, ^{125}I-EGF was rapidly endocytosed, with most ligand internalized after 10 min chase at 37 °C (Figure 2A, control). In cells overexpressing [K44A]dynamin the majority of the initially bound ^{125}I-EGF rapidly dissociated from the EGFR (Figure 2B, K44A). Of the ^{125}I-EGF that remained cell-associated, only a small amount was endocytosed and eventually

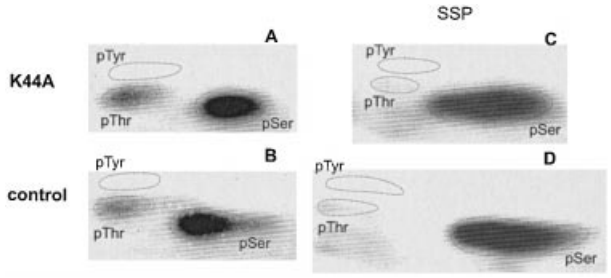

Figure 4 Phosphoamino acid analysis of ^{32}P-labelled EGFR

The Figure is an autoradiogram showing phosphoamino acids in EGFR following *in vivo* ^{32}P-labelling of HeLa cells with (**A** and **C**) or without (**B** and **D**) overexpression of [K44A]dynamin. HeLa cells with or without overexpression of [K44A]dynamin in 10-cm-diameter plates were incubated in phosphate-free medium for 24 h and labelled for 3 h at 37 °C with 1 mCi/ml [^{32}P]phosphate, as described in the Experimental section. SSP (100 nM) was added to the cells 30 min before lysis (**C** and **D**). Immunoprecipitated EGFR was subjected to SDS/PAGE, electrotransferred to a PVDF membrane and subjected to acid hydrolysis. Phosphoamino acids were separated by electrophoresis and chromatography as described in the Experimental section. The migration of phosphoserine ('pSer'), phosphothreonine ('pThr') and phosphotyrosine ('pTyr') standards is indicated by the dot-outlined ellipses.

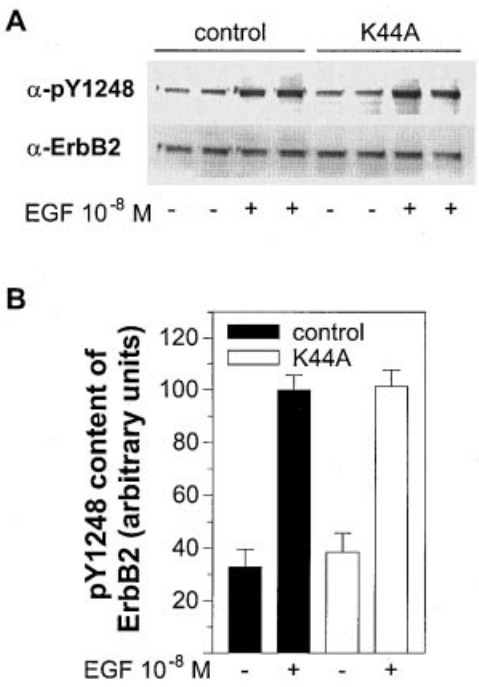

Figure 5 Effect of [K44A]dynamin on the level of ErbB2 and on the activation of ErbB2

EGF (10 nM) was added to HeLa cells with ('K44A') or without ('control') overexpression of mutant dynamin. The cells were incubated on ice for 15 min before the cells were lysed and subjected to Western blotting, as described in the Experimental section, using an antibody to ErbB2 and to activated ErbB2 (pY1248) (**A**). The intensity of the immunobinding was measured as described in the Experimental section. The intensity of the pY1248 band was normalized to the intensity of ErbB2 (**B**). The data in (**B**) represent the means ± S.E.M. for three independent experiments.

degraded. There was no significant effect on endocytosis of pretreating cells overexpressing [K44A]dynamin with SSP (Figure 2C, K44A, + SSP). This demonstrates that SSP does not restore high-affinity EGF binding by counteracting the inhibitory effect of mutant dynamin on clathrin-dependent endocytosis.

