The mitogenic signaling pathway for fibroblast growth factor-2 involves the tyrosine phosphorylation of cyclin D2 in MCF-7 human breast cancer cells

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Abstract Fibroblast growth factor-2 (FGF-2) is mitogenic for the human breast cancer cell line MCF-7; here we investigate some of the signaling pathways subserving this activity. FGF-2 stimulation of MCF-7 cells resulted in a global increase of intracellular tyrosine phosphorylation of proteins, particularly FGF receptor substrate-2, the protooncogene product Src and the mitogen-activated protein kinase (MAP kinase) cascade. A major increase in the tyrosine phosphorylation of a 30-kDa protein species was also found. This protein was identified as cyclin D2 by mass spectrometry after trypsin digestion. Immunoprecipitation of cyclin D2 and immunoblotting with anti-phosphotyrosine antibodies confirmed that the tyrosine phosphorylation of cyclin D2 was indeed induced by FGF-2 stimulation. In addition, pharmacological inhibition of Src (with herbimycin A and PP2), and of the MAP kinase cascade (with PD98059), confirmed that Src activity is required for the FGF-2-induced phosphorylation of cyclin D2 whereas MAP kinase activity is not. Thus, tyrosine phosphorylation of cyclin D2 may be a key regulatory target for FGF-2 signaling.

Key words: Fibroblast growth factor-2; Signaling pathway; Cyclin D2; Breast cancer cell

1. Introduction

Fibroblast growth factor-2 (FGF-2), the most studied member of the FGF family of growth factors, has been isolated from a large variety of tissues and various effects of this molecule have been reported for the control of cellular growth, differentiation and migration [1]. FGF-2 activates its target cells by binding to specific tyrosine kinase receptors (FGFRs) on the cell surface, which immediately induces transient phosphorylation on their tyrosine residues [2]. The activated FGFR further associates with, and phosphorylates a set of cytoplasmic signaling molecules containing SH2 domains, which in turn activate several pathways, and in particular the mitogen-activated protein kinase (MAP kinase) cascade. The MAP kinase pathway ultimately leads to the regulation of gene expression essential for proliferation [3]. However, a variety of signaling pathways has been described in the diverse cellular systems used to study FGF intracellular signaling, and a complete model for one defined cell type has yet to be established.

The role of FGFs in tumor progression includes the stimulation of cancer cell growth and metastasis as well as angiogenesis [1,4]. In breast cancer, the overexpression of FGF family members as well as FGFRs has been reported in a significant percentage of breast tumors [5–10]. Moreover, high levels of FGF have been detected in sera from patients with breast cancer [11]. We and others have found that FGF-2 is a strong mitogen for the prototypic MCF-7 breast cancer cell line [12–14] and that this activity involves a recruitment of G0 cells into the cell cycle, as well as a shortening of the G1 phase [15]. Under other experimental conditions, FGF-2 has been shown to inhibit breast cancer cell growth [16,17], underlining the pleiotrophic activities of this growth factor. However, the transfection of MCF-7 cells by FGFs induces a progression towards an aggressive and metastatic phenotype, supporting a role for autocrine and paracrine involvement of FGFs during malignant progression [18,19]. Interestingly, Liu et al. (1999) have shown that functional Rac-1 and Nck are required for FGF-2-induced proliferation in MCF-7 cells [20]. However, to date, the intracellular signaling pathways for the FGF-2 stimulation of breast cancer cells remain largely unknown.

In the present study, we have explored the signaling pathways involved in the mitogenic effect of FGF-2 in MCF-7 cells. Our results show that FGF-2 induces the activation of FGFR, FRS2, Src and the MAP kinase. Moreover, pharmacological inhibition indicated that the activation of both Src and the MAP kinase cascade is required for FGF-2 mitogenicity. Interestingly, a strong increase in the tyrosine phosphorylation of a 30-kDa protein was found to be induced by FGF-2; this protein was identified as cyclin D2 by mass spectrometry. The induction of tyrosine phosphorylation of

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Abbreviations: ERK, extracellular regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FRS2, fibroblast growth factor receptor substrate-2; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MAP kinase, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3 kinase, phosphatidyl-inositol 3′ kinase; PMA, phorbol 12-myristate 13-acetate

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cyclin D2 by FGF-2 stimulation was further confirmed using specific antibodies.

