

Polycythaemia-inducing mutations in the erythropoietin receptor (EPOR): mechanism and function as elucidated by epidermal growth factor receptor–EPOR chimeras

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Summary

Primary familial and congenital polycythaemia (PFCP) is a disease characterized by increased red blood cell mass, and can be associated with mutations in the intracellular region of the erythropoietin (EPO) receptor (EPOR). Here we explore the mechanisms by which EPOR mutations induce PFCP, using an experimental system based on chimeric receptors between epidermal growth factor receptor (EGFR) and EPOR. The design of the chimeras enabled EPOR signalling to be triggered by EGF binding. Using this system we analysed three novel EPOR mutations discovered in PFCP patients: a deletion mutation (Del1377-1411), a nonsense mutation (C1370A) and a missense mutation (G1445A). Three different chimeras, bearing these mutations in the cytosolic, EPOR region were generated; Hence, the differences in the chimera-related effects are specifically attributed to the mutations. The results show that the different mutations affect various aspects related to the signalling and metabolism of the chimeric receptors. These include slower degradation rate, higher levels of glycan-mature chimeric receptors, increased sensitivity to low levels of EGF (replacing EPO in this system) and extended signalling cascades. This study provides a novel experimental system to study polycythaemia-inducing mutations in the EPOR, and sheds new light on underlying mechanisms of EPOR over-activation in PFCP patients.

Keywords: myeloproliferative disorder, erythrocytosis, erythropoietin receptor, chimeric receptors.

Absolute erythrocytoses are rare disorders characterized by a significant increase of the red blood cell mass, which is reflected by an increased number of circulating erythrocytes, high haematocrit and haemoglobin (Lorberboym *et al*, 2005; Cario *et al*, 2013). Primary familial and congenital polycythaemia [PFCP, familial erythrocytosis type 1, Online Mendelian Inheritance in Man (OMIM) number 133100] is an inherited form of this disease, caused by erythropoietin (EPO) hypersensitivity of erythroid precursor cells (Sokol *et al*, 1995; Kralovics & Prchal, 2001; Maran & Prchal, 2004; Perrotta *et al*, 2010). Patients with PFCP have relatively low serum levels of EPO (Rives *et al*, 2007), normal leucocyte and platelet counts, hypersensitivity of erythroid progenitors to EPO and normal oxygen affinity to haemoglobin (Jedlickova *et al*, 2003; Maran & Prchal, 2004). The clinical symptoms of PFCP include headaches, muscle aches, dizziness, epistaxis and an increased risk of thrombotic and

haemorrhagic events leading to premature morbidity (Percy *et al*, 1998; Al-Sheikh *et al*, 2008; Perrotta *et al*, 2010). The common treatment for PFCP is phlebotomy (Perrotta *et al*, 2010).

A number of mutations in the cytosolic region of the EPO receptor (EPOR) have been found in family members suffering from PFCP (de la Chapelle *et al*, 1993). EPO is the major cytokine that induces erythropoiesis *via* growth, differentiation and maturation of erythroid progenitors (Budarf *et al*, 1990; Casadevall *et al*, 2002; Perrotta *et al*, 2010). EPO is produced mainly in the kidney under hypoxic conditions. It acts on erythroid progenitors in the adult bone marrow after binding to EPOR, a homodimeric transmembrane receptor. EPOR belongs to the superfamily of cytokine receptors (Bazan, 1989; Halaby & Mornon, 1998; Constantinescu *et al*, 1999; Watowich, 2011) and the mature protein is 508 amino-acids long (Smith *et al*, 2003; Perrotta *et al*, 2010).

EPOR lacks kinase activity, hence the signalling cascade is initiated through its association with Janus kinase 2 (JAK2). EPO binding to the extracellular domain of EPOR induces dimerization of the receptor and transphosphorylation of the associated JAK2 on both receptor monomers (Argetsinger *et al*, 1993; Hortner *et al*, 2002). JAK2 then phosphorylates tyrosine residues on EPOR which serve as docking sites for a variety of molecules, some of which participate in promoting signalling cascades and others serve as negative regulatory mediators (Argetsinger *et al*, 1993).

Most of the PFCP-inducing mutations in EPOR result in a C-terminal truncated receptor that lacks the negative regulatory domain (Kralovics & Prchal, 2001; O'Rourke *et al*, 2011). However, so far, no missense mutations have been identified as the cause of PFCP, despite of the fact that they are found in PFCP patients (Kralovics & Prchal, 2001; Gordeuk *et al*, 2005; Percy & Lee, 2008).

Our strategy was to explore the signalling and metabolism of such mutated EPORs in an *in vitro* system that is based on chimeric receptors which are constructed from the intracellular region of EPOR and the extracellular and transmembrane regions of the epidermal growth factor receptor (EGFR), a tyrosine kinase also known as HER1 or ErbB-1 (Yarden, 2001; Krampera *et al*, 2005; Normanno *et al*, 2006). Chimeric receptors containing EPOR or EGFR (Zou *et al*, 2008; Kuhns *et al*, 2010; Tachdjian *et al*, 2010; Cummings *et al*, 2013), as well as EGFR-EPOR chimeras (Yu *et al*, 2002; Buchse *et al*, 2006), were previously used as experimental tools to study specific receptor domains. Accordingly, EGF binding to the extracellular domain of the chimera triggers the EPOR signalling cascades. The working hypothesis was that the EPOR cytosolic region of the EGFR-EPOR chimera, when corresponding to mutations found in PFCP patients, would confer properties to the chimera that could explain the observed PFCP-associated manifestations. The constructed chimeras were transfected into the EPO-dependent UT7 erythroid cell line, which does not express EGFR and does not respond to EGF (data not shown). Thus, the differences in signalling and metabolism of the chimeric EGFR-EPORs, generated on the basis of the PFCF-associated EPOR mutations, could be discerned. The extracellular EGFR domain of the chimeras was HA-tagged, to facilitate immunological detection.

