

## Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFRvIII)

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The type-III deletion variant of the epidermal growth factor receptor (EGFRvIII) is frequently found in glioblastomas and other malignant human tumours. Although EGFRvIII confers ligand-independent oncogenic transformation of cell lines, the mechanism by which it promotes aberrant cellular proliferation is unknown. Using cell lines expressing comparable numbers of either wild-type receptor (EGFRwt) or EGFRvIII, we compared several parameters of receptor activation: dimerization, tyrosine phosphorylation and activation of intracellular signalling proteins. Like activated EGFRwt, EGFRvIII was phosphorylated and bound constitutively to the Shc adapter protein. Indeed, EGFRvIII-associated Shc had a higher phosphotyrosine content than Shc associated with stimulated EGFRwt. EGFRwt

dimerized in response to either EGF or transforming growth factor  $\alpha$ . Higher cross-linker concentrations and incubation at higher temperatures (37 °C) allowed detection of EGFRwt dimers even in the absence of exogenous ligand. In contrast, EGFRvIII failed to dimerize under any conditions studied. Moreover, neither mitogen-activated protein kinase nor phospholipase C $\gamma$  were phosphorylated in EGFRvIII-expressing cells. We conclude that the deletion of 267 amino acids from the 621-amino-acid N-terminal domain of EGFR does not result simply in a constitutively activated receptor, but alters the spectrum of signalling cascades utilized. Furthermore the ligand-independent transforming activity of EGFRvIII is independent of receptor dimerization.

### INTRODUCTION

Growth factors and their receptors play a central role in regulating both developmental and neoplastic processes. In particular, the epidermal growth factor receptor (EGFR) has been extensively implicated in tumorigenesis following its identification as the cellular homologue of *v-erbB* [1]. EGFR is a single-transmembrane-domain glycoprotein (170 kDa) with an extracellular ligand-binding regulatory domain of 621 amino acids, and an intracellular tyrosine kinase domain. EGFR overexpression has been detected in many human tumours. In glioblastoma isolates, EGFR gene amplification is often accompanied by gene rearrangement, resulting in several variant EGFR forms (reviewed in [2]).

Of the naturally occurring variant forms, the type-III EGFR variant (EGFRvIII) is the most common. It is present in 50–60% of high-grade gliomas, 70% of breast carcinomas, 73% of ovarian neoplasms and 16% of large-cell lung carcinomas, but not in normal tissues [3–6]. Structurally, the *EGFRvIII* gene is missing 801 coding bases, spanning exons 2–7 of the wild-type gene [3,7,8]. The deletion of amino acids 6–273 from the extracellular domain is accompanied by the appearance of a novel glycine residue at the fusion junction [9]. Several transfection experiments suggest that EGFRvIII is an oncoprotein [7,10–12], mediating soft agar focus formation and tumorigenesis in nude mice [12]. The mechanism by which EGFRvIII expression transforms cells is unknown.

One prominent model of wild-type EGFR (EGFRwt) activation involves an equilibrium between two aggregation states. Receptor ligation shifts the equilibrium towards the dimerized state, which promotes tyrosine kinase activity [13–15]. Receptor tyrosine phosphorylation regulates a series of interactions with intracellular signalling proteins, particularly SH2-domain-containing proteins such as the Shc adapter protein, STAT transcription factors and phospholipase C $\gamma$  (PLC- $\gamma$ ), which are themselves phosphorylated. A cascade of serine/threonine kinases ensues from recruitment of Ras-GTP. Eventually, activation of transcriptional factors occurs through involvement of mitogen-activated protein kinase (MAPK) or the Jak-STAT system (reviewed in [16]). Concurrently, down-regulating or desensitizing systems are initiated by receptor ligation [17,18]. Conceptually, constitutive receptor activation and/or impairment of down-regulating systems at any level can result in unregulated growth stimulation and oncogenesis.

Because the experimental addition of exogenous EGF to cells overexpressing EGFRwt induces a transformed phenotype [19,20], we compared the activation state of the EGFRvIII oncoprotein with that of stimulated and unstimulated EGFRwt using EGF and transforming growth factor  $\alpha$  (TGF- $\alpha$ ), another ligand that is more widely synthesized and more potent than EGF in a variety of systems [21,22]. Our results suggest that the deletion in EGFRvIII interferes with its ability to form dimers. Moreover, although EGFRvIII is phosphorylated and associates with Shc, it does not appear to activate PLC- $\gamma$  or MAPK. These

Abbreviations used: BS<sup>3</sup>, bis(sulphosuccinimidyl)suberate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride; EGF, epidermal growth factor; EGFRwt, the wild-type receptor for EGF; EGFRvIII, the type-III variant of EGFR; HBSS, Hanks balanced-salt solution; MAPK, mitogen-activated protein kinase; NR6M, an NR6 transfectant expressing EGFRvIII; NR6W, an NR6 transfectant expressing wild-type EGFR; PLC- $\gamma$ , phospholipase C $\gamma$ ; TGF- $\alpha$ , transforming growth factor  $\alpha$ .