[K44A]Dynamin-induced disruption of high-affinity EGF–EGFR interaction is not caused by altered phosphorylation of the EGFR

As phosphorylation on serine and threonine residues in the cytoplasmic tail of the EGFR has been suggested to account for loss of high-affinity EGF–EGFR interaction in A431 cells [23], we wanted to investigate whether the kinase activated by overexpression of [K44A]dynamin caused hyperphosphorylation of the EGFR. To study the EGF-independent phosphorylation of the EGFR, HeLa cells with or without overexpression of [K44A]dynamin were incubated with [^{32}P]phosphate in phosphate-free medium in the absence of ligand. The cells were lysed, the EGFR was immunoprecipitated, and radioactivity associated with the EGFR was quantified as described in the Experimental section. As demonstrated in Figure 3(A), the EGFR was slightly and equally phosphorylated regardless of overexpression of [K44A]dynamin. SSP reduced the specific phosphorylation of the EGFR to the same extent in cells both with and without overexpression of [K44A]dynamin. When cells with or without overexpression of [K44A]dynamin were incubated with PMA for 15 min, the ^{32}P content of the immunoprecipitated EGFR was strongly increased. The phosphorylation was increased to the same extent with or without overexpression of [K44A]dynamin (Figure 3A). When pre-incubating control HeLa cells for 15 min with PMA, as described in Figure 3(A), high-affinity EGF binding was almost completely disrupted when analysed by Scatchard analysis (Figure 3B), in accordance with previously published results [10,11].

We also analysed HeLa cells with (K44A) or without (control) overexpression of [K44A]dynamin by Western blotting, using anti-pTyr, anti-pY1173 and an antibody to EGFR. The results demonstrate that there was no PMA-induced tyrosine phosphorylation of the EGFR in the absence of EGF (Figure 3C). This demonstrates that PMA-induced phosphorylation occurs on threonine and serine residues only. In contrast, EGF induced strong tyrosine phosphorylation of the EGFR (Figures 3C and 3D). No tyrosine phosphorylation of the EGFR could be detected in the absence of EGF (Figure 3C). The phosphorylation of EGFR observed in the absence of ligand (Figure 3A) therefore probably represents serine and threonine phosphorylation only.

Overexpression of [K44A]dynamin induced an increased level of EGFR in HeLa cells, but a reduction in EGF-induced specific tyrosine phosphorylation of the EGFR (Figures 3C and 3D), consistent with previous findings [41,43]. In Figure 3(D) the band representing the EGFR, which was phosphorylated on Tyr^{1173}, was normalized to the EGFR band. The results obtained by using the antibody to pY1173 are consistent with the results obtained when using an antibody to phosphorylated tyrosine (Figure 3C and [41,43]).

To exclude fully the possibility that overexpression of [K44A]-dynamin altered the phosphorylation of the EGFR, we did phosphoamino acid analysis of ^{32}P-labelled EGFR. As demonstrated in Figures 4(A) and 4(B), we found no differences between control cells and cells overexpressing [K44A]dynamin in the absence of ligand. Phosphorylated tyrosine could not be detected under these conditions. This supports the findings demonstrated in Figure 3(C), where no tyrosine phosphorylation of the EGFR could be detected in the absence of ligand, using an antibody to pTyr or to pY1173. There was also no detectable difference between control cells and cells overexpressing [K44A]dynamin with respect to receptor phosphorylation after treatment with

