Extracellular point mutations in FGFR2 result in elevated ERK1/2 activation and perturbation of neuronal differentiation

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INTRODUCTION

FGFRs [FGF (fibroblast growth factor) receptors] are receptor tyrosine kinases that play important roles in various cellular processes, such as mitogenesis, growth regulation, differentiation and specific developmental processes. FGFRs contain an extracellular ligand-binding region, a single membrane-spanning region and an intracellular region containing the tyrosine kinase domain (reviewed in [1–3]). The FGFR family comprises four different genes (fgfr1–4), which are alternatively spliced to create numerous isoforms that portray some tissue-specific expression (reviewed in [4]). Upon ligand binding in the presence of auxiliary glycosaminoglycans (such as heparan sulfate), these receptors autophosphorylate intracellular tyrosine residues. This phosphorylation event facilitates the recruitment of numerous signalling proteins [5,6] which subsequently activate various signalling pathways downstream of the FGFR, including activation of the ERK1/2 (extracellular-signal-regulated kinase 1/2) pathway.

Two independent gain-of-function point mutations (S252W and P253R) in the extracellular region of the FGFR2 (fibroblast growth factor receptor 2) increase the binding affinity for the growth factor. The effect of this enhanced growth factor binding by these mutants is expected to be an increase in activation of regular signalling pathways from FGFR2 as a result of more receptors being engaged by ligand at any given time. Using PC12 (pheochromocytoma) cells as a model cell system we investigated the effect of these mutations on protein phosphorylation including the receptor, the activation of downstream signalling pathways and cell differentiation. Our results show that the effects of both of these extracellular mutations have unexpected intracellular phenotypes and cellular responses. Receptor phosphorylation was altered in both the ligand-stimulated and unstimulated states. The mutants also resulted in differential phosphorylation of a number of intracellular proteins. Both mutations resulted in enhanced ERK1/2 (extracellular-signal-regulated kinase 1/2) activation. Although ERK1/2 activation is believed to transduce signals resulting in cell differentiation, this response was abrogated in the cells expressing the mutant receptors. The results of the present study demonstrate that single extracellular point mutations in the FGFR2 have a profound effect on intracellular signalling and ultimately on cell fate.

Key words: Apert syndrome, differentiation, extracellular-signal-regulated kinase 1/2 (ERK1/2), fibroblast growth factor receptor 2 (FGFR2), green fluorescent protein (GFP), PC12 cell.

Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; GFP, green fluorescent protein; Grb2, growth-factor-receptor-bound protein 2; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NGF, nerve growth factor; PI3K, phosphoinositide 3-kinase.

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EXPERIMENTAL

Materials

FGF9 was from R&D systems and NGF (nerve growth factor) was from Sigma. The anti-(phospho-ERK1/2) antibody was purchased from Cell Signaling Technology, anti-ERK2 antibody was from Santa Cruz, anti-GFP (green fluorescent protein) antibody was from Rockland and the anti-Grb2 (growth-factor-receptor-bound protein 2) antibody was from BD Biosciences. Horseradish-peroxidase-conjugated secondary antibodies were obtained from Sigma. The biotinylation kit for cell-surface protein isolation was purchased from Pierce.

Constructs

The cDNAs encoding FGFR2(IIIc) and FGFR2(IIIc)-S252W-S252W-S252W-S252W-S252W were gifts from John Heath (School of Biosciences, University of Birmingham, Birmingham, U.K.) and were PCR-amplified and cloned in-frame into the pEGFP vector (Clontech). The P253R mutation was introduced using the QuickChange® mutagenesis kit (Stratagene) with appropriate primers encoding the single base-pair change according to the manufacturer’s protocol.

Cell culture

Parental PC12 cells (ECACC accession number 88022401) were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 5% foetal bovine serum and 10% horse serum. Prior to stimulation, cells were serum-starved for 18 h in DMEM containing 0.1% horse serum. Cells were individually transfected with the three receptor constructs by electroporation (300 V, 960 μF in PBS in a 4 mm electroporation cuvette) and stable cells were selected in 800 μg/ml G418 for 2 weeks with regular medium changes. Individual clones were isolated by dilution cloning in 96 well plates and screening for fluorescence.