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observations suggest a different pathway for transformation by EGFRvIII from that proposed for overexpressed EGF-stimulated wild-type receptors.

## EXPERIMENTAL

### Cell lines

Human epidermoid carcinoma A431 cells (cell line 329; passage 135–139) were obtained from ATCC (Rockville, MD, U.S.A.). Using the murine fibroblast NR6 line, a Swiss 3T3 mutant that does not express endogenous EGFR [23], a matched pair of transfectants stably expressing either EGFRwt (NR6W; passage 47–66) or EGFRvIII (NR6M) were produced [12]. NR6M clones expressing higher receptor numbers were selected by flow cytometry (NR6Msel3; passage 11–22). A Swiss 3T3 transfectant, HC2 20d2/c (HC2; passage 81–91), that expresses high levels of EGFRvIII ( $10^6$ ) along with low levels of EGFR ( $10^3$ – $10^4$ ) [5] was provided by Dr. Albert J. Wong (Thomas Jefferson University, Philadelphia, PA, U.S.A.). Cell lines were maintained in improved Zinc Option minimal essential medium (Gibco-BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum or Dulbecco's modified Eagle's medium with 10% fetal calf serum and 600  $\mu\text{g}/\text{ml}$  G418 for signalling studies.

### Quantitative flow cytometry

The number of EGFRwt or EGFRvIII expressed by cell populations used in the assays was determined by quantitative flow cytometry using the Quantum Simply Cellular™ microbead standard system (Flow Cytometry Standards Corporation, San Juan, Puerto Rico). Microbeads or cells were labelled for 2 h at 4 °C with 300  $\mu\text{l}$  of fluoresceinated monoclonal antibody (5–10  $\mu\text{g}/\text{ml}$ ), washed twice with ice-cold PBS containing 1% BSA, and resuspended in 0.5 ml of 0.5% paraformaldehyde for analysis on a FACSort equipped with Lysys software (Becton Dickinson, San Jose, CA, U.S.A.). Receptor density was analysed by interpolation with microbead standard curves using QuickCal™ software (Flow Cytometry Standards Corp.). Assuming a 1:1 stoichiometry of antibody to receptor, this allows calculation of the receptor number, expressed as a population mean.

Direct fluoresceination of monoclonal antibodies was performed by dialysis against 115 mM sodium phosphate buffer, pH 7.4, adjustment to 1 mg/ml protein, and addition of 70  $\mu\text{g}$  of *N*-hydroxysuccinimide ester of fluorescein isothiocyanate (Pierce) in 70  $\mu\text{l}$  of DMSO. After a 4 h incubation at room temperature, the solution was dialysed at 4 °C against PBS. L8A4, a monoclonal antibody specific for EGFRvIII [5], EGFR-1 (Boehringer-Mannheim, Mannheim, Germany), which recognizes EGFRwt but not EGFRvIII [10], and H11, which recognizes both wild-type and variant receptors [5], were used. Fluoresceinated irrelevant isotype controls (IgG1 and IgG2b) and negative cell controls (NR6W for L8A4; NR6M for EGFR-1) were used for background binding. The estimated cell receptor numbers obtained by quantitative flow cytometry were consistent with those obtained in both concurrent and historical series using iodinated antibodies or EGF in standard Scatchard analysis (see Table 1).

### Glycosidase digestion of cell lysates

Cell lysates containing 50  $\mu\text{g}$  of protein were denatured and digested with 24 units/ml recombinant N-glycosidase F (Boehringer-Mannheim) overnight at 37 °C in 25  $\mu\text{l}$  of 100 mM

sodium phosphate, pH 7.5, containing 25 mM EDTA, 0.1% SDS, 0.8% Triton X-100 and 1% 2-mercaptoethanol.

### Dimerization assay

A431 cells, NR6W cells, NR6M cells and HC2 cells were grown to more than 85% confluence in six-well tissue culture plates (Costar, Boston, MA, U.S.A. and Falcon, Oxnard, CA, U.S.A.). The monolayers were washed with Hanks balanced-salt solution (HBSS)/20 mM Hepes, pH 7.5, and incubated with human recombinant EGF (Collaborative Research, Bedford, MA, U.S.A. and Promega, Madison, WI, U.S.A.) or human recombinant TGF- $\alpha$  (Collaborative Research) in HBSS/20 mM Hepes/0.5% BSA, pH 7.5, for 1 h at 0 °C or 5 min at 37 °C. The binding medium was rapidly replaced with 0.5 ml of HBSS/20 mM Hepes, pH 7.5, containing various concentrations of freshly prepared bis(sulphosuccinimidyl) suberate (BS<sup>3</sup>; Pierce, Rockford, IL, U.S.A.) or 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide hydrochloride (EDC; Pierce), and the incubation continued with gentle shaking (20 min at 0 °C for BS<sup>3</sup> and 45 min at room temperature or 30 min at 37 °C for EDC). The reaction was quenched by the addition of 20 mM (final) Tris/HCl, pH 7.5. The cells were washed with 25 mM Hepes, pH 7.5, containing 150 mM NaCl and 5 mM EDTA, scraped off the plate, and lysed for 20–60 min in 30–50  $\mu\text{l}$  of 25 mM Hepes, pH 7.5, containing 150 mM NaCl, 1% Triton X-100 and 10% glycerol, and also freshly added serine proteinase inhibitor 3,4-dichloroisocoumarin (0.15 mM), cysteine proteinase inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (0.1 mM) and EDTA (5 mM) as a metalloproteinase inhibitor, all from Sigma Chemical (St. Louis, MO, U.S.A.). The lysates were stored at –80 °C until use.