Here we report on using this experimental system to explore three novel EPOR mutations, found in PFCP patients, which were not previously explored (Fig 1):

- 1 C1370A: a nonsense mutation (c.1235C>A; p.(Ser412*)), found in a 73-year-old male with erythrocytosis (unknown if hereditary or not, because other family members were not reported) (Bento *et al*, 2013). The mutation results in the transformation of TCG to TAG, generating a stop codon, which leads to the truncation of 97 C-terminal amino acids of EPOR.
- 2 Del1377-1411: deletion of 35 nucleotides (c.1242_1276del; p.(Ser415Hisfs*18)). The mutation results in a frameshift,

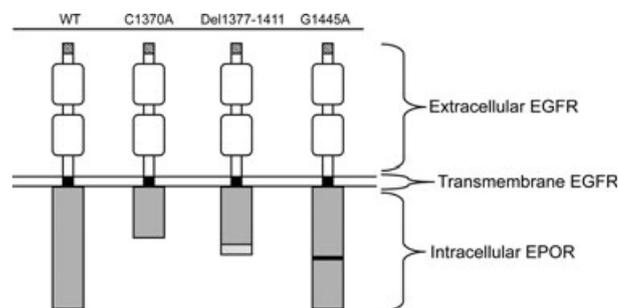


Fig 1. Schematic illustration of the wildtype (WT) and mutated EGFR-EPOR chimeras. Diagonal stripes: HA tag. White boxes: EGFR. Large white boxes in EGFR extracellular region: cysteine-rich sites. Black boxes: EGFR transmembrane region. Grey: WT EPOR intracellular domain. Light grey: amino acid alteration. Black horizontal line in G1445A: location of amino acid substitution. WT, wildtype; EGFR, epidermal growth factor receptor; EPOR, erythropoietin receptor.

alteration of 18 amino acids and truncation of 65 C-terminal amino acids. This mutation has not previously been reported.

- 3 G1445A: a missense mutation (c.1310G>A; p.(Arg437His)), discovered in two families with PFCP, one of them suffering also from Hereditary Spherocytosis (HS), a type of haemolytic anaemia characterized by sphere-shaped erythrocytes. This substitution results in the replacement of arginine 437 by histidine and has no effect on the size of EPOR. This mutation has not previously been reported.

Materials and methods

Cell culture

UT7 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 10 u/ml penicillin, 10 µg/ml streptomycin and 10 ng/ml EGF (Biotest, Solihull, UK) at 37°C and 5% CO₂.

Construction of the wild type (WT) EGFR-EPOR chimera

Human EGFR-HA in pcDNA3 was a gift from Prof. R. Pinkas-Kramarski (Tel Aviv University, Israel). Human EPOR was kindly provided by Prof. Y. Henis (Tel Aviv University, Israel) and cloned into pcDNA3. Primers: Extracellular region (5' end) of EGFR – sense 5'ATGCCACCCCTCCGGGACGGC 3'; Transmembrane region (3' end) of EGFR and Intracellular region (5' end) of EPOR – anti-sense 5' CCCGGCGGTGC ATGAAGAGGCCGATC 3'. Intracellular region (3' end) of EPOR – sense 5'CTAAGAGCAAGCCACATAGC 3' Intracellular region (5' end) of EPOR and transmembrane region (3' end) of EGFR – anti-sense 5' CCTCTTCATGCACCGCCGG GCTCTGA 3'.

Mutagenesis

Mutagenesis of the WT EGFR-EPOR chimera was performed using QuikChange Site-Directed Mutagenesis kit (Bio-Lab, Jerusalem, Israel). All primers were ordered from HyLabs (Rehovot, Israel).

UT7 transfection

UT7 cells were transfected using electroporation, as described (Ghaffari *et al*, 2003). The electroporation mixture contained 20 mmol/l PIPES, 128 mmol/l Glutamate, 10 μ mol/l Ca-acetate, 2 mmol/l Mg-acetate and 3:4 IMDM medium. Electroporation parameters were 300 mV, 20 ms. The electroporation device was an ECM 830 (BTX HARVARD APPARATUS, Holliston, MA, USA). Selection of the transfected clones was performed in the presence of 10 ng/ml EGF for at least 3 weeks.

EPOR signalling

Transfected UT7 cells were grown for 24 h in IMDM, in the absence of serum and growth factor (EGF or EPO). Cells were then collected and activated with 100 ng/ml EGF and lysed in lysis buffer containing 50 mmol/l Tris/HCl, 1% Triton, 150 mmol/l NaCl, 5 mmol/l EDTA and freshly-added Protease Inhibitor Cocktail (PIC; Roche Diagnostics, Basel, Switzerland).

Degradation assay

Transfected UT7 cells were incubated for different time intervals in IMDM in the absence of EGF and EPO, in the presence of 0.35 mmol/l cycloheximide (Sigma, St. Louis, MO, USA). Cells were then lysed, using the above-mentioned cell lysis buffer.