Membrane fractionation and cell-surface biotinylation

Membrane fractionation was carried out as described previously [15] using three 10 cm dishes per sample. Biotinylation and isolation of cell-surface proteins was carried out using the Pierce Cell Surface Protein Isolation Kit. Cells (1 x 10^6) were treated according to the manufacturer’s protocol; however, the biotinylation time was increased to 90 min and the cell lysate was incubated with the immobilized NeutrAvidin gel for 2 h at 4 °C.

Stimulation, differentiation and proliferation assays

For Western blot analysis, cells were seeded on tissue-culture dishes coated with poly-D-lysine and serum-starved overnight. Cells were stimulated with 10 ng/ml FGF9 in the presence of 1 μg/ml heparan sulfate, lysed in 20 mM Tris/HCl (pH 7.5), 138 mM NaCl, 1 mM EGTA, 20 mM β-glycerophosphate, 10% (w/v) glycerol, 1 mM sodium orthovanadate and 20 mM sodium fluoride, supplemented with 1% (v/v) protease inhibitor cocktail III (Calbiochem) and subjected to SDS/PAGE and Western blotting (Figure 1A). Those expressing similar amounts of each receptor (wild-type-C6, S252W-C2, P253R-C3) were selected for further experiments after determining that cellular responses did not vary between clones (results not shown). Confocal imaging revealed that all three GFP-tagged receptors localized primarily to the plasma membrane. A small amount of receptor was observed in intracellular compartments presumably undergoing biosynthetic processing (Figure 1B).

Despite the total expression levels being similar in all three cell lines (Figure 1A), to confirm that any alterations in signalling from the wild-type and mutant receptors were not due to differential expression at the cell surface we performed subcellular fractionation and biotinylation followed by isolation of membrane

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proteins (Figures 1C and 1D). Subcellular fractionation and biotinylation experiments revealed that all three receptors are located in the membrane, thereby confirming correct post-translational targeting of the mature receptor. Separation of the receptors expressed at the cell surface from those located within the cell interior using biotinylation revealed that the overall expression of the three different receptors in the plasma membrane was comparable (Figure 1D).

The two mutant FGFR2s are differentially phosphorylated

PC12 cells were stimulated with FGF9 to analyse changes in overall cellular tyrosine phosphorylation. FGF9 is specific for FGFR2(IIC) [19,20] and does not activate the endogenously expressed FGFR1 [21]. Since PC12 cells do not express FGFR2 [21], the use of FGF9 therefore allows more specific activation of the expressed FGFR2–GFP constructs. Previous reports have shown that FGF9 binds to the mutant receptors with greater affinity [7,8]. In addition it has been reported that FGF9 induces mitogenesis to a greater extent in cells expressing the mutant receptors compared with the wild-type FGFR2 [22]. An increase in receptor phosphorylation would generally be assumed to be the result of the increased affinity, since the overall population of receptors which are activated is increased.

Assessment of FGFR2 phosphorylation in the presence and absence of the two mutations revealed that all three variants of the FGFR2 displayed a stimulation-dependent increase in phosphorylation (Figure 2), confirming that the GFP tag did not interfere with the kinase activity of the receptor. Interestingly, a straightforward increase in ligand-stimulated receptor phosphorylation that is concomitant with the reported increase in ligand binding was not observed (Figure 2). All three receptors displayed high basal phosphorylation. Interestingly, the level of basal receptor phosphorylation was lower in the presence of the S252W mutation, whereas it was most prominent in the presence of the P253R mutation. This indicated that the mutations have more diverse effects on the FGFR2 than simply resulting in an increased ligand affinity. It was further noticeable that whereas phosphorylation of the wild-type FGFR2 did not increase further upon prolonged exposure of cells to FGF9, both mutant receptors showed increased receptor phosphorylation up to 60 min (Figure 2). This observation is commensurate with the increased ligand binding affinity of the mutant receptors. This increased receptor phosphorylation at later time points is likely to affect recruitment of signalling proteins to the FGFR2 and subsequent activation of downstream signalling pathways.

