bFGF and aFGF induce membrane ruffling in breast cancer cells but not in normal breast epithelial cells: FGFR-4 involvement

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Acidic and basic fibroblast growth factors (aFGF and bFGF) are growth factors which may have a physiological role in the normal breast and in breast cancer. A study of the effects of aFGF and bFGF on a variety of breast cell lines and epithelial cells purified from normal breast organoids showed that whereas normal breast cells did not exhibit membrane ruffling in response to either of these growth factors, some breast cancer cell lines did. This difference was not due to lack of receptor since all the cell lines tested were mitogenically stimulated by bFGF. Dominant negative mutations of FGF receptor 3 (FGFR-3) and the small GTP-binding protein p21rac inhibited membrane ruffling, showing that receptor dimerization and phosphorylation and p21rac activation are prerequisites for membrane ruffling in response to aFGF and bFGF. Transient transfection of individual FGFRs into cos-7 cells showed that FGFR-1, FGFR-2 and FGFR-3 could not mediate a membrane ruffling response whereas FGFR-4 could. These studies elucidate one signalling mechanism of FGF and point to differences in the response of normal and cancer breast epithelial cells which may be important in cell motility.

INTRODUCTION

The fibroblast growth factors (FGFs) belong to a family of growth regulatory proteins which induce the proliferation and differentiation of a wide range of cells of epithelial, mesodermal and neuroectodermal origin [1–4]. Nine members of the FGF family have been identified, sharing 35–50% homology. Of these, int-2, hst/K-FGF, FGF-5, FGF-6 and KGF are synthesized with an N-terminal hydrophobic signal sequence leading to the normal secretory pathway, whereas acidic FGF (aFGF) and basic FGF (bFGF) have no such secretory signal [5,6]. There is evidence for release of aFGF and bFGF possibly through leakage from damaged cells but perhaps from viable cells through a novel mechanism [7–9].

The response of cells to FGF is mediated through formation of a ternary complex of growth factor, proteoglycan and high-affinity receptor [10–14]. A family of tyrosine kinase receptors which bind to FGFs including FGF receptor 1 (FGFR-1) [15,16], FGFR-2 [16–18], FGFR-3 [19] and FGFR-4 [20] has emerged. There is extensive diversity within the high-affinity FGF receptors with splice variation producing alternative exon usage and truncated forms of receptor [21–25].

bFGF and aFGF are both present in the breast. bFGF has been localized to the myoepithelial cells of normal breast by immunocytochemistry of paraffin sections [26]. bFGF has also been detected in breast cancer cell lines by Western blotting and immunocytochemistry (Bansal, G. S., Yiangou, C., Coope, R., Gomm, J. J., Johnston, C. L. and Coombes, R. C., unpublished work). aFGF is released from breast cancer biopsies and can be detected by Western blotting and a soft-agar bioassay [27]. Receptors for aFGF and bFGF are found in breast cancer cells. FGFR-1 and FGFR-2 were detected in normal epithelial and breast cancer cell lines by PCR of cDNA made from cellular mRNA [28]. However, Northern blotting revealed that FGFR-4 is expressed at higher levels than FGFR-1 in breast cancer cell lines [29]. A study of 30 primary breast tumours showed 10% of the samples having 2–4-fold amplification of FGFR-4 DNA by Southern blotting, indicating that FGFR-4 may have a role in breast tumourigenesis [30]. An analysis of FGF receptor mRNAs expressed in breast cancer cell lines revealed the presence of FGFR-1, FGFR-3 and FGFR-4 [31]. Since breast cancer cell lines contain receptors for aFGF and bFGF and will be exposed to both these growth factors in the breast, it is of interest to examine how these growth factors will effect the breast cancer cells. bFGF has been shown to be mitogenic to human breast cancer cell lines and cultured epithelial cells derived from normal and malignant breast biopsies [32,33]. bFGF also stimulates plasminogen activator in breast tumour cell lines [34]. This enzyme is thought to be important in angiogenesis and may also be involved in the degradation of the basement membrane and subsequent tumour invasion into the surrounding stromal tissue [35]. We wished to investigate whether bFGF and aFGF could affect motility of breast cells and we used the phenomenon of membrane ruffling to assess this.

Membrane ruffles are specialized plasma membrane ultrastructures of mammalian cells which are thought to have roles in growth, development and locomotion [36,37]. Migrating fibroblasts form membrane ruffles at their leading edges and this is believed to play a fundamental role in directional migration. Membrane ruffles contain fine actin filaments and may be visualized using a fluorescently labelled phalloidin. A variety of growth factors and the oncogene p21ras are able to transiently induce circular membrane ruffling in serum-starved cells [38–40]. In addition, microinjection of the small GTP binding protein p21rac into fibroblasts was found to induce membrane ruffling [41]. We show here that aFGF and bFGF do not induce membrane ruffling in normal breast cells, however some of the metastatic breast cancer cell lines tested did ruffle in response to both growth factors. We have investigated the receptors through which FGFs elicit membrane ruffling and used dominant negative

Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; FGFR-1, fibroblast growth factor receptor 1; CALLA, common acute lymphoblastic leukaemia antigen; FITC, fluorescein isothiocyanate; PDGF, platelet-derived growth factor; TRITC, tetramethyl-rhodamine isothiocyanate.