### SDS/PAGE and immunodetection

Lysates were analysed by electrophoresis through 5% acrylamide gels using a Tris/glycine/SDS discontinuous buffer system. Equal amounts of protein, by Coomassie Plus (Pierce) assay, were loaded per lane. Molecular-mass markers were non-reduced  $\alpha_2$ -macroglobulin dimers purified from outdated human plasma (~360 kDa) [24] and the Bio-Rad prestained SDS/PAGE standards myosin (~206 kDa),  $\beta$ -galactosidase (~117 kDa) and phosphorylase *b* (~98 kDa). The proteins were electroblotted to nitrocellulose and the membranes blocked overnight at 4 °C in PBS, pH 7.4, containing 5% instant non-fat dry milk. Membranes were probed with the monoclonal antibody H11 (2–3  $\mu\text{g}/\text{ml}$ ), which recognizes the extracellular domain of both EGFRvIII and EGFRwt [5], and developed using horseradish peroxidase-conjugated sheep anti-mouse serum and an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL, U.S.A.).

### Intracellular signalling assays

Before ligand stimulation, cells were grown to approx. 80% confluency and incubated in serum-free medium for 24 h. Human recombinant TGF- $\alpha$  was added to 20 ng/ml and the cells incubated at 37 °C for 0–20 min. The cells were washed twice in cold PBS. RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% doxycholate, 0.1% SDS, 50 mM Tris/HCl, pH 8.0) containing proteinase and phosphatase inhibitors was added to the plates and the lysates incubated on ice for 30 min before centrifugation (4000 *g* for 20 min at 4 °C). Cleared lysates were stored at –70 °C. For immunoprecipitations, aliquots of cell extract were incubated at 4 °C for 24 h with antibodies specific for human EGFR (sheep polyclonal; UBI), Shc (rabbit polyclonal; UBI), MAPK (rabbit polyclonal; Santa Cruz) or phosphotyrosine

(mouse monoclonal; UBI). Immune complexes were precipitated with Protein A–Sepharose or Protein G–Sepharose (ZY MED) and washed three times with RIPA buffer. Control immunoprecipitation reactions were performed using Protein G–Sepharose and RIPA buffer alone. Immunoprecipitates were separated by reducing SDS/PAGE and transferred to Immobilon-P. Non-specific binding sites were blocked by incubation in 5% BSA/TBST for 1 h. Primary antibody–antigen complexes were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection as described above. Blots to be reprobed were stripped in 5 M NaI for 30 min. Band densities were analysed using an Ultrascan XL laser densitometer (LKB).

## RESULTS

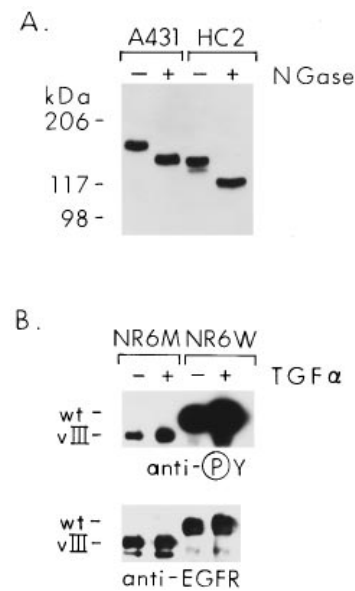
### Characterization of cell surface receptors

Since expression of receptor proteins can vary with passage number in NR6M cells [12], we performed quantitative flow cytometry on the passages of each cell line used in the assays. Appropriate surface receptor expression was confirmed for each of the four cell lines using antibodies specific for either the wild-type or variant EGFR. The results verified that all four cell lines expressed comparable densities of receptors (Table 1).

Immunoblotting cell lysates with an antibody that recognizes both wild-type and variant EGFR revealed a single 170 kDa band in lysates from EGFRwt-expressing cells (Figure 1A). In EGFRvIII-expressing cells, there was a doublet migrating at 140–145 kDa. N-Glycosidase F digestion of HC2 lysates to remove N-linked sugars resulted in the disappearance of the doublet and the appearance of a single band migrating at about 118 kDa, indicating carbohydrate heterogeneity of EGFRvIII (Figure 1A). The phosphotyrosine content of both EGFRvIII and EGFRwt increased with stimulation, although the baseline phosphorylation of EGFRvIII was approximately ten times lower than that of unstimulated EGFRwt (Figure 1B).