Effects of the mutations on FGFR2 signalling pathways

Several proteins were found to be differentially tyrosine phosphorylated in cells expressing the S252W or P253R receptors...
using densitometry and the data were represented graphically. The S.E.M. calculated from two independent experiments is indicated by error bars.

(200 µg level of P253R receptor phosphorylation (Figure 2B). The S252W cells to FGF9 (Figure 3). This effect mirrored the grossly increased phosphorylation, which increased with prolonged exposure of and 3C). The P253R mutant showed the highest level of ERK1/2 greatly enhanced in cells expressing the mutants (Figures 3B and 3C). The P253R mutant showed the highest level of ERK1/2 phosphorylation, which increased with prolonged exposure of cells to FGF9 (Figure 3). This effect mirrored the grossly increased level of P253R receptor phosphorylation (Figure 2B). The S252W receptor also caused enhanced activation of ERK1/2 at all time points observed, which is interesting in light of the much lower receptor phosphorylation in cells expressing the S252W receptor compared with the P253R receptor. Additionally, the onset of ERK1/2 phosphorylation was faster in cells expressing either of the mutant receptors compared with the wild-type (Figure 3B; 3 min).

Expression of constitutively active MEK1 or Ras has been shown to be sufficient for induction of neurite outgrowth, and the ERK1/2 pathway is therefore a major determinant of the decision as to whether to undergo proliferation or differentiation in PC12 cells. The PI3K (phosphoinositide 3-kinase) pathway is also activated downstream of the FGFR; however it has been shown to be more important for cell survival than differentiation in PC12 cells [24,25]. Assessment of the phosphorylation levels of Akt revealed minor differences in the activation of PI3K in cells expressing the wild-type FGFR2 or one of the two mutant receptors (Figure 3B). Whereas the levels of Akt phosphorylation seemed relatively unchanged in cells expressing the S252W mutant FGFR2, its phosphorylation was slightly up-regulated upon FGF9 stimulation of cells expressing the P253R receptor. This mirrors the changes in FGFR2 phosphorylation in the presence of the two mutations to some extent and further highlights the various effects that the S252W and P253R mutations have on signalling from the FGFR2.

Decreased neurite outgrowth in response to FGF9 in the presence of the mutations

It is commonly accepted that the ERK1/2 pathway plays a major role in differentiation of PC12 cells and it has been shown that sustained ERK1/2 activation is required and sufficient for the stimulation of neurite outgrowth [26,27]. We therefore investigated whether the enhanced ERK1/2 activation in the

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**Figure 3** Effect of the S252W and P253R mutations on ERK1/2 activation

(A) Untransfected PC12 cells were stimulated with 10 ng/ml FGF9 and 1 µg/ml heparan sulfate for 10 min or left unstimulated following overnight serum-starvation and lysed. Whole cell lysate (200 µg) was subjected to SDS/PAGE and immunoblotting with an anti-(phospho-ERK1/2) antibody. The membrane was stripped and re-probed with an anti-ERK2 antibody as a loading control. (B) PC12 cells expressing the wild-type (WT), S252W or P253R FGFR2 were stimulated with FGF9 and heparan sulfate for various time periods as indicated and subjected to SDS/PAGE and immunoblotting with anti-(phospho-ERK1/2) and anti-(phospho-Akt) antibodies. The membrane was stripped and re-probed with anti-ERK2 and anti-GFP antibodies as loading and receptor expression controls respectively. (C) The amount of phospho-ERK1/2 following stimulation of the wild-type (solid line), S252W (broken line) or P253R (dotted line) FGFR2 in (B) was quantified using densitometry and the data were represented graphically. The S.E.M. calculated from two independent experiments is indicated by error bars.
Effect of extracellular mutations on FGFR2 signalling