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inhibitors to show that phosphorylation of high-affinity tyrosine kinase receptors and the activation of the small GTP-binding protein p21rac are required.

MATERIALS AND METHODS

Cell culture

Most of the cell lines were grown in RPMI1640 supplemented with 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum. MCF-10A cells were grown in a medium based on equal amounts of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 and supplemented with 5% equine serum, 100 units/ml penicillin, 100 μg/ml of streptomycin, 10 μg/ml of insulin, 1.4 nM cortisol, 100 ng/ml of cholera enterotoxin and 20 ng/ml of epidermal growth factor.

Purification of epithelial cells from normal breast organoids

Epithelial cells were prepared from normal breast organoids using an immunomagnetic separation method. Mouse monoclonal antibodies to common acute lymphoblastic leukaemia antigen (CALLA) (Sera-lab) were bound to anti-mouse antibody coated magnetic beads (Dynabeads) overnight at 4 °C and then washed four times with cold medium. Breast organoids were prepared from reduction mammoplasty tissue by a modification of the methods of Stampfer et al. [42] and Easty et al. [43]. Single-cell suspensions were prepared by digestion of organoids in PBS containing trypsin/EDTA (0.05%/0.02%) and 0.4 mg/ml of DNase for 15 min at 37 °C. Cells were washed three times in medium and filtered through 56 μm gauze. The cells were preincubated with anti-mouse antibody coated beads for 40 min at 4 °C. Unbound cells were collected and then bound to anti-CALLA coated beads in a ratio of 10 beads per target cell. Three separate incubations of 15 min at 4 °C were performed to collect all myoepithelial cells. Myoepithelial cells bound to the magnetic beads were removed leaving a population of myo-depleted epithelial cells. After culture, the cells were stained for the epithelial cell markers keratin 18 and keratin 19, and 100% of cells stained for each of these markers confirming that the cells were purified epithelial cells. Cells were cultured for a week before being treated with trypsin and plated onto glass coverslips for membrane ruffling assays.

Membrane ruffling assay

Cells were grown on glass coverslips until semi-confluent. The cells were serum starved by incubating overnight in serum-free medium. They were stimulated with 1–30 ng/ml aFGF or bFGF (Farmitalia Carlo Erba) and 1 μg/ml of heparin in serum-free medium for 10 min, rinsed briefly with PBS and fixed in 3% formaldehyde in PBS for 10 min. The cells were permeabilized by treating with 0.2% Triton-X100 in PBS for 5 min and cells were then treated with 50 mM ammonium chloride in PBS for 10 min. Polymerized actin filaments were visualized by incubating with phalloidin–tetramethyl-rhodamine isothiocyanate (TRITC) for 1 h. The cells were washed extensively, mounted and viewed using a fluorescence microscope.

Immunofluorescence

Cells grown on glass coverslips were fixed in 3% formaldehyde in PBS for 10 min, permeabilized by treating with 0.2% Triton-X100 in PBS for 5 min and treated with 50 mM ammonium chloride in PBS for 10 min and blocked with 10% goat serum in PBS. They were then incubated with a monoclonal antibody against FGFR-1 (UBI), or purified rabbit antisera against FGFR-2, FGFR-3 or FGFR-4 for 1 h. After washing, the cells were incubated with goat anti-(rabbit-FITC) or rabbit anti-(mouse-FITC) (FITC = fluorescein isothiocyanate; Pierce) for 1 h. After extensive washing the cells were mounted and viewed using a fluorescence microscope.

[^{3}H]Thymidine incorporation assay

Cells (5×10^3) cells were seeded into each well of a 24-well plate and were grown in medium containing 10% fetal calf serum for a day. The cells were starved for 24 h in serum-free medium. Cells were treated with 1–30 ng/ml of growth factor and 1 μg/ml of heparin in serum-free medium and 0.5 μCi of [^{3}H]thymidine was added for the final 4 h of incubation. Heparin was included to assist binding of FGF to its receptor since a ternary complex of growth factor, receptor and proteoglycan is required for effective receptor activation [10–14]. Heparin (1 μg/ml) was included in negative controls. After 24 h the cells were lysed in 1% SDS and DNA was precipitated by the addition of trichloroacetic acid to a final concentration of 7.5%. The samples were filtered, dried and counted.

Site-directed mutagenesis

A truncated FGFR-3 cDNA was made by mutating codon 405 of FGFR-3 from GGC to TGA, thus introducing a stop codon. The receptor encoded by this construct would contain the three immunoglobulin domains, the transmembrane domain and eight further amino acids. Site-directed mutagenesis was achieved using an Amersham mutagenesis kit following the recommended protocol. The resulting construct was sequenced and subcloned into a pSG5 plasmid.