### EGFRwt dimerization

All previous studies of EGFR dimerization utilized A431 cells or membrane preparations [13,15,26]. We compared dimerization patterns of A431 cells with those of NR6W, an EGFRwt transfectant. In all respects, transfected EGFRwt on NR6W dimerized in response to EGF in a manner indistinguishable from that of endogenous EGFRwt on the A431 carcinoma line. A BS<sup>3</sup> cross-linker concentration of 1 mM mediated detection of



**Figure 1** Immunoblot characterization of EGFRwt and EGFRvIII cell lines

(A) Cell lysates from A431 (EGFRwt) and HC2 (EGFRvIII) were treated with buffer or N-glycosidase F (NGase) overnight as described in the Experimental section. Protein bands were resolved by SDS/PAGE, transferred to nitrocellulose, and probed using the H11 monoclonal antibody that recognizes both EGFRwt and EGFRvIII. Note that H11 recognizes only a single 170 kDa band corresponding to EGFRwt in crude A431 lysates, and a tight doublet at 140–145 kDa in crude lysates from the EGFRvIII transfectant HC2. Digestion of N-linked carbohydrates causes the EGFRvIII doublet to collapse into a single faster migrating band. (B) Serum-starved NR6M cells (EGFRvIII) and NR6W cells (EGFRwt) were treated with or without 20 ng/ml TGF- $\alpha$  for 10 min at 37 °C. Cell lysates were immunoprecipitated using an anti-EGFR polyclonal antibody as described in the Experimental section, and subjected to immunoblot analysis. Blots were probed with anti-phosphotyrosine (anti-PY), then stripped and reprobed with anti-EGFR. Note the marked ligand-sensitive increase in EGFRwt phosphorylation that is markedly less for EGFRvIII (see also Figure 4B, right).

EGF-elicited dimers (approx. 360 kDa) (Figure 2A). Increasing BS<sup>3</sup> concentrations resulted in stabilization of more dimers; however, the highest concentration also promoted the appearance of a 360 kDa EGFR-containing band in buffer-treated cells, consistent with a previously described low level of constitutive dimerization [13,26,27]. Since both EGF and TGF- $\alpha$  require Ca<sup>2+</sup> for receptor ligation, control experiments were performed

**Table 1** Receptor number determined by quantitative flow cytometry

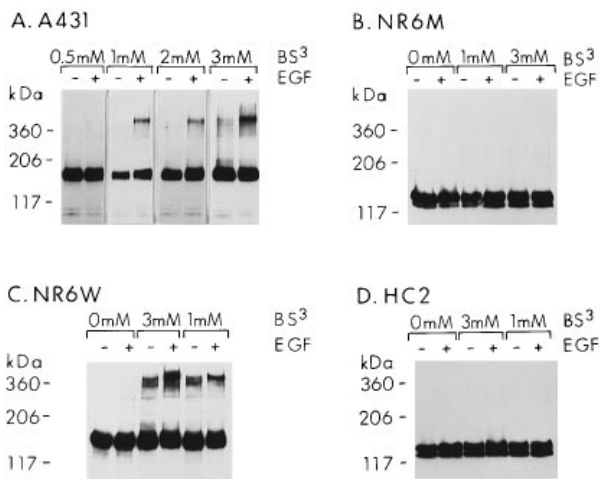
The numbers of receptors/cell are given as means with range in square brackets. The Scatchard values are means for the number of determinations given in parentheses.

Cell line	Passage no.	Receptor	Antibody used	Receptors/cell	No. of independent determinations	Scatchard
NR6M	sel 3: 17–28	vIII	L8	$7.9 \times 10^5$ [(0.42–1) $\times 10^6$ ]	5	$7.4 \times 10^5$ (3)
NR6W	55–67	wild-type	H11, EGFR-1	$1.1 \times 10^6$ [(0.65–1.5) $\times 10^6$ ]	6	$1.5 \times 10^6$ (3)*
HC2	81–97	vIII	L8, H11	$2.5 \times 10^6$ [(1.5–4.1) $\times 10^6$ ]	5	$2.6 \times 10^6$ (16)†
A431	137–139	wild-type	EGFR-1	$3.7 \times 10^6$ [(2.4–5) $\times 10^6$ ]	2	$2.6 \times 10^6$ ‡

\* As reported previously using iodinated EGF [12].

† Including data from a previously reported series using iodinated monoclonal antibodies ( $2.1 \times 10^6$ /cell;  $n = 9$ ) [5].

‡ As reported in the literature using iodinated EGF [25].