2. Materials and methods

2.1. Materials

FGF-2 was from Amersham (France). The pharmacological inhibitors PD98059, K252-a, herbimycin A and PP2 were from Calbiochem (France) and genistein was from Sigma (France). All the cell culture products were from Bio-Whittaker (Belgium) except insulin (Organon, France), fibronectin (Falcon-Biocoat, France) and transferrin (Sigma, France). The petri dishes were purchased from Greiner (Fisher, France). The antibodies against phosphotyrosine (PY99), FRS2, ERK-1, and cyclin D2 were purchased from Santa Cruz Biotechnology (Tebu, France). The rabbit polyclonal anti-FGFR (nominally against R1) was from UBI (Euromedex, France). Trypsin was purchased from Promega (France).

2.2. Cell culture

MCF-7 human breast cancer epithelial cells were obtained from the American Type Culture Collection. They were cultured between passages 15–25 in minimal essential medium (Earle's salts) supplemented with 20 mM HEPES, 2 g/l sodium bicarbonate, 10% fetal calf serum (FCS), 1% non-essential amino acids, 2 mM L-glutamine, 5 μg/ml insulin, 40 U/ml penicillin–streptomycin, 50 μg/ml gentamicin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

2.3. Cell growth assay

MCF-7 cells were seeded at 30 000 cells/ml in 35-mm dishes in complete medium. When 40% confluence was reached, cells were washed twice and starved in a serum-free medium containing fibronectin (2 μg/ml) and transferrin (30 μg/ml) for 24 h. Cells were then treated for 48 h with FGF-2 (10 ng/ml) in fresh serum-free medium in the presence of various inhibitors at different concentrations: 1–100 μM genistein, 5 nM K-252a, 1–100 μM PD98059, 25–100 nM herbimycin A, 5–15 nM PP2. After 48 h of treatment, cell numbers were determined after trypsinization of the monolayer culture with 0.25% trypsin/EDTA solution. In parallel with the experiments with FGF-2, MCF-7 cells were treated with the same inhibitors in control serum-free medium to ensure that the effect of each compound on FGF-2-induced proliferation was not due to a non-specific toxicity.

2.4. Cell lysate preparation

Cells were seeded in 100-mm dishes at 500 000 cells per dish. They were grown to pre-confluency and then made quiescent in serum-free MEM for 24 h. Pretreatment of cells by 10 μM pervanadate before addition of FGF-2 was performed for 10 min. FGF-2 was then directly added to the medium (10 ng/ml) for another 10 min. When inhibitors were used, they were added directly into the medium at the required concentration 50 min before pretreatment with pervanadate. Cells were rinsed with cold PBS containing 100 μM orthovanadate. Cell lysates were prepared by adding 200 μl of immunoprecipitation (IP) lysis buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% Nonidet P-40, 0.1% SDS, supplemented with the following inhibitors: orthovanadate, PMSF, and $Na_4P_2O_7$ at 1 mM, leupeptin and aprotinin at 10 μg/ml, pepstatin at 1 μg/ml) to each dish, kept 15 min on ice, transferred to 1.5 ml Eppendorf tubes, boiled for 10 min, and centrifuged for 2 min at $10\,000 \times g$. The supernatants were stored at −80°C. Protein content in cell lysates was measured with the Bio-Rad protein assay.

2.5. Immunoprecipitation and Western blot analysis

Immunoprecipitations were performed by incubation of cell lysates (500 μg protein) at 4°C for 2 h with the appropriate antibody, followed by incubation of the immunocomplexes with protein A-agarose 10% (Sigma) beads for 1 h at 4°C. The beads were then washed three times with the IP buffer. The immunocomplexes were resuspended in Laemmli sample buffer, boiled for 5 min and subjected to 10% SDS–PAGE. Proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell). The membranes were immersed for 2 h at room temperature in blocking buffer: Tris-buffered saline, 0.1% Tween-20 containing 5% non-fat dry milk (for detection of FGFR, ERK, and cyclin D2), 1% non-fat dry milk (for detection of FRS2) or 3% BSA (for detection of phosphotyrosine), and were then incubated overnight at 4°C with the appropriate antibody. The membranes were then incubated with the appropriate secondary antibody for 1–2 h and, after extensive washing, antibody complexes were detected by the enhanced chemoluminescence (ECL) Western blotting detection system according to the manufacturer's instructions (Amersham).