Antibodies

For Western blot analysis: anti-HA (MMS-101R; Covance, Princeton, NJ, USA), actin (MAB1501; Millipore, Billerica, MA, USA), pSTAT5 (9351; Cell Signaling, Danvers, MA, USA), pMAPK (4377; Cell Signaling) (which detects only pERK1/2), STAT5 (sc-836; Santa Cruz Biotechnology, Dallas, TX, USA), ERK (sc-154; Santa Cruz Biotechnology) were used. Anti-HA (MMS-101R; Covance) and fluorescein isothiocyanate (FITC)-conjugated Affinipure Goat anti-Mouse IgG (115-095-003; Jackson Immunoresearch, West Grove, PA, USA) were used for fluorescence-activated cell sorting (FACS) analysis.

Western blot analysis

Samples were loaded onto 7.5% polyacrylamide gels and separated *via* electrophoresis. Gels were transferred onto nitrocellulose membrane filters and probed with the relevant

antibodies. The enhanced chemiluminescence (ECL) method was used for detection (Pollard-Knight *et al*, 1990).

Flow cytometry

Transfected UT7 cells were washed in cold phosphate-buffered saline and incubated for 1 h with 1:100 anti-HA antibody. Cells were washed again and incubated for 1 h with 1:100 FITC-labelled anti-mouse antibodies. Fluorescence detection was performed by FACSsort flow cytometer [Becton Dickinson (BD), Franklin Lakes, NJ, USA] and results were analysed using WINMDI 2.8 Software (J.Trotter; free download).

Glycan-maturation

Cells were lysed in the whole-cell lysis buffer and incubated for 1 h with 500 units Endoglycosidase H (Endo H, p0703L; Bio-labs) at 37°C.

Cell viability

Transfected UT7 cells were seeded in triplicates in 96-well, flat-bottom culture plates (5×10^3 cells per well) and incubated at 37°C with EGF (at the indicated concentrations) for 0, 24, 48 and 72 h. Cell viability was determined using the colourimetric methyl-thiazole-tetrazolium bromide (MTT) (Sigma) assay (Gerlier & Thomasset, 1986). After incubation, 5 μ g/ml MTT was added to the cells and they were further incubated at 37°C for 3 h. The dye was completely solubilized with 0.04 N HCl in 2-propanol and plates were read at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader (SpectraMAX 190; Molecular Devices, Sunnyvale, CA, USA).

Results

Viability and growth patterns of cells expressing the EPOR mutations

To address whether the EPOR cytosolic mutations affect the sensitivity of UT7 cells, expressing the WT and chimeric receptors, to EGF, we performed the MTT cell viability assay (Fig 2). UT7 cells expressing chimeric receptors with EPOR portions derived from the PFCP patients exhibited different growth patterns than cells expressing the chimera containing the WT EPOR cytosolic domain (Fig 2). Cells expressing the mutated chimeric receptors were more viable, proliferated faster, and were responsive even to very low (0.01 ng/ml) EGF concentrations. In contrast, cells expressing the WT EGFR-EPOR chimera did not proliferate at all in the lowest EGF concentration, 0.01 ng/ml, while all other three cell lines, expressing the chimeras containing EPOR mutations from the PFCP patients, exhibited different growth patterns (Fig 2A). At 0.1 ng/ml EGF (Fig 2B) all three chimeras of