**Figure 2** Effects of ligand, cross-linker concentration and temperature on receptor dimerization

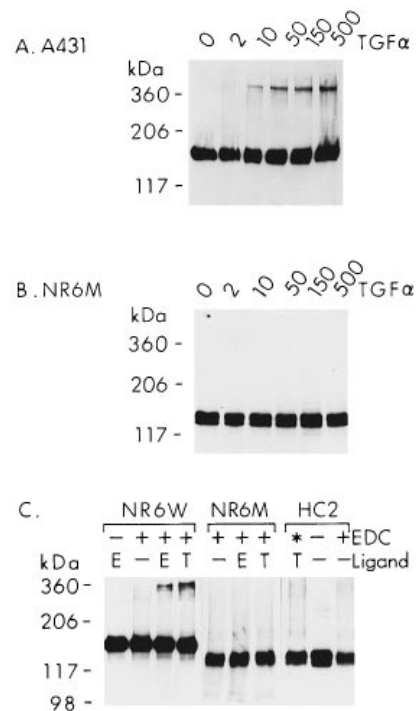
After treatment of cell monolayers with the indicated ligands and cross-linkers, cell lysates were analysed by SDS/PAGE and immunodetection using the H11 antibody as described in the Experimental section. (A, B) A431 cells, NR6W cells, NR6M cells or HC2 cells were incubated for 1 h at 0 °C with buffer or 150 nM EGF, followed by cross-linking with 0–3 mM BS<sup>3</sup> for 20 min. The results for NR6W were indistinguishable from those of A431 (A) and the results for HC2 indistinguishable from those of NR6M (B). Parallel experiments were performed using 150 nM TGF- $\alpha$ , with identical results (not shown). Note the ligand and cross-linker-dependent appearance of EGFRwt dimers (approx. 360 kDa; A) that is absent for EGFRvIII (B). (C, D) NR6W cells (C), NR6M cells or HC2 cells (D) were treated with buffer or 150 nM EGF for 5 min at 37 °C before cross-linking with 0–3 mM BS<sup>3</sup>. The results for NR6M cells were identical with those of HC2 cells. Note the enhanced dimerization of EGFRwt at this higher temperature with readily detectable EGFRwt dimers even in unstimulated cells (C), compared with the absence of dimerization in cells expressing EGFRvIII (D).

using HBSS lacking bivalent cations. Under these conditions, there was no dimerization response, verifying that the observed 360 kDa band was dependent on specific receptor–ligand interaction.

The ability of TGF- $\alpha$  to elicit receptor dimerization has not been previously studied. Although TGF- $\alpha$  competes with EGF for receptor ligation, eliciting similar cellular responses, there are differences in biological effects, intracellular trafficking, and aspects of the physical interaction with the receptor [21,22,28]. We found that TGF- $\alpha$  effectively stimulated dimerization in both A431 and NR6W cells, in a ligand-, Ca<sup>2+</sup>- and crosslinker-dependent manner indistinguishable from results using EGF. A dose–response study showed that 10 nM TGF- $\alpha$  elicited clear-cut dimerization in A431 cells (Figure 3A). This 10 nM detection limit is comparable with the  $K_d$  of binding (approx. 1–5 nM) and the concentration shown to mediate ligand-dependent transformation of cells overexpressing EGFRwt (3.3 nM) [12,19,20,22].

#### Lack of dimerization in EGFRvIII

After optimizing assay conditions using wild-type cell lines, we studied dimerization of EGFRvIII using NR6M and HC2. There was no dimerization response to EGF (Figure 2B) or TGF- $\alpha$ , even at 500 nM concentrations (Figure 3B). Given the ligand-independent nature of EGFRvIII-transforming activity [11,12], however, the level of constitutive dimerization is more pertinent. In contrast with unstimulated EGFRwt, in which high cross-linker concentrations resulted in detection of low-level constitutive dimerization, no dimers were detected for EGFRvIII at



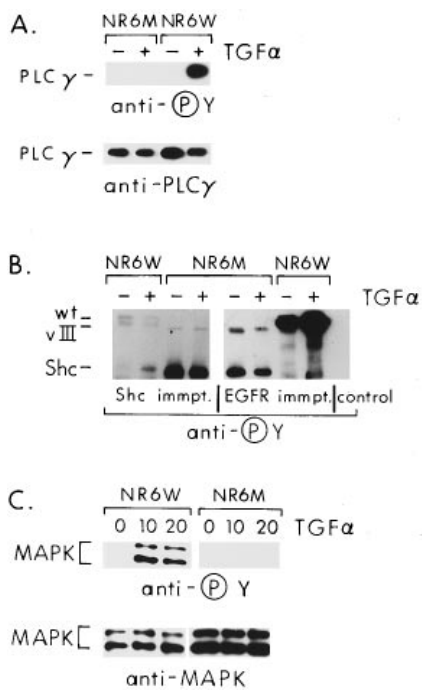
**Figure 3** Effects of ligand dosage and an alternative cross-linking agent on dimerization