2.6. Src kinase assay

Src kinase activity was determined by immunoprecipitation with the anti-Src monoclonal antibody 327 [21]. The antibody complexes were washed 3 times with lysis buffer and once with kinase buffer (30 mM Tris pH 7.4, 10 mM $MnCl_2$), and were subsequently incubated in 50 μl of kinase buffer containing 10 μCi of (γ-^{32}P)-ATP and 2 μg acid-denatured enolase (Sigma) at room temperature for 10 min. The phosphorylated proteins were resolved by 7.5% SDS–PAGE, transferred to nitrocellulose, visualized by autoradiography and bands quantitated using NIH Image software. Subsequently, immunoblot analysis was performed using the anti-Src-2 antibody [21] and quantitated by fluorimaging (Molecular Dynamics) with the ECL+reagent (Amersham Pharmacia) procedure. The results of at least six separate experiments were averaged and the standard deviation calculated.

2.7. Mass spectrometry analysis

Protein identification was performed as described [22,23] with modifications. Silver nitrate-stained proteins were cut out from the gel and washed three times with 400 μl of a 125 mM ammonium carbonate/acetonitrile (ACN) 1:1 (v/v) solution for 20 min with shaking. The wash solution was discarded and the pieces of gel were dried at room temperature for 2 h. Enzymatic cleavage was initiated by re-swelling the gel in ammonium carbonate solution (125 mM); 50 mM acetic acid was then added, and finally the digestion was initiated by adding 50 mM acetic acid containing 7–7.5 units of trypsin (Promega). After absorption of the protease solution, aliquots (5 μl) of pure water were added sequentially. The gel slices were placed in an Eppendorf tube and a minimum volume of water was added to totally immerse the gel pieces. The digestion was carried for 12–16 h at 37°C. The liquid was collected and the resulting peptides recovered after two extractions with a solution containing 45% acetonitrile and 10% formic acid. To recover very hydrophobic peptides, a third extraction with 95% acetonitrile, 5% formic acid was performed. The total extract was then dried using a Speed Vac-concentrator (Savant).

MALDI-TOF analysis of trypsin digests was performed on a Vision 2000 (Finnigan, Bremen, Germany) reflector instrument in positive ion mode at an accelerating voltage of 6 kV. Peptides were resuspended in CH_3CN/H_2O (1:1) containing 0.5% formic acid of which 0.5 μl were mixed directly onto the target with 1 μl of 2,5-dihydroxy benzoic acid matrix solution (10 mg/ml in CH_3OH/H_2O, 7:3). Between 30 and 50 laser shots were accumulated to obtain the final spectrum. Mass measurements were then finalized after peak smoothing and internal calibration using the monoisotopic mass of the two autolysis trypsin fragment and DHB matrix ions respectively at 2211.1, 842.8 and 273.04, resulting in mass accuracy better than 0.2 Da. Protein database searching was performed using MS-Fit (http://prospector.ucsf.edu/htmlucsf3.0/msfit.htm) according to the average molecular weight of $[M+H]^+$ peptide ions.

3. Results

3.1. Pharmacological inhibition of FGF-2 mitogenic effect

MCF-7 cells were treated with each inhibitor in the presence of FGF-2, and the cell number measured after 2 days of treatment. For each inhibitor used we determined the dose–response for the inhibition of FGF-2 mitogenic activity (data not shown). Genistein inhibited the proliferation induced by FGF-2 (Fig. 1), confirming that FGF-2 stimulates cell growth through tyrosine kinase pathways. In contrast, a specific inhibitor of the NGF receptor (TrkA) tyrosine kinase, K-252a, which inhibits NGF-induced proliferation in MCF-7 cells [24], had no effect on FGF-2-induced proliferation (Fig. 1). Herbimycin A and PP2 are known to inhibit Src and Src-related kinases by binding to their SH2 domains. These two inhibitors markedly decreased the mitogenic activity of FGF-2 (Fig. 1),