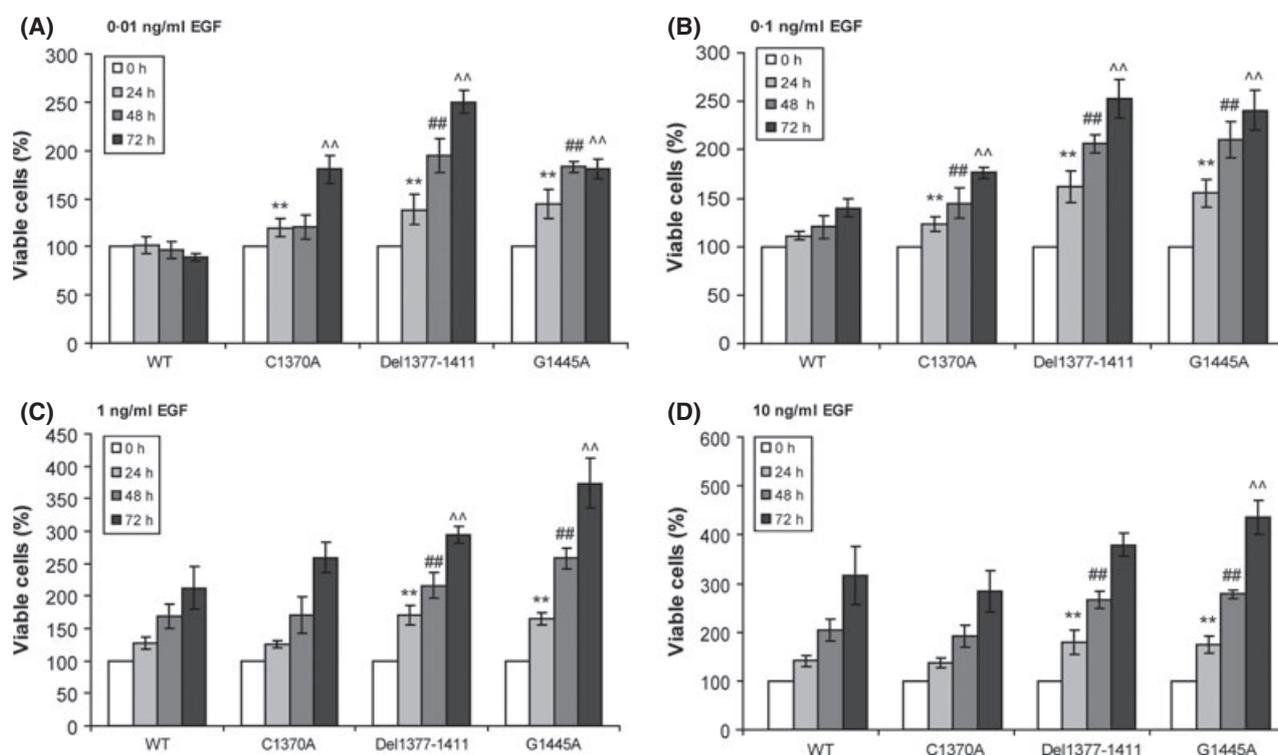


Fig 2. Viability of UT7 cells expressing EGFR-EPOR chimeras. UT7 cells, expressing WT or mutated EGFR-EPORs, were incubated for 0, 24, 48 and 72 h with the indicated concentrations 0.01 ng/ml (A), 0.1 ng/ml (B), 1 ng/ml (C) and 10 ng/ml (D) of EGF. Cell viability was determined using the MTT method. OD₅₇₀ nm at time 0 for each mutant was considered 100%. Proliferation of UT7 cells expressing each EGFR-EPOR chimera was calculated with respect to the corresponding 100% (time 0). Bars indicate mean \pm standard deviation, $N = 4$, each experiment was performed in triplicates. ** $P < 0.05$ compared to WT levels at 24 h, ## $P < 0.05$ compared to WT levels at 48 h, ^^ $P < 0.05$ compared to WT levels at 72 h. WT, wildtype; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPOR, erythropoietin receptor; MTT, methylthiazole-tetrazolium bromide; OD, optical density.

the EPOR PFCP mutants conferred significantly increased EGF-dependent growth, compared to WT, at all times. At 1 ng/ml EGF (Fig 2C), no difference in growth between WT and C1370A expressing cells was observed. Cells expressing the Del1377-1411 or G1445A chimeras showed higher levels of proliferation for all time periods tested. The proliferation rates of C1370A expressing cells at 10 ng/ml EGF (Fig 2D) were similar to those of cells expressing the WT chimera, while Del1377-1411 expressing UT7 cells proliferated at a higher rate for 24 and 48 h. However this difference disappeared after 72 h. Once again, G1445A expressing cells showed higher levels of proliferation at all times. The ability of the EGFR-EPOR chimeras to confer EGF-mediated proliferation was reflected in enhanced EGF-mediated proliferative responses of the chimeras, indicating the reliability of this experimental system. Notably, all mutated EGFR-EPOR chimeras conferred proliferation of UT7 cells even when cultured in the absence of growth factor (data not shown).

All three EPOR mutations increased the sensitivity of the cells to EGF, yet variations in the amplitude of responses between the chimeras were detected – thus pointing out distinct EGF-mediated growth capacity of the WT and mutated chimeras. Consequently, all EPOR mutations hereby tested

can be linked to higher proliferation rates of the cells, including the missense EPOR mutation (G1445A) which, to date, has not been linked to a polycythaemic phenotype.

Cell surface expression and glycan maturation of the EGFR-EPOR chimeras

Given that differences in the growth patterns of cells expressing the WT and mutated chimeric receptors have been observed, a panel of experiments was initiated in order to detect the possible mechanisms which attribute to the enhanced proliferation of cells expressing the EGFR-EPOR chimeras that harbour PFCP-associated EPOR mutations. These possible mechanisms include modulation of cell surface levels of the chimeras, changes in their metabolism (e.g. degradation, glycan maturation) and altered EGF-driven signalling cascades.

To measure the cell surface level of the chimeras under various EGF concentrations, flow cytometry analysis was performed (Fig 3). The results showed the following: (i) The cell surface levels of the WT EGFR-EPOR chimera following culture of the cells in 0.01 ng/ml EGF were significantly lower, compared to those on the surface of cells cultured in 10 ng/ml

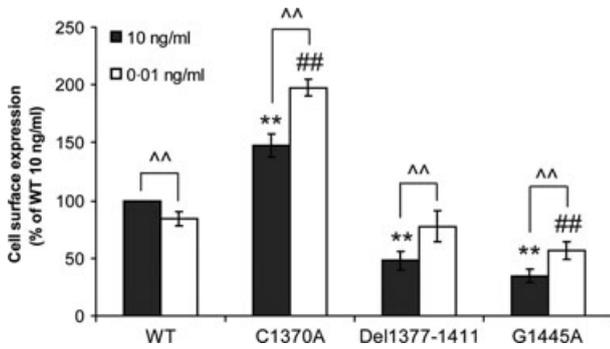


Fig 3. Levels of WT and mutated chimeric receptors on the cell surface. Cell surface levels of WT and mutated EGFR-EPOR were measured by flow cytometry analysis. Cells were grown on media containing 0.01 ng/ml or 10 ng/ml EGF for 24 h. Results were normalized to cell surface levels of WT chimera expressing cells grown in 10 ng/ml EGF. Bars indicate mean \pm standard error of the mean, $N \geq 3$. ** $P < 0.05$ compared to WT chimera with 10 ng/ml EGF, ## $P < 0.05$ compared to WT chimera with 0.01 ng/ml EGF, ^^ $P < 0.05$ comparison between surface levels of the chimeras at 10 ng/ml and 0.01 ng/ml EGF. WT, wildtype; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPOR, erythropoietin receptor.