After treatment of cell monolayers with the indicated ligands and cross-linkers, cell lysates were analysed by SDS/PAGE and immunodetection using the H11 antibody as described in the Experimental section. (A, B) A431 cells (A), NR6W cells, NR6M cells (B) or HC2 cells were treated with 0–500 nM TGF- $\alpha$  for 1 h at 0 °C, followed by cross-linking with 2 mM BS<sup>3</sup>. The results for NR6W were similar to those for A431, and the results for HC2 identical with those for NR6M. Note that whereas a 10 nM concentration of TGF- $\alpha$  induces detectable EGFRwt dimerization (A), EGFRvIII does not dimerize (B). (C) NR6W cells, NR6M cells or HC2 cells were treated with buffer or 150 nM ligand for 1 h at 0 °C, followed by cross-linking with 0, 15, 30 or 60 mM EDC for 45 min at room temperature or 20 min at 37 °C. The composite Figure shows representative results using 15 mM EDC, except for the lane marked with \* in which 60 mM EDC was used. The presence or absence of ligand (E, EGF; T, TGF- $\alpha$ ) is indicated. Note the ligand-dependent dimerization (approx. 360 kDa) of EGFRwt (NR6W), and the complete absence of receptor dimerization for EGFRvIII (NR6M, HC2).

any cross-linker concentration in either line (Figure 2B), even when the cross-linking volume was reduced fourfold in an effort to increase the effective concentration of cross-linker at the cell surface.

Dimerization of wild-type EGFR is more efficient at 37 °C than at 0 °C [27]. Furthermore the transforming effects of EGFRvIII were observed at 37 °C in both cell culture and animal models [10–12,29]. Thus we investigated the possibility that EGFRvIII might form temperature-sensitive dimers that were not stable at 0 °C. For wild-type EGFR, incubation at 37 °C not only increased ligand-induced dimerization, but also increased dramatically the level of constitutive dimerization (compare 1 mM lanes in Figures 2A and 2C). In contrast, variant receptors did not dimerize even at 37 °C (Figure 2D).

Although the amino acid targets for BS<sup>3</sup> are evenly distributed along the extracellular domain, it is possible that the region deleted in EGFRvIII coincidentally contained side chains cross-linked most efficiently after dimerization. Thus trying another cross-linker system was critical. Since several previous studies of EGFR dimerization used EDC, a cross-linker that is different from BS<sup>3</sup> in terms of amino acid targets, chemical mechanism, spacer distance and geometry, dimerization studies were



**Figure 4** Activation of intracellular signalling mediators PLC- $\gamma$ , Shc or MAPK by wild-type or variant receptors

(A) Serum-starved cells expressing EGFRvIII (NR6M) or EGFRwt (NR6W) were treated with 20 ng/ml (3.3 nM) TGF- $\alpha$  for 10 min. PLC- $\gamma$  was immunoprecipitated from cell lysates and subjected to immunoblot analysis. Blots were probed initially with anti-phosphotyrosine (anti-PY), then stripped and reprobed with anti-PLC- $\gamma$ . Note PLC- $\gamma$  (148 kDa) undergoes tyrosine phosphorylation only in cells expressing stimulated EGFRwt. (B) Serum-starved NR6W or NR6M was treated with 20 ng/ml TGF- $\alpha$  for 10 min. Proteins immunoprecipitated using either anti-Shc (left) or anti-EGFR (right) were subjected to Western-blot analysis using anti-phosphotyrosine (anti-PY) for immunodetection. Note the ligand-induced tyrosine phosphorylation of Shc (66 kDa isoform) precipitated from stimulated NR6W cells, and the constitutively high phosphorylation content of Shc precipitated from NR6M cells (left). Note co-precipitation of tyrosine-phosphorylated Shc in anti-EGFR immunoprecipitations (immpt.) of EGFRvIII and stimulated EGFRwt (right). Faint bands corresponding to EGFRvIII and EGFRwt were also observed in anti-Shc immunoprecipitants (left). The control lane represents immunoprecipitation performed in the absence of the specific primary antibody. (C) Serum-starved cells expressing EGFRwt (NR6W) or EGFRvIII (NR6M) were treated with 20 ng/ml TGF- $\alpha$  for 0, 10 or 20 min. MAPK was immunoprecipitated and analysed by immunoblotting. The blot was probed with anti-phosphotyrosine, then stripped and reprobed with anti-MAPK. The p44 and p42 forms of MAPK are indicated. Note the absence of MAPK phosphorylation in NR6M, in contrast with ligand-induced phosphorylation of MAPK in NR6W.

performed using 15–60 mM concentrations of this cross-linker. These concentrations of EDC resulted in stabilization of ligand-dependent dimers in NR6W cells (Figure 3C, left). However, neither NR6M nor HC2 cells exhibited receptor dimerization (Figure 3C, right).