Formation of cell lines expressing truncated FGFR-3

T47D or Swiss3T3 cells (10^7)were grown, trypsinized off the flask and washed in 20 mM Hepes (pH 7), 140 mM sodium chloride, 5 mM potassium chloride and 6 mM dextrose. The cells were resuspended in 0.5 ml of the above buffer and 100 μg of human placental DNA (Sigma), 0.5 μg of a puromycin resistance plasmid, pUC-puro, and 10 mg of pSG5-FGFR-3 were added. The cells were transferred to an electroporation cuvette and electroporated at 250 μFD, 250 V. The cells were returned to flasks for 2 days and then split into medium containing 2 μg/ml of puromycin. After 2 weeks puromycin-resistant colonies were picked and tested for FGFR-3 expression by immunofluorescence.

Transient transfection

T47D or Swiss3T3 or cos-7 cells (10^7) were grown, trypsinized off the flask and washed in 20 mM Hepes (pH 7), 140 mM sodium chloride, 5 mM potassium chloride and 6 mM dextrose. The cells were resuspended in 0.5 ml of the above buffer and 100 μg of human placental DNA (Sigma), 0.5 μg of pUC-puro and 30 μg of pSG5-FGFR-3 were added. The cells were transferred to an electroporation cuvette and electroporated at 500 μF, 250 V. Control cells were electroporated in the presence of the empty pSG5 vector. Cells were used in experiments 48 h after electroporation.

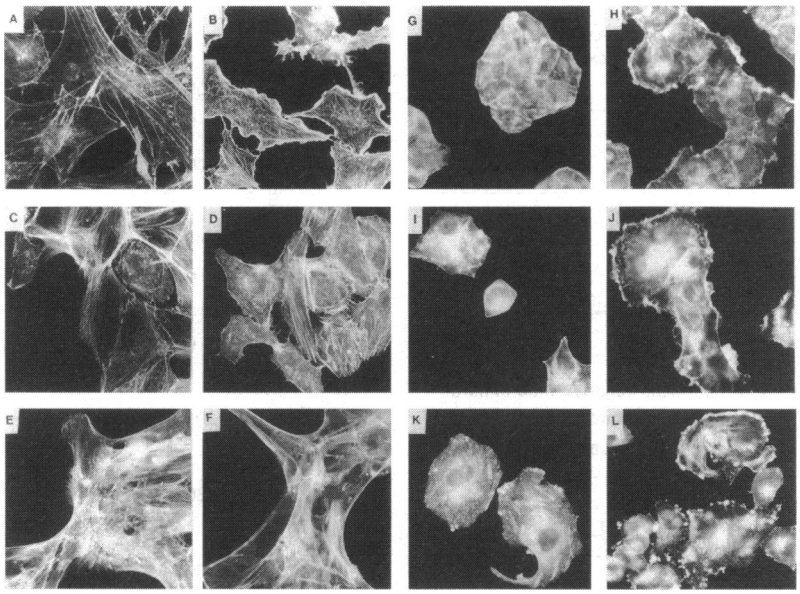


Figure 1 Membrane ruffling responses of normal and cancer breast cells and Swiss3T3 cells to bFGF

A, Unstimulated Swiss3T3 cells; B, Swiss3T3 cells stimulated with 30 ng/ml bFGF; C, unstimulated HBR cells; D, HBR-SV161 cells stimulated with 50 ng/ml of bFGF; E, unstimulated purified normal epithelial cells; F, purified normal cells stimulated with 50 ng/ml of bFGF; G, unstimulated T47D cells; H, T47D stimulated with 30 ng/ml of bFGF; I, unstimulated BT474 cells; J, BT474 cells stimulated with 30 ng/ml of bFGF; K, shows unstimulated SKBR3 cells; L, SKBR3 cells stimulated with 30 ng/ml bFGF (all × 400).

Table 1 Membrane ruffling responses of Swiss3T3 cells and breast cell lines to aFGF and bFGF

	Membrane ruffling responses									
	aFGF (ng/ml)					bFGF (ng/ml)				
Cell line	1	3	10	30	50	1	3	10	30	50
Swiss3T3 fibroblasts	−*	+†	++	++‡	+++§	+	++	+++	+++	+++
Normal breast										
HBL100	−	−	−	−	−	−	−	−	−	−
MCF10A	−	−	−	−	−	−	−	−	−	−
HBR-SV161	−	−	−	−	−	−	−	−	−	−
Purified epithelial cells	−	−	−	−	−	−	−	−	−	−
Cancer cells										
T47D	−	+	++	+++	+++	−	−	+	++	++
MCF7	−	−	+	++	+++	−	−	−	+	++
BT474	−	+	++	+++	+++	−	−	+	++	++
SKBR3	+	+	++	+++	+++	−	−	+	++	++
MDA-MB-157	+	+	++	+++	+++	−	−	+	+	+++
MDA-MB-231	−	−	−	−	−	−	−	−	−	−
ZR75	−	−	−	−	−	−	−	−	−	−

* No membrane ruffling response.
† Slight membrane ruffling response.
‡ Medium membrane ruffling response.
§ Strong membrane ruffling response.