EGF (Fig 3). (ii) The cell surface levels of EGFR-EPOR C1370A were higher when the cells were incubated in 0.01 ng/ml EGF as compared to their levels at 10 ng/ml EGF. At both high and low EGF concentrations, the cell surface levels of this EGFR-EPOR chimera were higher than those of the WT EGFR-EPOR. (iii) Lower levels of cell surface EGFR-EPOR Del1377-1411 compared to those of WT EGFR-EPOR were measured when the respective cells were grown in 10 ng/ml EGF. However, in 0.01 ng/ml EGF, the surface levels of EGFR-EPOR Del1377-1411 were elevated and approached those of the WT EGFR-EPOR chimera. (iv) G1445A expressing cells presented increased cell surface levels of the chimera at 0.01 ng/ml EGF, compared to the levels detected at 10 ng/ml EGF. Yet, at both EGF concentrations, the levels of G1445A were significantly lower than those of the WT EGFR-EPOR.

A possible explanation for the differences in cell surface levels of EGFR-EPOR in high and low EGF concentrations may be derived from a different pool of mature and immature chimeras in cells expressing the WT and mutated receptors. EndoH removes high mannose residues, which are a characteristic ER modification, thereby enabling the detection of ER-and-Golgi glycan immature and mature forms (Fig 4A). Lysates of the cells expressing the chimeras were subjected to EndoH digestion and Western blot analysis, and probed with anti-HA antibodies. Quantification of Western blots revealed that C1370A and Del1377-1411 have significantly higher levels of glycan mature chimeras and lower levels of glycan immature chimeras compared to the WT EGFR-EPOR, and G1445A is significantly less glycan-mature, as shown by the higher levels of immature chimera compared to WT EGFR-EPOR (Fig 4B).

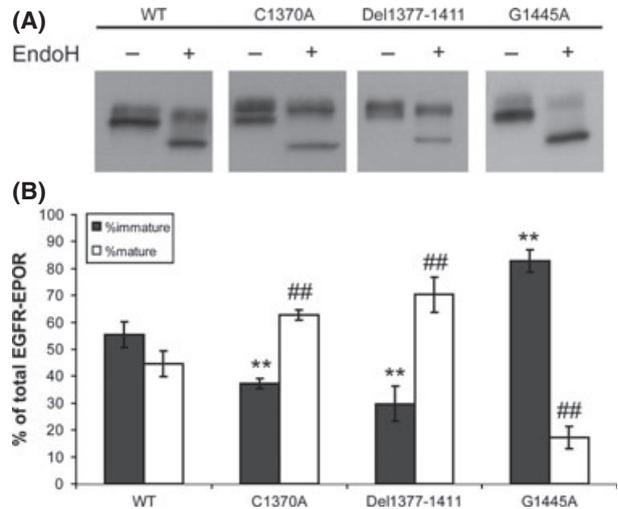


Fig 4. Glycan maturation of EGFR-EPOR chimeras. Sensitivity of the chimeric receptors to digestion by EndoH indicates their glycan maturation at steady state. (A) Representative Western blots of protein extracts from cells expressing WT, and mutated EGFR-EPOR chimeras. (B) Mean value of the quantifications of the Western Blots. Percentages of mature (upper band) and immature (lower band) forms of the chimeras were calculated with respect to the total amount of protein (the sum of mature and immature forms). Bars indicate mean \pm standard deviation, $N = 3$. ** $P < 0.05$ compared to the percentage of glycan-immature WT chimera. ## $P < 0.05$ compared to the percentage of glycan-mature WT chimera. WT, wild-type; EGFR, epidermal growth factor receptor; EPOR, erythropoietin receptor; Endo H, Endoglycosidase H.

Degradation of the EGFR-EPOR chimeras

To further elucidate the metabolism of the WT and mutated chimeric receptors, we measured the degradation rates of the chimeric receptors after inhibiting protein synthesis with cycloheximide for 0–5 h. The results showed the following: (i) Two chimeric receptors, C1370A and Del1377-1411 exhibited significantly slower degradation rates after 1, 2, 3 and 5 h or 2, 3 and 5 h respectively, compared to the WT chimera (Fig 5). (ii) G1445A did not exhibit any significant difference in degradation rates compared to WT EGFR-EPOR. A difference in the degradation patterns was also observed: (i) The WT and G1445A chimeras showed consistent degradation over the first 5 h with cycloheximide. (ii) C1370A and Del1377-1411 exhibited a considerably slower decline in the receptor levels.