Mutant-expressing cells could lead to increased neurite extension. Strikingly, our results revealed the opposite effect. In cells expressing the S252W or P253R FGFR2, differentiation was impeded, whereas cells expressing the wild-type receptor underwent rapid FGF9-stimulated differentiation which was evident within 24 h (Figure 4). The cells expressing the P253R receptor were able to induce formation of some neurites, but despite the greatly enhanced ERK1/2 activation, cells were not able to reach neurite maturation stages. By 48–72 h of FGF9 stimulation many cells were in the early stages of differentiation (i.e. flattened, less round and with short projections), but could not progress to form extended neurites even after 120 h of FGF9 stimulation (Figure 4B). Barely any neurite extension was evident in S252W-FGFR2-expressing cells even after 72 h of FGF9 stimulation (Figure 4B). Activation of the S252W receptor did not even induce flattening of the cells, which remained round and loosely attached to the surface. The ability of the wild-type FGFR2 to induce neurites was comparable with the response of untransfected cells to NGF stimulation. However, the neurites induced by FGF stimulation were less stable and retracted with prolonged exposure (beginning at 72 h), whereas NGF-stimulated outgrowth was sustained up to 120 h. It is unclear why this occurred, but seems to be in accordance with a previous report indicating decreased stability of FGF-induced compared with NGF-induced neurites [13].

To assess whether instead of initiating neurite outgrowth, cells expressing the mutant receptors were undergoing proliferation, the number of viable cells present was assessed using an MTT cell-proliferation assay following stimulation of cells with FGF9 for 24 or 72 h (Figure 5). Somewhat surprisingly, no difference was observed between any of the cell lines assessed, and all cells showed a similar increase in cell number (which is indicative of similar levels of proliferation). It is unclear why even those cells that began to differentiate after 24 h (i.e. cells expressing the wild-type FGFR2 stimulated with FGF9 and untransfected PC12 cells stimulated with NGF) showed similar levels of proliferation. This may be due to the residual horse serum contained in the differentiation medium. More importantly however, cells expressing either the S252W or the P253R FGFR2 did not show increased levels of cell proliferation despite portraying enhanced activation of the ERK1/2 pathway.

DISCUSSION

Mutations in the FGFR, including S252W and P253R in the FGFR2, have been previously described as gain-of-function when acting through various mechanisms such as constitutive activation, illegitimate splicing or, as in the case of the mutations used in the present study, through increased affinity and altered selectivity in ligand binding [12,28]. This term implies that the S252W and P253R mutations simply result in enhanced activation of the regular signalling pathways as a result of a greater number of receptors being engaged by ligand at any given time. In contrast, our results show that the presence of the mutations results in a more complex intracellular phenotype than can be described by simple up-regulation of FGFR2 signalling.

Our results suggest that the extracellular point mutations affect receptor phosphorylation in both the unstimulated and the FGF9-stimulated state. Although in the basal state the S252W receptor was found to be less phosphorylated than the wild-type FGFR2, phosphorylation of the P253R receptor was significantly more pronounced. These observations show that the two mutations not only affect ligand binding extracellularly, but also appear to alter the receptor intracellularly to result in differential tyrosine phosphorylation.

It is also particularly interesting to note that both mutant receptors displayed a further increase in tyrosine phosphorylation after prolonged (60 min) stimulation compared with the

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**Figure 4** Effect of mutations on neurite formation and differentiation

Untransfected PC12 cells, or those expressing the wild-type, S252W or P253R FGFR2 were seeded on to poly-<i>N</i>-lysine-coated plates, allowed to attach for 24 h with a subsequent change to differentiation medium containing 10 ng/ml FGF9 and 1 μg/ml heparan sulfate or 100 ng/ml NGF. **(A)** Pictures of the cells were taken as indicated. **(B)** At least 500 cells were scored for neurite outgrowth in each condition. The average data from two independent experiments (one for NGF stimulation) is represented as the percentage of cells that contained neurites more than two cell diameters in length. The S.E.M. is indicated as error bars. Wild-type (WT), solid line; S252W, dashed line; P253R, dotted line; PC12 FGF9, grey triangles; PC12 NGF, circles.