#### Activation of intracellular signalling cascades

Since EGFRvIII did not appear to dimerize like stimulated EGFRwt, we investigated its ability to associate with intermediate signalling molecules. The Shc adapter protein and PLC- $\gamma$  are two SH2-domain proteins known to be involved in EGFRwt signalling cascades. In contrast with cells expressing ligand-stimulated EGFRwt, cells expressing the constitutively transforming EGFRvIII lacked evidence of PLC- $\gamma$  phosphorylation (Figure 4A). EGFRvIII was, however, constitutively associated with Shc, as evidenced by mutual co-immunoprecipitation. Moreover, the phosphotyrosine content

of Shc immunoprecipitated by anti-Shc and co-precipitated by anti-EGFR was actually higher in cells expressing EGFRvIII than in stimulated EGFRwt-expressing cells (Figure 4B). Although activation of the Shc/Grb2/Ras pathway usually results in phosphorylation of MAPK, which in turn phosphorylates transcription factors, there was no appreciable tyrosine phosphorylation of MAPK in EGFRvIII transfectants (Figure 4C). This distinct difference between cells expressing EGFRvIII and those expressing EGFRwt was observed using two separate pairs of transfectants, verifying that the absence of detectable MAPK phosphorylation was not a clonal artifact.

#### DISCUSSION

Cell surface EGFRwt transduces extracellular growth factor signals to initiate intracellular signalling cascades. Although immunohistochemical studies suggest cell surface expression of EGFRvIII in primary lung carcinoma isolates, glioblastoma biopsies and xenografts [4,5,9], and antibody-mediated endocytosis of EGFRvIII has been visualized in HC2 cells [30], EGFRvIII appears to be unregulated by extracellular ligands [10–12]. Nevertheless, it functions as an oncoprotein, transforming transfected cells through an unknown mechanism [10,12,29]. In this study, we compared receptor dimerization and other parameters of cellular activation in cells expressing either EGFRwt or EGFRvIII.

Both EGFRwt-expressing cell lines displayed effective ligand-dependent dimerization in response to either EGF or TGF- $\alpha$ . In addition, certain conditions promoted the detection of a similar cross-linked species in the absence of added ligand (Figures 2A and 2C), as was observed in earlier studies [26]. This probably reflects low-level constitutive EGFRwt dimerization rather than the presence of residual growth factors, since constitutive EGFRwt dimers were also observed in systems using column-purified EGFRwt [13]. Cross-linking of EGFRwt to other membrane proteins is also possible, although the consistent absence of minor bands in the present and previous dimerization studies renders this unlikely. The lack of any high-molecular-mass bands from EGFRvIII lysates also argues against a non-specific cross-linking artifact (Figures 2B and 2D, 3B and 3C), although high-molecular-mass bands of similar size have been reported [29] using a less specific polyclonal antiserum that cross-reacted with multiple 100–170 kDa bands [6].

It has been suggested that the two cysteine-rich subdomains of EGFRwt may stabilize receptor dimers [13,31]. One of these domains is largely deleted in EGFRvIII, and it was not possible to demonstrate either constitutive or ligand-regulated dimerization in cell lines expressing EGFRvIII (Figures 2B and 2D and 3B and 3C). This was not due to inaccessibility of receptor to cross-linker or inadequate receptor densities. Although incomplete processing with intracellular retention of EGFRvIII and EGFRwt has been reported in some transfectants [6,32], flow cytometry studies demonstrated adequate cell surface receptor numbers in all cell lines used in this study (Table 1). In fact, receptor density of non-dimerizing HC2 cells was greater than that of dimerizing NR6W cells. Since two cross-linkers with different chemical mechanisms and linker geometries yielded the same result, steric or chemical inaccessibility of potential cross-linking sites is unlikely, particularly since the amino acid targets for both cross-linkers were abundantly located along the entire extracellular domain. Therefore the deletion of a portion of the extracellular domain in EGFRvIII appears to be associated with loss of receptor dimerization.

It is unknown whether or not EGFRvIII can form heterodimers with the wild-type receptor. Heterodimerization

**Table 2 Comparison of activities associated with EGFRwt or EGFRvIII**

	EGFRwt resting	EGFRwt stimulated	EGFRvIII constitutive	References
Ligand binding	+		—*	10–12
Ligand-induced degradation	—	+	—	32
Antibody-induced endocytosis	+		+	30
Receptor dimerization	+	++	—	Figures 2 and 3
Phosphotyrosine content	++	+++	+	Figure 1(B), 10–12
Tyrosine kinase activity	+	++	+	12
Shc association	—	+	++	Figure 4(B), 34
Grb2 association	—	+	+	29
Ras-GTP loading	—	+	+/-†	29,34
MAPK kinase	—	+	+	35
MAPK	—	+	—‡	Figure 4(C), 29,35
PLC- $\gamma$	—	+	—	Figure 4(A)
STAT transcription factors	—	+	—	H.-J. Kung and K. D. Everiss, unpublished work
Cytoplasmic phospholipase A <sub>2</sub>	—	+	+	35
Cellular transformation	—	+	++	7,10,12,29