Intracellular signalling mediated by the EGFR-EPOR chimeras

Another possible explanation for the enhanced proliferation of cells expressing the mutated chimeras might be attributed to extended signalling cascades triggered by the mutated forms of EPOR. Therefore, the kinetics of two signalling cascades, STAT5 and MAPK/ERK, activated upon EPO binding to EPOR, were explored. (i) STAT5 signalling was similar

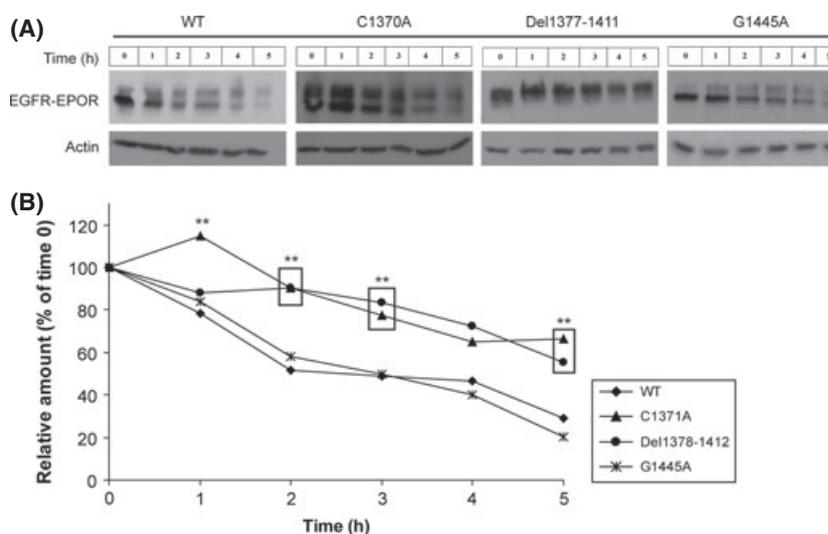


Fig 5. Degradation of EGFR-EPOR chimeras. UT7 cells stably expressing each of the three EGFR-EPOR chimeras were subjected to 0.35 mmol/l cycloheximide (CHX) for 5 h. Cell lysates were prepared at the indicated time intervals, and were further subjected to Western blot analysis with anti-HA antibodies. (A) Representative Western blots of protein extracts from cells treated with CHX. (B) Mean values of the quantifications of the Western blots. Degradation of each EGFR-EPOR chimera was calculated with respect to the corresponding time 0, which was considered 100%. $N \geq 3$. $**P < 0.05$ compared to WT levels at the same time. Boxes mark significantly different values. Standard errors of the means are not included due to visual constraints. WT, wildtype; EGFR, epidermal growth factor receptor; EPOR, erythropoietin receptor.

in cells expressing the WT and Del1377-1411 chimeras (Fig 6A–B). (ii) The basal level of phosphorylated-STAT5 (pSTAT5) in cells expressing C1370A was far higher than in cells expressing all other chimeric receptors, and higher levels of pSTAT5 were observed in cells expressing the C1370A chimera compared to cells expressing the WT chimera after 30–60 min. These results may be attributed to constitutive activation of the mutated chimeric receptor (Fig 6A–B), and correspond to our observation that cells expressing this mutated receptor show ligand-independent growth. (iii) This signalling cascade was delayed in cells expressing the G1445A chimera, but the activation and attenuation patterns were similar to the WT.

The ERK1/2 signalling cascade proved to be similar in WT, C1370A and Del1377-1411 expressing cells (Fig 6C–D), whereas the activation of this signalling cascade appeared to be delayed in cells expressing G1445A. However, higher levels of pERK persisted after 60 min in cells expressing G1445A than those in cells expressing the WT chimeric receptor. This result thus suggests also slower attenuation of this signalling cascade mediated by the G1445A substitution (Fig 6C–D).

Discussion

PFCP is characterized by the enhanced production of erythrocytes (Rives *et al*, 2007), and some PFCP cases have been attributed to mutations in the cytosolic region of the EPOR. The vast majority of these mutations result in a truncated receptor and the loss of its negative regulatory domain. (Kralovics & Prchal, 2001; O'Rourke *et al*, 2011).

Currently, the methods used to assess the mechanisms by which these EPOR mutations induce polycythaemia have been based on cells retrieved from PFCP patients or from polycythaemic mice (Maran & Prchal, 2004; Perrotta *et al*, 2010). As these cells do not readily grow and proliferate in culture, exploring EPO-driven signalling cascades in them is limited, and information derived from these methods is deficient. Furthermore, EPOR in mice and humans, though largely homologous, differ slightly in the amino acid sequence, hence mutations in a certain region in one receptor are not necessarily parallel to the same mutations in the other (Burns *et al*, 2002). Moreover, most of the studies regarding EPOR mutations have been performed on murine EPOR and/or on murine cell lines. In addition to the aforementioned differences between human and murine EPORs, some of these cell lines do not endogenously express EPOR and thus do not respond naturally to EPO.

Here we report on the generation of an experimental system based on chimeric human EGF-EPO receptors. The EPOR portion of these chimeras was derived from mutations found in EPORs of PFCP patients. The resulting chimeras were stably transfected into the EPO-dependent human erythroleukaemia UT7 cell line. This system enabled us to explore the mechanisms by which the EPOR mutations affect the cells, and showed that all three mutations could induce ligand-independent growth of cells expressing them and increase the sensitivity of cells expressing them to EGF, which replaces EPO in this system, and induce enhanced proliferation rates. It also enabled us to show, for the first time, that even missense mutations in the cytosolic portion of EPOR can confer enhanced proliferation.