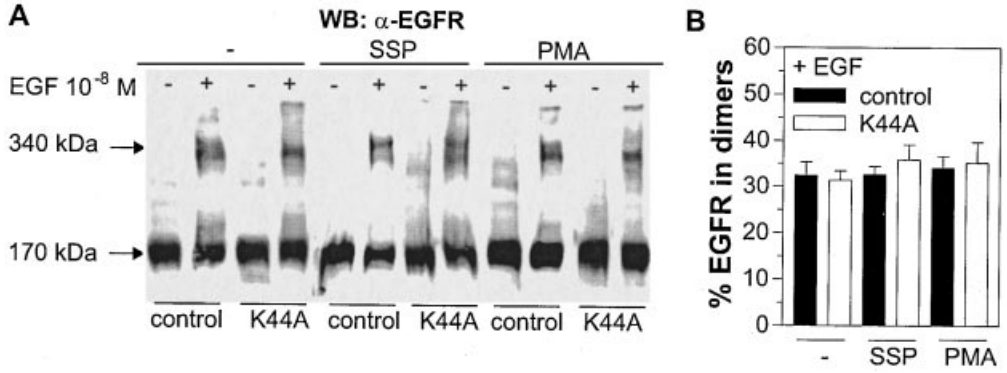

Figure 6 Effect of [K44A]dynamin, PMA, SSP and EGF on EGFR dimerization

(**A**) HeLa cells with ('K44A') or without ('control') overexpression of [K44A]dynamin were preincubated at 37 °C with or without 100 nM SSP for 30 min or 0.1 μg/ml PMA for 15 min before addition of 10 nM EGF on ice. The cells were incubated on ice for 15 min before cross-linking the EGFR using the cross-linker BS^3 as described in the Experimental section. The cells were lysed and subjected to SDS/PAGE and Western blotting (WB), using an antibody to EGFR as described in the Experimental section. (**B**) The intensity of the EGFR was measured as described in the Experimental section. The intensity of the 340 kDa bands (EGFR dimers) was normalized to the intensity of all EGFR [170 kDa band (EGFR monomers) + 340 kDa band]. The histogram shows the percentage of EGFR cross-linked in the presence of EGF and in the presence or absence of [K44A]dynamin, SSP or PMA (means ± S.E.M., $n = 3$).

100 nM SSP (Figures 4C and 4D). Threonine phosphorylation of the receptor was reduced in cells treated with SSP, and this most probably acounts for the reduction of the ^{32}P content of the EGFR upon incubation with SSP. Altogether our results show that disruption of high-affinity EGF binding cannot be explained by altered phosphorylation of the EGFR.

Effect of different kinase inhibitors on the affinity of the EGF–EGFR interaction in HeLa cells overexpressing [K44A]dynamin

As SSP is a non-specific kinase inhibitor, we also studied the effect of several other kinase inhibitors, the aim being to identify the kinase activated by overexpression of [K44A]dynamin. PKC can induce phosphorylation of the EGFR on Thr^{654} [49], and this has been observed to correlate with the disrupted high-affinity EGF binding [12–14]. Activation of a temperature-sensitive tyrosine kinase, $pp60^{v-src}$, reportedly inhibited high-affinity binding of EGF to the EGFR by PKC-independent mechanisms [15]. The cAMP-dependent protein kinase A (PKA) has also been demonstrated to phosphorylate the EGFR *in vitro* [16,17], and it was recently demonstrated that phosphorylation of the EGFR by PKA on serine residues leads to decreased tyrosine kinase activity and diminished autophosphorylation of the EGFR both *in vitro* and *in vivo* [18].

Ca^{2+}/calmodulin-dependent kinase II has been demonstrated to phosphorylate the EGFR on serine and threonine residues upon increase of cytosolic calcium and thereby to down-regulate the EGFR kinase activity [19–21]. Additionally, EGFR has been demonstrated to become phosphorylated on threonine residues in a mitogen-activated protein kinase (MAPK)-dependent manner [22].

HeLa cells overexpressing [K44A]dynamin were therefore incubated with a number of different kinase inhibitors, as described in the Experimental section. Then ^{125}I-EGF was added to cells on ice, and the binding was assayed by Scatchard analysis. Inhibitors of PKA (H89), PKC (bisindolylmaleimide, GF109203X), phosphoinositide 3-kinase (PI 3-kinase) (wortmannin), calmodulin (trifluoperazine), Src kinases (PD180970-0000), MAPK kinase (MEK) (PD 98059) or the non-specific tyrosine kinase inhibitor genistein did not restore high-affinity EGF binding in cells overexpressing [K44A]dynamin.