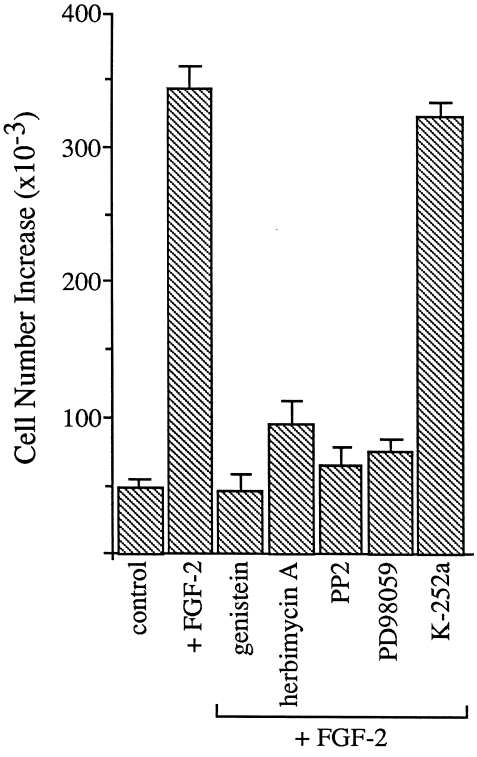

Fig. 1. Pharmacological inhibition of FGF-2 mitogenic activity on MCF-7 cells. Quiescent MCF-7 cells were stimulated with FGF-2 (10 ng/ml) for 48 h with or without inhibitors (genistein 10 μM, K-252a 5 nM, PD98059 10 μM, herbimycin A 25 nM, PP2 15 nM) and the cell number determined. For each inhibitor, a dose–response curve was generated and only the effect for the optimal concentration is presented here. Results represent the average of triplicate determinations for two independent experiments.

suggesting that Src kinase activity is involved in the signaling of this growth factor. In contrast, the PI3 kinase inhibitor wortmannin (5–15 nM) had only a slight inhibitory effect on the mitogenic activity of FGF-2, even when the medium was changed after 24 h of treatment (data not shown). This indicates that PI3 kinase is probably not crucial for the mitogenesis induced by FGF-2 in breast cancer cells. MAP kinase pathway involvement in the FGF-2 mitogenic effect in MCF-7 cells was investigated using the synthetic MEK-1 inhibitor, PD98059. At a concentration of 10 μM, the mitogenic effect of FGF-2 was completely inhibited (Fig. 1), demonstrating that MAP kinase activity is necessary for the mitogenic stimulation of MCF-7 cells by FGF-2.

3.2. Tyrosine phosphorylation induced by FGF-2

The tyrosine phosphorylation induced by FGF-2 in MCF-7 cells was studied after immunoprecipitation and Western blotting of cell lysates using an anti-phosphotyrosine antibody. Several bands with apparent molecular weights of 110, 95, 90, 85–70, 64, 60, 45 and 30 kDa were found phosphorylated after 10 min of stimulation with FGF-2 (Fig. 2). The 45-kDa protein has a molecular weight corresponding to the ERK-1/2 isoforms known to be regulated by growth factors; the 60–64-kDa band could correspond to ERK-4 and/or Src, which have a molecular mass of about 60 kDa and are also reported to be phosphorylated by MEK in response to growth stimuli [3].

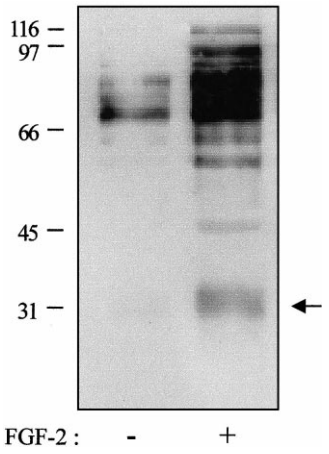

Fig. 2. FGF-2-induced tyrosine phosphorylation. Cells were stimulated with 10 ng/ml of FGF-2 in serum-free medium for 10 min as described in Section 2. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and subjected to immunoblot analysis with the same antibody. Molecular weight is indicated in kDa. The arrow indicates the position of the 30-kDa band which has been subjected to analysis by mass spectrometry.

3.3. FGFR, FRS2, Src and MAP kinase activation

We used a polyclonal antibody to immunoprecipitate FGFR-1, and its activation was detected by immunoblotting with anti-phosphotyrosine antibodies. Tyrosine phosphorylation of the FGFRs was observed after 10 min of stimulation by FGF-2 (Fig. 3A). Differences observed in FGFR phosphorylation were not due to differences in protein quantity, as we reprobed the membrane with the anti-FGFR antibody. Similarly, FGF-2 induced the tyrosine phosphorylation of the FGF receptor substrate FRS2, a membrane-anchored docking protein involved in the activation of the Ras/MAP kinase cascade through FGFR (Fig. 3B).