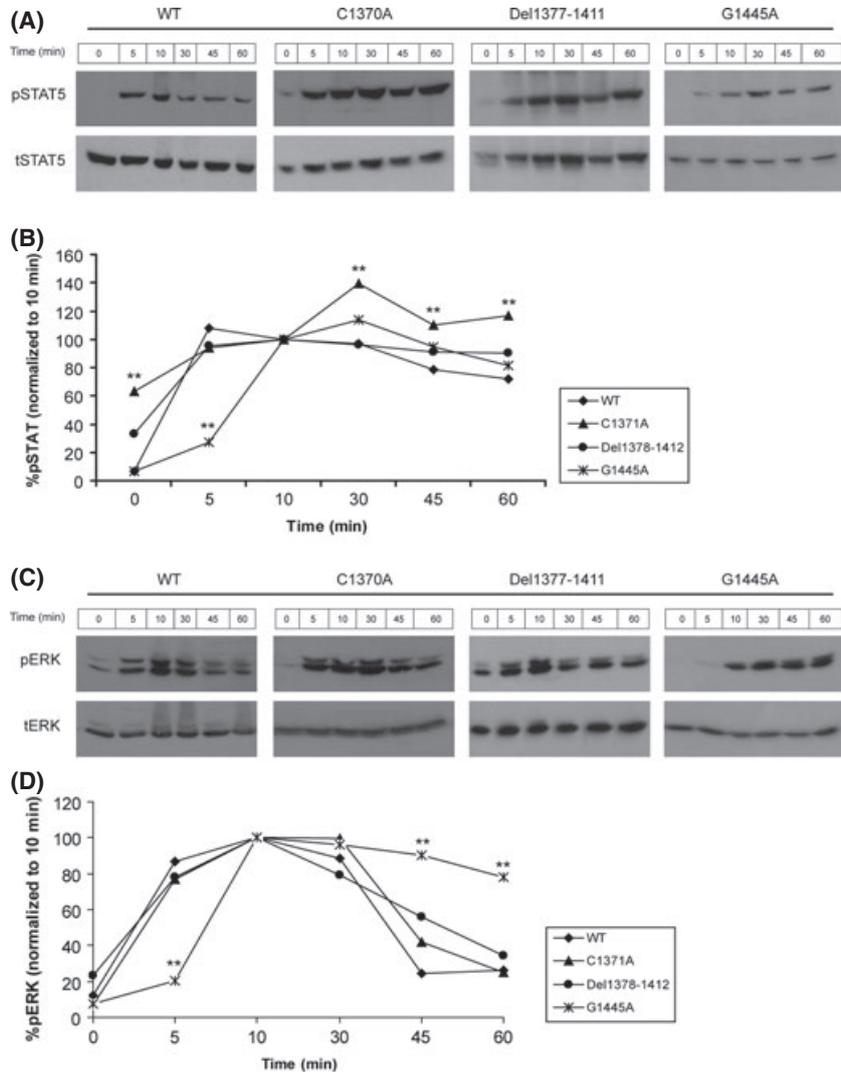


Fig 6. STAT5 and ERK signalling cascades triggered by EGFR-EPORs. Cells expressing the WT or mutated chimeras were EGF-starved for 24 h and subsequently activated with 100 ng/ml EGF for the indicated time periods. Cell lysates were subsequently subjected to Western blot analysis. (A) Representative Western blots showing pSTAT5 and STAT5 levels. (B) Quantification of pSTAT levels measured in the Western blots. (C) Representative Western blots showing pERK1/2 and ERK1/2 levels. (D) Quantification of pERK1/2 levels measured in the Western blots. Relative pSTAT or pERK levels of each EGFR-EPOR chimera was calculated with respect to the corresponding STAT or ERK levels respectively, at time 10 min, which was considered 100%. Graphs indicate mean values, $N \geq 3$. $**P < 0.05$ compared to WT levels at the same time points. Standard errors of the means are not included due to visual constrains. WT, wildtype; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPOR, erythropoietin receptor; t, total; p, phosphorylated; STAT5 Signal Transducer and Activator of Transcription 5; ERK, extracellular-signal-regulated kinase.

A unique property of the EPOR is its high intracellular retention and low surface expression levels (Ravid *et al*, 2007). In that respect, although the expression levels of all EGFR-EPOR chimeras were similar, the truncated chimeric receptors (C1370A and Del1377-1411) had an increased level of the glycan-mature form compared to both the WT chimera and the chimera harbouring the G1445A missense mutation. This may indicate that the shorter forms of the receptor fold better, and emphasize the role of the cytosolic region of EPOR in proper – or improper – folding of the protein. This phenomenon can also indicate different interactions of the WT and mutated EPOR cytosolic domains with the cytosolic transport proteins. In the case of the C1370A chimeric receptor, the higher levels of the glycan mature form were in correlation with higher cell surface levels in both high and low EGF concentrations, compared to WT chimeric receptors. These findings may indicate that in the case of the C1370A mutation, the enlarged pool of glycan mature receptors enables more mature receptors to be

integrated in the membrane at steady state and following stress, due to low levels of growth factor.