Overexpression of [K44A]dynamin does not affect the constitutive and EGF-induced tyrosine phosphorylation of ErbB2

It has been reported that overexpression of ErbB2 affects both EGF binding and the activity of EGFR [32,37] and that a kinase-deficient ErbB2 mutant reduced the ligand binding affinity of the EGFR [50]. We therefore investigated whether [K44A]dynamin affected expression of ErbB2 or the EGF-induced activation of ErbB2. Activation of ErbB2 was measured by Western blotting using an antibody to phosphorylated ErbB2. As Figure 5(A) shows, the level of ErbB2 was unaltered in cells overexpressing [K44A]dynamin (K44A) compared with that in control cells (control). Moreover, the specific EGF-induced tyrosine phosphorylation of ErbB2 was the same regardless of overexpression of [K44A]dynamin (Figure 5B). It should further be noted that the ErbB2 was constitutively and equally autophosphorylated in HeLa cells with or without overexpression of [K44A]dynamin.

Since ErbB3 and ErbB4 could potentially interfere with binding of EGF, we studied the expression of these proteins in HeLa cells with or without overexpression of [K44A]dynamin. While we clearly detected ErbB3 and ErbB4 in A431 cells, we could not detect ErbB3 and ErbB4 in HeLa cells under either condition (results not shown).

Overexpression of [K44A]dynamin does not cause a reduction in the number of EGFR–ErbB2 heterodimers

In order to examine EGFR dimerization, we initially took advantage of a chemical cross-linking reagent previously used for this purpose [51]. Following incubation with or without EGF (100 nM) on ice for 15 min, the cells were incubated with the membrane-impermeable cross-linking reagent BS^3 before lysis of the cells, as described in the Experimental section. The cell lysates were analysed by SDS/PAGE and Western blotting, using

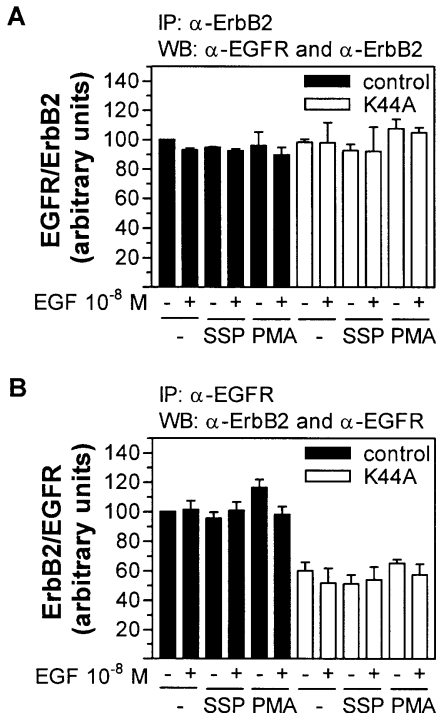

Figure 7 Effect of [K44A]dynamin, SSP, PMA and EGF on the heterodimerization of EGFR and ErbB2

HeLa cells with ('K44A') or without ('control') overexpression of [K44A]dynamin were preincubated with 100 nM SSP for 30 min or with 0.1 μg/ml PMA for 15 min. Then the cells were incubated with or without 10 nM EGF on ice for 15 min as indicated, before lysis and immunoprecipitation of the ErbB2 (**A**) or EGFR (**B**) as described in the Experimental section. The immunoprecipitated (IP) proteins were subjected to SDS/PAGE and Western blotting (WB) with an antibody to EGFR (**A**) or to ErbB2 (**B**). The membranes were re-blotted with an antibody to ErbB2 (**A**) or to EGFR (**B**). The intensity of the ErbB2 and EGFR bands was measured as described in the Experimental section. The intensity of the EGFR band was normalized to the intensity of the ErbB2 band (**A**), and the intensity of the ErbB2 band was normalized to the intensity of the EGFR band (**B**). Results are means $\pm$ S.E.M. for three independent experiments.

antibody to the EGFR. The 170 kDa band, representing monomeric EGFR, and the 340 kDa band, representing dimeric EGFR, were quantified as described in the Experimental section. The intensity of the EGFR dimers was normalized to the intensity of the total amount of EGFR (monomeric and dimeric EGFR). As demonstrated in Figure 6(A), EGF increased the dimerization of the EGFR under all conditions tested, and the dimerization increased percentage-wise to the same extent regardless of overexpression of [K44A]dynamin and of treatment with SSP and PMA (Figure 6B). The lack of effect of PMA on dimerization is in agreement with previously published findings [52].