\* Minimal low-affinity binding is observed in some systems [12].  
† Conflicting results have been reported using different transfectants.  
‡ Dephosphorylated, with suppression of phorbol ester-inducible activity, although weak phosphorylating activity comparable with that of chronically stimulated EGFRwt may be seen [35].

has been reported among several other wild-type and mutant members of the EGFR family, often with distinct effects on receptor function (reviewed in [33]). There was no evidence for heterodimerization with other major cell surface constituents expressed on either NR6M or HC2 cell lines, although the low numbers of EGFRwt expressed by HC2 cells were probably below the detection limit. In any case, the fact that NR6M, a cell line that completely lacks EGFRwt, is transformed by EGFRvIII indicates that heterodimerization with wild-type receptor is not important to EGFRvIII-transforming activity.

Since even unstimulated EGFRwt displays low-level activities that are dramatically enhanced on ligand binding, we reasoned that finding similar low-level activity in the variant could not by itself explain its transforming potential. Instead, EGFRvIII ought to demonstrate activities comparable with stimulated EGFRwt to be considered constitutively activated. EGFRvIII, although not dimerized, is constitutively active in its association with intermediate signalling molecules such as Shc and its ability to mediate transformation (Table 2). Indeed, Shc immunoprecipitated from cells expressing EGFRvIII had a higher phosphotyrosine content than Shc immunoprecipitated from ligand-stimulated EGFRwt-expressing cells (Figure 4B). This correlates with the recent proposal that tumorigenesis mediated by EGFRvIII may proceed through the Shc/Grb2/Ras pathway [34].

Interestingly, MAPK was apparently not activated in transformed cells expressing EGFRvIII (Figure 4C), despite its involvement in the Ras pathway and its central role in transformation by ligand-stimulated EGFRwt. This suggests that phosphorylation of Shc is not sufficient to activate the Ras/Raf/MAPK kinase/MAPK pathway in EGFRvIII transfectants, or that an inhibitor is induced in these cells that interferes with the sustained activation of MAPK. This apparent down-regulation of MAPK has been observed in other studies of EGFRvIII [29,35]. It has been suggested that MAPK may be

dephosphorylated in EGFRvIII-transfected cells through induction of an unidentified phosphatase, and that the residual MAPK activity may be sufficient to induce transformation [35]. Although our data are in general agreement with this interpretation, the lack of MAPK phosphorylation in two EGFRvIII transfectants suggests that a MAPK-independent pathway may be responsible for transformation. Genetic dissection using dominant negative signalling mutants will be needed to test these hypotheses. Irrespective of the detailed mechanism, it is clear that MAPK activation is different in EGFRvIII and wild-type transformed cells. The observations that PLC- $\gamma$  (Figure 4A) and Stats 1, 3 and 5 (H.-J. Kung and K. D. Everiss, unpublished work) are not activated in EGFRvIII-expressing cells further support the notion that the oncoprotein EGFRvIII utilizes an altered spectrum of signalling cascades.

It is interesting to note that, although EGFRvIII is constitutively phosphorylated, the level of phosphorylation is much less than that of stimulated EGFRwt (Figure 1B) [12]. The fact that EGFRvIII nevertheless acts as a potent oncoprotein is not surprising since autophosphorylation sites are not essential for mitogenesis [36,37]. EGFRvIII may thus be differentially phosphorylated with respect to EGFRwt in accord with its function as a constitutively activated monomer, altering its substrate specificities and signalling pathways. An analogous situation can be found in the multiple endocrine neoplasia-associated mutations of the *RET* proto-oncogene. The RET-MEN2B mutant functions as an activated monomer, displaying differential autophosphorylation and substrate phosphorylation patterns relative to the dimerizing RET proteins [38]. Perhaps other less studied aspects of EGFRwt signalling may prove pertinent, as several dimerization-independent effects of EGFRwt ligation have been reported [39–43] and continuously stimulated EGFRwt loses the ability to dimerize [44].

Although receptor dimerization is believed to form a common theme in the normal regulated activities of several growth factor receptors, these studies indicate that dimerization is not essential for oncogenic transformation by EGFRvIII, a naturally occurring deletion mutant of EGFR. The immunoprecipitation experiments demonstrate that EGFRvIII interacts with the adapter protein Shc in a ligand-independent and constitutive manner. However, the lack of detectable tyrosine phosphorylation on MAPK or PLC- $\gamma$  indicates a diversion from the major wild-type signalling pathway. Although the full mechanism of transformation mediated by EGFRvIII remains to be elucidated, we have shown that it is not merely behaving like ligand-activated EGFR. Our observations suggest a different pathway for transformation by EGFRvIII from that proposed for EGF-stimulated overexpressed wild-type receptors.

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