RESULTS

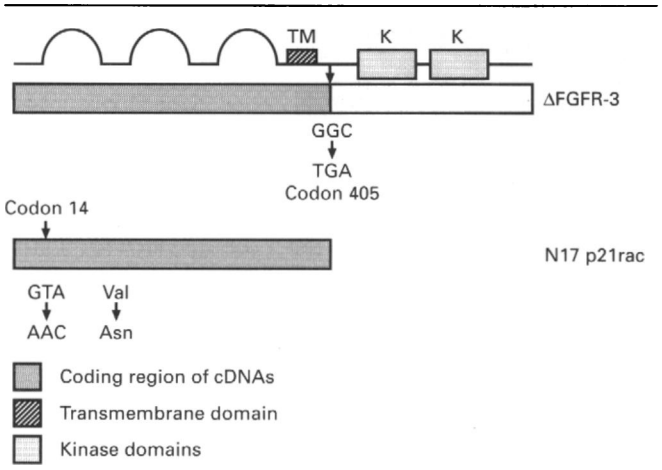
bFGF and aFGF induce membrane ruffling in Swiss3T3 cells and T47D breast cancer cells but not in normal breast cells

Swiss3T3cells were treated for 10 min with aFGF or bFGF at concentrations between 1 and 30 ng/ml. After staining with phalloidin–FITC, membrane ruffling could be seen in response to both growth factors (Figure 1, A and B and Table 1). A variety of breast cell lines comprising both normal and cancer cells were tested for ruffling in response to aFGF or bFGF. As seen in Figure 1 and Table 1, none of the normal breast cells, including both epithelial and myoepithelial cells, responded to either of the growth factors at concentrations up to 50 ng/ml. The breast cancer cell lines ZR75 and MDA-MB-231 also failed to respond to either aFGF or bFGF at concentrations up to 50 ng/ml. However, the breast cancer cell lines T47D, BT474, MDA-MB-

Table 2 [^{3}H]thymidine incorporation assay showing the response of breast cell lines to 10 ng/ml of bFGF. Results shown represent the percentage increase in [^{3}H]thymidine incorporation after stimulation with 10 ng/ml of bFGF compared with unstimulated cells

Cell line	HBL-100	HBR-SV161	MCF-10A	ZR-75	MDA-MB-231	T47D
[^{3}H]thymidine incorporation stimulation (%)	261 ± 22	212 ± 16	234 ± 19	294 ± 36	210 ± 24	157 ± 12

Table 3 Diagramatic representation of dominant negative mutations of FGFR-3 and rac and the number of puromycin resistant colonies achieved after transfection into Swiss 3T3 and T47D cells



Plasmids	No. of colonies T47D	Swiss 3T3
pUC-puro	119	84
pUC-puro pSG5 ΔFGFR-3	105	97
pUC-puro pSG5 N17 rac	34	21

157, SKBR3 and MCF7 exhibited membrane ruffling in response to both FGFs. These cells gave some response after treatment with 3 ng/ml of aFGF but maximal stimulation required 30 ng/ml of aFGF and aFGF elicited a more pronounced response than bFGF. However, the converse situation was true for Swiss3T3 cells where bFGF promoted the greater response.

Normal epithelial cells were purified from an organoid preparation from a normal breast by immunomagnetic separation. The purified epithelial cells were tested for membrane ruffling in response to aFGF and bFGF and, similarly to all the normal breast cell lines tested, did not respond at concentrations of growth factor up to 50 ng/ml (Figure 1, E and F). In summary, no normal breast cells exhibited membrane ruffling in response to growth factor, however some cancer cell lines did respond in this way.

In order to investigate whether the lack of membrane ruffling in HBR-SV161, HBL-100, MCF10A, ZR-75 and MDA-MB-231 cell lines is due to the absence of FGFRs in these cell lines, the cells were stimulated with bFGF and their mitogenic response assessed using a [^{3}H]thymidine incorporation assay. As shown in

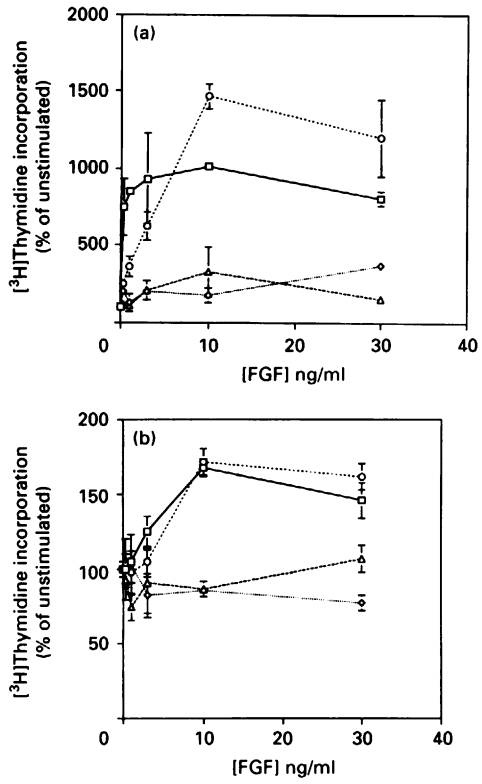


Figure 2 [^{3}H]Thymidine incorporation assay showing the response of Swiss3T3 cells (a) and T47D cells (b) to aFGF and bFGF and the inhibitory effect of truncated FGFR-3 expression