As we have shown an inhibition of FGF-2-induced proliferation by herbimycin A and PP2, we further studied Src

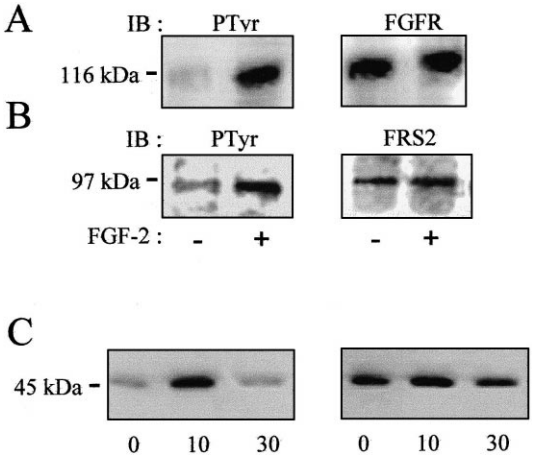

Fig. 3. Activation of FGFR, FRS2, and MAP kinase. Cells were exposed to 10 ng/ml of FGF-2 for 10 min. Cell lysates were prepared as described in Section 2 and immunoprecipitated with anti-FGFR (A) or anti-FRS2 antibodies (B). The immunocomplex was resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibodies. C: MAP kinase activation. Cells were stimulated for the indicated times with FGF-2 at 10 ng/ml. Lysates were immunoprecipitated with anti-ERK1 antibodies and immunoblotted with anti-phosphotyrosine or anti-ERK1 antibodies.

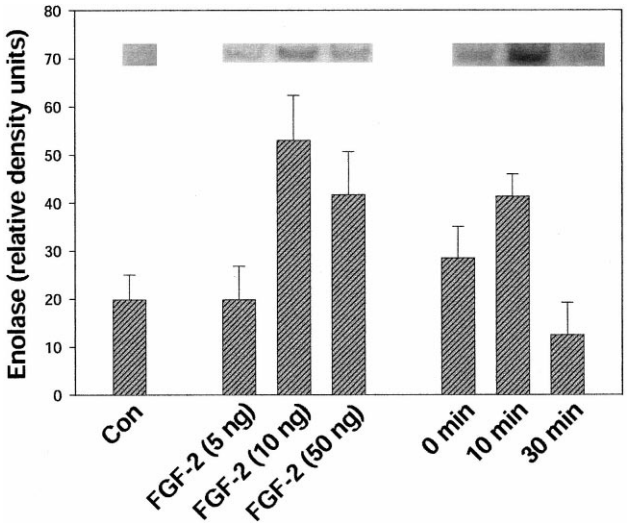

Fig. 4. Quantitation of Src kinase activity. MCF-7 cells were exposed to different concentrations (0, 5, 10, 50 ng/ml) of FGF-2 and for 0, 10 or 30 min with 10 ng/ml FGF-2. Src kinase activity was determined by immunoprecipitation with the anti-Src monoclonal antibody 327 and in vitro kinase assays performed using enolase as a substrate. The density of the enolase bands was then quantitated using calibrated densitometry. Representative enolase bands appear above the graph. Each bar represents the mean+S.D. of six replicate cell preparations.

activation. Because the Src protein can be phosphorylated on tyrosine in an inactive, as well as an active state [25], we used an in vitro kinase assay in the manner of LaVallee and coworkers [21] to determine the effects of FGF-2 on Src activity. The activity of Src was not stimulated by 5 ng/ml FGF-2 in the MCF-7 cells, but was markedly stimulated by 10 ng/ml; at 50 ng/ml it was already starting to be inhibited (Fig. 4). Maximal activity for the FGF-2 was reached within 10 min, whereafter it declined. Quantitative fluorimaging results using the ECL+visualizing reagent demonstrated that although there were changes in Src activities brought about by FGF-2, there were no major changes in the general Src pool at any of the times or concentrations tested (data not shown).