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experiments. Error bars represent the S.E.M. the percentage increase from point zero. Similar trends were observed in two independent 
100 ng/ml NGF. The relative number of viable cells was assessed using an MTT cell-proliferation 
μ change to differentiation medium containing 10 ng/ml FGF9 and 1 
g/ml heparan sulfate or

Activated receptors, then all FGFR2-induced signalling pathways 
affected by the presence of both the S252W and the P253R (Z. Ahmed, A.C. Schüller 
and J.E. Ladbury, unpublished work) suggesting that the early 
signalling complex recruited to the receptors are fundamentally 
and PI3K pathway remained relatively unaffected by the presence of the mutations, although slight 
changes in Akt phosphorylation in the three different cell lines could also be observed. Furthermore, analysis of total tyrosine 
phosphorylation of proteins revealed that not all proteins were 
phosphorylated to a greater extent in cells expressing the mutant 
receptors when compared with the wild-type receptor (Figure 2, 
as indicated by arrows). These observations further support the 
notion that the effects of these mutations on the receptor and 
resulting downstream response are more complex than a simple 
gain-of-function effect as described previously.

The mutant-receptor-induced up-regulation of the ERK1/2 
pathway was not sufficient to result in differentiation of PC12 
cells, further demonstrating that both mutations have profound 
effects on the response of cells to FGF stimulation. The up-
regulation of the ERK1/2 pathway is in agreement with the pre-
viously reported increase in mitogenesis in cells expressing the 
S252W FGFR2 [22] and seems to reflect directly the increased 
ligand-binding affinity of this receptor. However, the fact that 
avtivation of this pathway led to neither PC12 cell differentiation 
or increased proliferation in cells expressing the S252W or 
P253R receptors indicates that activation of other pathways 
downstream of the FGFR2 must render these cells incapable of 
the expected phenotypic response.

Previous studies have implicated prolonged ERK1/2 activation 
as sufficient to cause neurite outgrowth in this cell line [26,30,31]. 
Overexpression of, for example, the EGFR (epidermal growth 
factor receptor) and the insulin receptor, which also lead to 
enhanced and prolonged ERK1/2 activation is able to induce PC12 
cell differentiation [32,33]. The present observation therefore 
not only indicates that the S252W and P253R mutations must 
 affect other FGFR2-activated signalling pathways that contribute 
to the observed lack of neurite outgrowth, but also show that 
the commonly accepted dogma that prolonged ERK1/2 activation 
is sufficient for differentiation [27] may not be correct. It is likely 
that more complex control mechanisms are in place to provide the 
changes that prevent neurite outgrowth despite enhanced levels of 
ERK1/2 activation. For example, we have shown that recruitment 
of the docking protein FR52 to FGFR2 is altered in the pre-
sence of both S252W and the P253R (Z. Ahmed, A.C. Schuller 
and J.E. Ladbury, unpublished work) suggesting that the early 
signalling complex recruited to the receptors are fundamentally 
affected by the extracellular mutations. Alterations in the 
recruitment of proteins to the receptor, probably as a result of 
the altered receptor phosphorylation, would be expected to affect 
not only one signalling pathway, but the activation and interplay of 
all FGFR2-activated pathways. Further investigation is required to 
determine the mechanism by which neurite outgrowth is prevented 
response to activation of the two mutant receptors despite the 
enhanced activation of the ERK1/2 pathway.

In conclusion, we have shown the importance of the recognition 
of extracellular ligands to the specific signal generation 
downstream of tyrosine kinase receptors such as the FGFR2. This 
has a profound effect on maintenance of the integrity of cell-
ular responses to a stimulus which are reflected in the ultimate 
cellular phenotype such as neuronal differentiation. By perturbing 
the normal signal generation from the FGFR2, the S252W and 
P253R mutations result in enhanced ERK1/2 activation. However, 
changes in signal activation downstream of the FGFR2 were found 
to prevent differentiation of PC12 cells despite this enhanced 
ERK1/2 activation.

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