□, The response of cells to bFGF; ○, the response of cells to aFGF; ◇, the response of cells expressing truncated FGFR-3 to bFGF; and △, the response of cells expressing truncated FGFR-3 to aFGF.

Table 2, all the cell lines tested showed a modest increase in [^{3}H]thymidine incorporation. The cell lines MCF-7 and T47D, which exhibit membrane ruffling in response to FGF, showed similar mitogenic stimulation to the cell lines which had failed to show membrane ruffling. Therefore the non-ruffling cell lines contain FGF receptors. However, their failure to show membrane ruffling could be a result of their containing a different complement of FGFRs or could be due to inhibition of processes downstream from the receptor.

FGF-induced membrane ruffling is inhibited by a dominant negative FGFR-3

In order to show that high-affinity FGFRs are involved in transducing membrane ruffling, a dominant negative FGFR-3 construct was made. A stop codon was introduced eight amino acids after the transmembrane domain of FGFR-3 so that a truncated receptor would be translated (Table 3). FGFR-3 was chosen as the receptor to be mutated in preference to FGFR-1 since FGFR-1 has been reported not to mediate membrane ruffling [44]. No information was available as to which of the remaining FGFRs might mediate membrane ruffling so FGFR-3 was chosen at random from this group. Since heterodimerization between different classes of FGFR has been observed, it was hoped that a truncated FGFR-3 might inhibit signalling through more than one class of receptor [45,46]. The truncated FGFR-3 was subcloned into a pSG5 eukaryotic

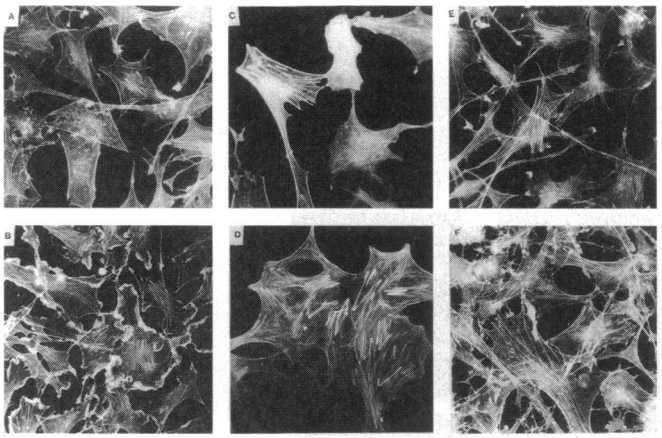


Figure 3 Membrane ruffling in Swiss3T3 cells expressing truncated FGFR-3 and N17rac

A, Unstimulated Swiss3T3 cells; B, Swiss3T3 cells stimulated with 10 ng/ml of bFGF; C, unstimulated Swiss3T3 cells expressing truncated FGFR-3; D, Swiss3T3 cells expressing truncated FGFR-3, stimulated with 10 ng/ml of bFGF; E, unstimulated Swiss3T3 cells expressing N17rac; F, Swiss3T3 cells expressing N17rac, stimulated with 10 ng/ml of bFGF (all × 400).

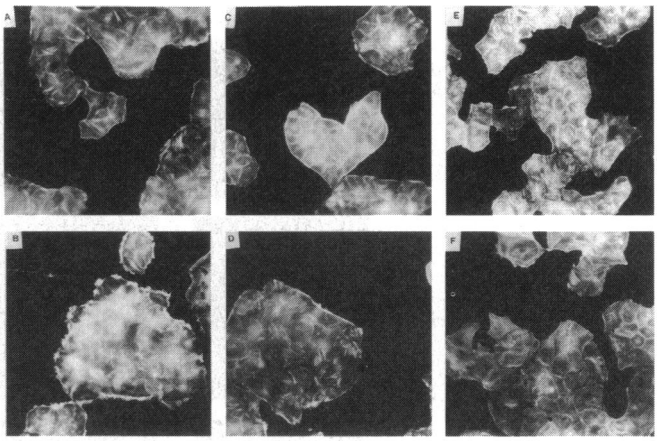


Figure 4 Membrane ruffling in T47D cells expressing truncated FGFR-3 and N17rac

A, Unstimulated T47D cells; B, T47D cells stimulated with 10 ng/ml of bFGF; C, unstimulated T47D cells expressing truncated FGFR-3; D, T47D cells expressing truncated FGFR-3, stimulated with 10 ng/ml of bFGF; E, unstimulated T47D cells expressing N17rac; F, T47D cells expressing N17rac stimulated with 10 ng/ml of bFGF (all × 400).