The differences in glycan maturation, however, cannot solely explain the differences observed in the cell surface levels of the WT and mutated chimeric receptors. Notably, higher surface levels of the chimeras may also indicate less endocytosis. In that respect, previous studies show that mutated EPORs which lack Tyr430, 432 and 480 do not internalize upon stimulation with EPO (Sulahian *et al*, 2009). The surface levels of the WT chimeric receptor declined when the cells were cultured in 0.01 ng/ml EGF, as opposed to increased levels at the low EGF concentration observed in the C1370A, Del1377-1411 and G1445A EGFR-EPORs. In addition, the MTT proliferation assay revealed that cells expressing WT chimera did not proliferate at 0.01 ng/ml EGF, while cells expressing all other three mutated receptors displayed proliferation under these conditions. Given that the increase in surface levels of the mutated chimeras at low EGF concentration is in correlation with increased EGF sensitivity of cells

expressing these mutated chimeras, the results may indicate more effective activation or initiation of signalling cascades of these mutated receptors.

Extended EPOR signalling often leads to increased cell proliferation. Most of the information obtained regarding the role of each phosphorylated tyrosine residue of EPOR was collected from experiments performed with murine EPOR. Despite the differences in the human and murine EPORs, analogous tyrosine residues are found on both receptors. Based on information retrieved from studies on murine EPOR, the following is known: (i) STAT5 binds to phosphorylated Tyr344 (pTyr344) and pTyr402 (Wojchowski *et al*, 1999) of the human EPOR, (ii) STAT1 and STAT3 associate with pTyr432 (Kirito *et al*, 2002), (iii) AP-1 transcription factor binds to pTyr344 and pTyr465 (Bergelson *et al*, 1998), (iv) shc-Grb2, which activate the MAPK cascade, bind mostly to pTyr465 though they can also be associated with other tyrosine residues (Barber *et al*, 1997) and (v) the p85 subunit of the PI-3Kinase binds to pTyr480 (Wojchowski *et al*, 1999). Simultaneously, negative regulatory mechanisms inhibit EPO-induced signalling, causing it to return its level to basal levels after 30–60 min of EPO stimulation (Kralovics & Prchal, 2001; Hortner *et al*, 2002). Among these negative regulatory molecules are SHP-1, which binds to pTyr430 and pTyr432, dephosphorylates JAK2 and shuts down the signalling cascades triggered by EPOR (Maran & Prchal, 2004; Minoo *et al*, 2004), and SOCS3, which acts as E3 ubiquitin ligase (Hortner *et al*, 2002) and down-regulates EPOR-induced signal transduction.

Here we examined two signalling cascades, STAT5 and MAPK/ERK, which were both activated by all the chimeric receptors. One of the chimeric receptors, C1370A, lacks all tyrosine residues apart from the first one, Tyr344. Given that phosphorylation of ERK was observed in cells expressing the EGFR-EPOR bearing this mutation, following activation by EGF, we have concluded that, apart from its known role in STAT5 binding, pTyr344 it is also able to facilitate the MAPK/ERK signalling cascade. C1370A was found to be constitutively active, because levels of pSTAT5 were detected even after 24 h of serum and EGF starvation (time 0, Fig 6A–B). This finding correlates with our observation that, unlike cells expressing the WT chimera, cells expressing EGFR-EPOR C1370A display ligand-independent growth (data not shown). This may be explained by the shorter form of the receptor and the lack of most of the tyrosine residues that are involved in the negative regulation of the receptor (Sokol *et al*, 1995; Kralovics *et al*, 1997; Perrotta *et al*, 2010), and is, to date, the first description of a mutation in the cytosolic region of EPOR which confers constitutive activation of STAT5 signalling. The MAPK/ERK cascade appears to be similar in the WT and the mutated EGFR-EPOR chimeras despite a slower attenuation of the signal conferred by the G1445A chimera. This prolonged activation of the MAPK/ERK cascade may be one of the underlying mechanisms responsible for the

elevated proliferation rates displayed by cells expressing chimeras bearing that missense mutation. In addition, a different activation pattern has been observed in G1445A for both STAT5 and MAPK/ERK signalling cascades. Their activation is delayed and lower levels of phosphorylated STAT5 or ERK are observed after 5 min of EGF stimulation, compared to their levels in cells expressing the WT chimera at the same time.

The proteasome complex prevents the renewal of surface EPOR by inhibiting the insertion of newly-synthesized EPOR into the cell membrane (Verdier *et al*, 2000). The main signal for proteasomal degradation is lysine-related ubiquitination. The cytosolic region of the murine EPOR contains 5 lysine residues (Yosha *et al*, 2011). The human EPOR cytosolic domain contains 6 lysines at positions 255, 257, 277, 349, 389 and 429. Two of the EPOR mutations, C1370A and Del1377-1411, lack only Lys 429, but C1370A lacks also Lys389. This latter EPOR mutant displays a slower degradation rate, implying possible involvement of the proteasome-related degradation pathway.

In summary, the EGFR-EPOR chimeras enable us to explore mechanisms underlying PFCP-inducing mutations in the cytosolic region of EPOR at a better resolution than currently available. We have found that all the three novel EPOR mutations increased the sensitivity of UT7 cells to low growth factor concentration and conferred enhanced proliferation. Each mutation influenced unique aspects regarding signalling and metabolism of the corresponding EGFR-EPOR chimera. Despite the clustered proximity of the mutations, they all induced the polycythaemic phenotype, yet *via* different mechanisms.

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Authorship contributions

DN was the principal investigator; MG performed the research, analysed the data and wrote the paper; NBC constructed the chimeras; DN, MG and NBC interpreted the data; MFM, MJP, CB, HC and MM identified the EPOR mutations and reviewed the manuscript.

Conflict of interest

The authors report no potential conflicts of interest.

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