Dimerization could in the described experiments be the result of EGFR homodimerization and of heterodimerization of the EGFR and ErbB2. ErbB2 has been shown to be the preferred dimerization partner for EGFR, as well as for ErbB3 and ErbB4 [30–34]. To investigate whether overexpression of [K44A]-dynamin could alter heterodimerization of EGFR and ErbB2, EGFR and ErbB2 were immunoprecipitated from HeLa cells with or without overexpression of [K44A]dynamin and with or without incubation with EGF. Western blotting with antibodies to ErbB2 and EGFR was performed to analyse the co-immunoprecipitated proteins. The intensity of the ErbB2 and EGFR bands was quantified as described in the Experimental section.

When ErbB2 was immunoprecipitated and the intensity of the EGFR band was normalized to the intensity of the ErbB2 band, heterodimerization was seen not to be affected by overexpression of [K44A]dynamin (Figure 7A). When the EGFR was immunoprecipitated, and the intensity of the ErbB2 band was normalized to the intensity of the EGFR band, heterodimerization of EGFR and ErbB2 was apparently reduced by 40% in cells overexpressing [K44A]dynamin compared with that in control cells (Figure 7B). However, this apparent reduction can be explained by the increased levels of EGFR, but not of ErbB2, in cells overexpressing [K44A]dynamin (see Figure 5).

Heterodimerization was not affected by incubation with either SSP or PMA (Figures 7A and 7B). Furthermore, addition of EGF did not increase the formation of EGFR–ErbB2 heterodimers (Figures 7A and 7B). The fact that no EGFR dimerization could be observed when cross-linking the EGFR in the absence of EGF (Figure 6), combined with the fact that EGFR–ErbB2 heterodimerization was not affected by the addition of ligand (Figure 7) indicates that heterodimers are pre-formed and constitute a very small fraction of the total dimers observed upon addition of ligand.

DISCUSSION

In the present study we have investigated the mechanisms whereby overexpression of [K44A]dynamin can possibly disrupt high-affinity EGF binding in HeLa cells [41]. The finding that SSP normalized high-affinity binding of EGF suggests that activation of an SSP-inhibitable kinase is involved in the observed disruption of high-affinity EGF–EGFR interaction. We were unable to identify the activated kinase, as a number of more specific kinase inhibitors had no effect on EGF binding. On the basis of these findings we can probably exclude PKC, PKA, Src, Ca/calmodulin-dependent kinases, MEK, PI 3-kinases and tyrosine kinases as responsible for the disrupted high-affinity binding of EGF upon overexpression of [K44A]dynamin.

SSP has been shown to increase the number of high-affinity EGFRs in A431 cells, and this increase in high-affinity EGF binding was observed to correlate with reduced serine and threonine phosphorylation of the cytoplasmic tail of the EGFR [23]. We could not detect differences in serine and threonine phosphorylation of the EGFR in control cells and cells overexpressing [K44A]dynamin. Tyrosine phosphorylation of the EGFR could only be detected in the presence of ligand. In HeLa cells SSP reduced the basal phosphorylation of the EGFR to the same extent both in cells overexpressing [K44A]dynamin and in control cells. However, in contrast with in A431 cells, SSP did not increase the number of high-affinity EGFRs in HeLa cells not overexpressing [K44A]dynamin. Basal serine and threonine phosphorylation of the EGFR in the presence of SSP was the same in control cells and in cells overexpressing [K44A]dynamin.