Table 4 Summary of the effect of dominant negative inhibitor expression on membrane ruffling responses

Transfected plasmids	Swiss 3T3 aFGF	bFGF	T47D AFGF	bFGF
pUC-puro	++*	+++†	+++	++
pUC-puro + pSG5 ΔFGFR-3	−‡	−	−	−
pSG5	++	+++	+++	++
pSG5 N17 rac	−	−	−	−

* Medium membrane ruffling response.
† Strong membrane ruffling response.
‡ No membrane ruffling response.

expression vector and was introduced together with pUC-puro into Swiss3T3 and T47D cells by electroporation. The number of puromycin-resistant colonies achieved was similar to that produced by the puromycin resistance plasmid alone, showing that the truncated FGFR-3 was not toxic to either cell line (Table 3). Puromycin-resistant colonies were tested for overexpression of FGFR-3 by immunoflorescence (results not shown) and two positive cell lines were selected.

The ability of the truncated FGFR-3 to inhibit the action of aFGF and bFGF in the transfected cell lines was assessed by using a [^{3}H]thymidine incorporation assay. As shown in Figure 2(a), both aFGF and bFGF induced increased [^{3}H]thymidine incorporation in Swiss3T3 cells transfected with a puromycin resistance gene, with a concentration of 10 ng/ml giving maximal stimulation. In Swiss3T3 cells expressing truncated FGFR-3, there was a large reduction in [^{3}H]thymidine incorporation stimulation by aFGF and bFGF. Control transfected T47D cells showed a smaller mitogenic response to bFGF and aFGF, again with maximal activity at 10 ng/ml. Expression of truncated FGFR-3 in T47D cells led to almost complete inhibition of the small response to aFGF and bFGF (Figure 2b). The complete inhibition of growth in response to FGF indicates that the truncated FGFR-3 may inhibit signalling through FGFR-1, 2 and 4 as well as FGFR-3. This would be achieved through heterodimer formation between different classes of FGFR.

We tested whether the dominant negative FGFR-3 was able to inhibit membrane ruffling. As shown in Figure 3, A and B and Table 4, extensive ruffling occurred in control transfected Swiss3T3 cells in response to 10 ng/ml of bFGF and aFGF. In Swiss3T3 cells expressing truncated FGFR-3, a much smaller amount of membrane ruffling resulted from aFGF or bFGF stimulation (Figure 3, C and D). When control transfected T47D cells were used in a similar experiment, treatment with aFGF and bFGF resulted in membrane ruffling, however expression of truncated FGFR-3 completely inhibited this effect (Figure 4, C and D and Table 4). Therefore it would be predicted that FGFR phosphorylation is required for membrane ruffling to occur. The actual member(s) of the FGFR family mediating this effect cannot be predicted, however either FGFR-3 or a receptor able to dimerize with it must be involved.

A dominant negative mutant of p21rac inhibits membrane ruffling in Swiss3T3 and T47D cells

An N17 mutation of p21rac has previously been shown to act as a dominant negative [41]. N17 rac cDNA, obtained from Dr. A. Hall's laboratory (MRC Laboratory for Molecular Cell Biology, University College London), was subcloned into a eukaryotic expression vector (pSG5) and was introduced together with a puromycin resistance vector into Swiss3T3 and T47D cells by electroporation. As shown in Table 3, co-expression of N17p21rac with a puromycin resistance plasmid substantially reduced the number of puromycin resistant colonies. This indicates that N17p21rac may be toxic to cells. Owing to the difficulty in achieving N17p21rac-expressing cells, a transient expression system was used to study the effect of N17p21rac.

As shown in Table 4, Swiss3T3 cells electroporated in the presence of pSG5 vector exhibited membrane ruffling when

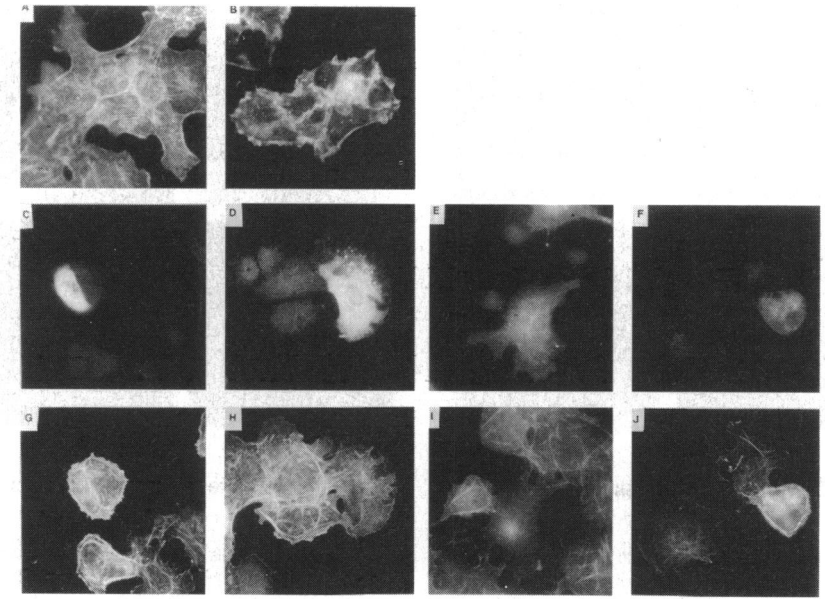


Figure 5 Membrane ruffling in cos-7 cells transiently transfected with FGFRs

A, The membrane ruffling response of control transfected cos-7 cells to EGF; B, the membrane ruffling response of control transfected cos-7 cells to aFGF and bFGF in the presence of 1 μg/ml of heparin. C, D, E and F show expression of, FGFR-1, 2, 3 and 4 respectively in transfected cos-7 cells treated with 1 μg/ml of heparin, 20 ng/ml of aFGF and 20 ng/ml bFGF (FITC label); G, H, I and J show the same cells stained with phalloidin–TRITC, showing that a membrane ruffling response is achieved only when FGFR-4 is expressed (all × 400).

stimulated with 10 ng/ml of aFGF or bFGF. However, transient expression of N17p21rac was able to inhibit membrane ruffling in response to aFGF and bFGF (Figure 3, E and F). Similarly for T47D cells, electroporation in the presence of pSG5 vector allowed cells to exhibit membrane ruffling in response to aFGF and bFGF but expression of N17p21rac was able to inhibit this effect (Figure 4, E and F). This indicates that, similarly to platelet-derived growth factor (PDGF) signalling, activation of p21rac is required for membrane ruffling in response to FGFs.

FGFR-4 is the receptor involved in the membrane ruffling response

In order to investigate which of the FGFRs is involved in the membrane ruffling response, cos-7 cells were transiently transfected with individual receptors and tested for their ability to give a membrane ruffling response after treatment with heparin and a mixture of aFGF and bFGF. Cos-7 cells were selected for this experiment since they are able to give a membrane ruffling response to EGF but not to aFGF or bFGF even in the presence of heparin (Figure 5, A and B). This suggests that their failure to give this response is due to lack of receptors that recognise human aFGF or bFGF. Cells shown to be expressing FGFR-1, FGFR-2 or FGFR-3 by immunofluorescence, failed to show membrane ruffling after treatment with 1 μg/ml of heparin, 20 ng/ml of aFGF and 20 ng/ml bFGF (Figure 5, C, D, E, G, H and I). However, cells expressing FGFR-4 could give a membrane ruffling response (Figure 5, F and J). Therefore of the four receptor classes tested, only FGFR-4 appears to transduce the membrane ruffling response.

DISCUSSION

A number of growth factors cause changes in the state of actin polymerization in cells. One consequence of this is the phenomenon of membrane ruffling in which actin polymerizes in fine filaments at the plasma membrane. Membrane ruffling is associated with cell motility and therefore could be important in determining the metastatic potential of cells. Both bFGF and aFGF are present in the breast with bFGF being produced in myoepithelial cells [26] and aFGF being associated with endothelial cells present especially in vascularized tumours [27,47]. We were interested to see whether aFGF and bFGF could affect cells by causing membrane ruffling. Using Swiss3T3 cells both these growth factors were able to do so.

A range of breast cell lines were tested for membrane ruffling and although none of the normal cell lines exhibited membrane ruffling, some of the breast cancer cell lines did. This presents the possibility that growth factors present in the breast may be able to affect some breast cancer cells causing them to be more motile and more likely to metastasize. It is possible that the additional response of the T47D, BT474, MDA-MB-157, SKBR3 and MCF7 cells could be due to the transfer of the original cells to culture and subsequent passaging, however no similar phenomenon was seen with any of the normal cells. The use of purified primary culture cells gives better access to the situation and we have used epithelial cells purified from normal breast. These cells confirmed that normal epithelial cells do not ruffle in response to aFGF and bFGF. It is more difficult to grow primary cultures of breast cancer cells and we are unable at present to perform similar experiments on these cells.

The lack of a membrane ruffling response to FGF in normal breast cells could be due to any one of a number of factors. It is unlikely to be due to a complete absence of FGFRs since receptor mRNA has been detected in normal breast cells [31]. However, the complement of FGFRs in breast cancer cell lines may be more suitable for mediating membrane ruffling. We have shown that of the four classes of FGFR, only FGFR-4 was able to mediate membrane ruffling in cos-7 cells, and there is evidence of FGFR-4 gene amplification in breast cancer [30]. Alternatively, proteins required for membrane ruffling may be absent from

normal breast cells, or downregulatory elements which are present in normal cells may be lost in breast cancer cells. Whichever mechanism is involved, the ability of breast cancer cells to ruffle in response to growth factors which they will be exposed to within the breast may indicate a greater motility and a greater ability to metastisize. It will be of interest to find the basis of this difference.