The possibility therefore exists that the observed effect of SSP is due to inhibition of a kinase, which does not phosphorylate the EGFR itself, but rather a substrate responsible for modulating the affinity of the EGFR. This is in accordance with the hypothesis of Walker and Burgess [28] and Van der Heyden et al. [29], who proposed the existence of affinity-modulating proteins. Walker and Burgess [28] suggested that proteins which negatively modulate EGF binding are phosphorylated. They observed that, in plasma membranes isolated from EGFR-expressing cells, the high-affinity EGF binding was disrupted when the cells had been pretreated with EGF or platelet-derived growth factor. During the pretreatment, several tyrosine phosphorylated proteins were found to be released from the plasma membrane, and when these proteins were dephosphorylated *in vitro* and added back, high-

affinity EGF binding was restored [28]. Van der Heyden et al. [29] suggested that, on the basis of the observation that a deletion in this subdomain disrupted high-affinity binding [29], an affinity-modulating protein binds to subdomain XI of the EGFR within the receptor tyrosine kinase domain.

In the present study we observed that EGFR–ErbB2 heterodimers formed independently of EGF. Neither the amount of ErbB2 nor EGFR–ErbB2 heterodimerization was affected by overexpression of [K44A]dynamin, while the amount of EGFR increased. ErbB2 has been shown to be the preferred dimerization partner for EGFR, ErbB3 and ErbB4 [30–34], and EGFR–ErbB2 heterodimerization has been proposed to be relevant for high-affinity binding of EGF in cells transfected with cDNAs encoding EGFR and ErbB2 [36]. However, we now demonstrate that high-affinity binding of EGF was disrupted upon overexpression of mutant dynamin and upon incubation with PMA, while the actual number of EGFR–ErbB2 dimers was unaltered. This clearly shows that EGFR–ErbB2 heterodimers do not in themselves constitute high-affinity EGF-binding sites.

In the presence of EGF, the amount of dimerized EGFR increased in cells both with and without overexpression of mutant dynamin. As EGFR–ErbB2 heterodimers were preformed and not influenced by EGF, and neither ErbB3 nor ErbB4 was detected, the EGF-induced dimers are most likely EGFR homodimers. The ratio of EGF-induced EGFR dimers compared with the total amount of EGFRs in cells overexpressing [K44A]dynamin was the same as in control cells. Consequently, as the number of EGFR was increased in HeLa cells overexpressing [K44A]dynamin, the actual number of EGFR homodimers at the cell surface was also increased upon incubation with EGF. The fact that high-affinity binding of EGF was disrupted under these conditions demonstrates that EGF-induced EGFR homodimerization is not sufficient for high-affinity binding of EGF.

HeLa cells overexpressing [K44A]dynamin contained percentage-wise a smaller amount of EGFR–ErbB2 heterodimers, while the proportion of EGFR dimers in the presence of EGF was the same as in control cells. Therefore the proportion of EGFR–ErbB2 heterodimers compared with all dimers in the presence of ligand was reduced in cells overexpressing [K44A]-dynamin. Consistently we observed a clearly reduced specific tyrosine phosphorylation of the EGFR, while the EGF-induced activation of ErbB2 was unaltered. The reduced specific tyrosine phosphorylation of EGFR upon overexpression of mutant dynamin can be explained by the proportional reduction of heterodimers and the more potent kinase activity of EGFR–ErbB2 heterodimers than of EGFR homodimers [53].

It should be emphasized that formation of EGFR–ErbB2 dimers was independent of EGF, indicating high stability of such dimers. We found that PMA and overexpression of [K44A]-dynamin disrupted high-affinity EGF binding without changing EGFR–ErbB2 dimerization. While normal binding of EGF was restored when treating HeLa cells overexpressing [K44A]-dynamin with SSP, EGFR–ErbB2 heterodimerization was not altered. Our data therefore strongly suggest that EGFR–ErbB2 heterodimerization and changes in EGF-binding affinity can be regulated independently and indicate that an SSP-inhibitable kinase is involved in modulation of EGF–EGFR affinity.

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