We wished to investigate the pathway by which aFGF and bFGF produce membrane ruffling. Most of the cells' responses to FGFs are mediated through the family of high-affinity receptors, however some proteoglycans like syndecan have some biological activity after binding of growth factor [48]. The cytoplasmic domain of syndecan associates with the actin cytoskeleton and it could be envisioned that membrane ruffling could be mediated through this interaction, without the involvement of high-affinity receptors. A dominant negative mutation of FGFR-3 was used to address this question. A truncated FGFR-1 containing the extracellular and transmembrane domains only, has previously been shown to act as a dominant negative inhibitor of FGF action [46]. It presumably works by dimerizing with full length receptor and preventing tyrosine phosphorylation from occurring. There is some evidence indicating that different classes of FGFR can dimerize with each other, allowing the possibility that one class of truncated receptor can inhibit signalling through different FGFRs [45,46]. The ability of FGFR-3 to completely inhibit growth in response to aFGF and bFGF suggests that this is indeed the case, so that truncated FGFR-3 would inhibit a signal mediated through FGFR-1, 2 and 4 as well as FGFR-3. Our results indicate that membrane ruffling is mediated through high-affinity receptors since a truncated FGFR-3 is able to inhibit this effect.

We have used transiently transfected cos-7 cells to address the question of which FGFR is responsible for the membrane ruffling response and we find that FGFR-4 is the only receptor class tested that can produce this response. A previous report showed that FGFR-1 does not mediate membrane ruffling and our results are in agreement with this [44]. Various studies have indicated that PI3 kinase activation may be required for membrane ruffling since mutation of receptor tyrosines to which p85 binds leads to loss of membrane ruffling [49], and wortmannin, an inhibitor of PI3 kinase, inhibits membrane ruffling [50]. The SH2 domain of p85 recognises the phosphotyrosine motif YXXM [51]. Such a motif is present near the C-terminus of all four classes of FGFR, however in the case of FGFR-1 it has not been reported as one of the tyrosine phosphorylation sites [52]. It is possible that the ability of FGFRs to elicit a membrane ruffling response will be dependent on the ability of the YXXM motif to be phosphorylated efficiently. Gene amplification and relatively high expression levels for FGFR-4 have been found in breast cancer cells [29,30] It is possible that increased levels of FGFR-4 account for the membrane ruffling ability of breast cancer cell lines. The involvement of FGFR-4 would also explain the greater ruffling response to aFGF since FGFR-4 has a higher affinity for aFGF than bFGF [29]. However, in the case of Swiss3T3 cells, bFGF gave a greater ruffling response indicating that in these cells an additional receptor may be important.

p21rac has previously been shown to be involved in the signal transduction pathway leading to membrane ruffling [41]. We wanted to know whether FGF stimulation uses a similar pathway to that already demonstrated for PDGF. To ascertain this, we used a dominant negative mutant of p21rac. Overexpression of N17p21rac in both Swiss3T3 and T47D cells led to cell death, showing that this protein is toxic to cells when present in high amounts. It is unlikely that inhibition of membrane ruffling alone is responsible for the observed toxicity, however p21rac may control a number of different activities and one of these may be responsible for the toxic effect. p21rac has a second function in neutrophils where it controls the production of superoxide [53]. It is of interest to note that the *Saccharomyces cerevisiae* CDC42, similar to p21rac, has a role in positioning bud formation before cell division in budding yeast [54,55]. It is possible that p21rac has some similar role in cell division which may account for a dominant negative inhibitor preventing cells from growing. Alternatively, the toxicity of p21rac could result from inhibition of ras activation by sequestering an important exchange factor. One exchange factor for ras has two separate exchange factor domains, one which activates p21ras and a second having homology with exchange factors which activate proteins in the rho-like family of small GTPases which includes p21rac [56]. Sequestering of such an exchange factor by N17rac could affect GDP/GTP exchange of p21ras as well as p21rac.

Cells transiently expressing N17p21rac showed an inhibition of membrane ruffling in response to aFGF and bFGF, indicating that FGF receptors probably use a similar signalling pathway to PDGF receptors with p21rac lying downstream of the receptor. The steps linking tyrosine kinase receptors to p21rac and those linking p21rac to membrane ruffle formation remain to be elucidated.

In summary, we have shown that membrane ruffling may occur in some cell types following stimulation with aFGF or bFGF and that FGFR-4 activation and p21rac activation occur upstream of membrane ruffle formation. In normal breast cells, this series of events does not lead to membrane ruffling and either the FGFR complement of such cells is not able to transduce the signal or there are blocks in the signalling pathway to prevent this response. However, in some of the breast cancer cells tested, FGFs were able to produce a ruffling response and this may affect metastatic behaviour of cancer cells.

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