To visualize MAP kinase activation, extracts of MCF-7 cells were immunoprecipitated with an ERK1-specific antibody and immunoblotted for phosphotyrosine. MAP kinases (p42, p44) were slightly phosphorylated in control, unstimulated cells, whereas treatment with FGF-2 resulted in a rapid and transitory activation of MAP kinase (Fig. 3C). To demonstrate equal loading of proteins, the blot was stripped and reprobed with a MAP kinase antibody, which recognized both the phosphorylated and non-phosphorylated forms. The use of specific ERK2 antibodies showed the same result, although the signal was less strong (data not shown).

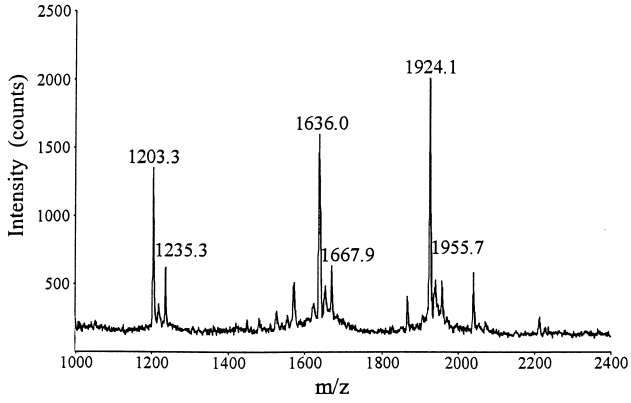

Fig. 5. MALDI-TOF peptide mass fingerprint spectrum of the 30-kDa phosphotyrosyl protein regulated by FGF-2. The band corresponding to the 30-kDa phosphotyrosyl protein that we found regulated by FGF-2 (indicated by an arrow in Fig. 2) was cut out from a silver-stained gel of anti-phosphotyrosine immunoprecipitated material and submitted to trypsin digestion and MALDI-TOF mass spectrometry as described.

3.4. Detection of cyclin D2 tyrosine phosphorylation induced by FGF-2

As shown in Fig. 2, there was a major increase in the tyrosine phosphorylation of a 30-kDa protein induced by FGF-2 stimulation. None of the antibodies that we used recognized this 30-kDa protein. Consequently, we used mass spectrometry to identify this protein. The band corresponding to the 30-kDa protein was cut out and submitted to in-gel trypsin digestion. MALDI-TOF analysis revealed a spectrum of tryptic peptides (Fig. 5). This allowed the identification of the 30-kDa protein as the cyclin D2, with 4/6 matched tryptic peptides covering 10% of the protein sequence (Table 1). MALDI-TOF mass spectrometry is a powerful method for characterization of proteins after database searching [22,23,26]. However, this method does not monitor the entire population of peptides resulting from the tryptic digestion of a protein. This results from differences in the charge and mass of the ion species generated after trypsin digestion, which leads to the preferential protonation of the low mass, and more basic peptides. It should be emphasized that only small or basic peptides can be detected in MALDI-TOF. Thus, the identification of a protein is based on the fingerprint of the molecule obtained after trypsin digestion, and not on the characterization of the entire molecule. Differences in amino acid sequence between the different categories of cyclin D (1, 2 or 3) are important and the fragments that we have obtained specific identify the D2 form. In particular, we have observed a tryptic fragment of 1667.90 Da corresponding to the N-terminal end of cyclin D2, which does not exist in cyclins D1 and D3. The tyrosine phosphorylation of cyclin D2 by FGF-2 was further

Table 1
Peptide mass fingerprint of the 30-kDa phosphotyrosyl protein

Masses submitted	MH^+ matched	Delta Da	Start amino acid	End amino acid	Peptide sequence consistent with mass	Modifications
1636.00	1636.8157	0.2157	180	193	(K) HAQTFIALCATDFK (F)	–
1667.90	1667.8362	0.0638	1	13	(-) MELLCHEVDPVRR (A)	–
1924.10	1923.9131	0.1869	57	71	(R) RMVATWMLEVCEEQK (C)	–
1955.70	1955.9029	−0.2029	57	71	(R) RMVATWMLEVCEEQK (C)	2 Met-ox

Tryptic fragment masses obtained by MALDI-TOF analysis (Fig. 5) are listed. The amino acid sequences shown are deduced from the trypsin fragments after database searching. Abbreviations: Met-ox: oxidated methionine.

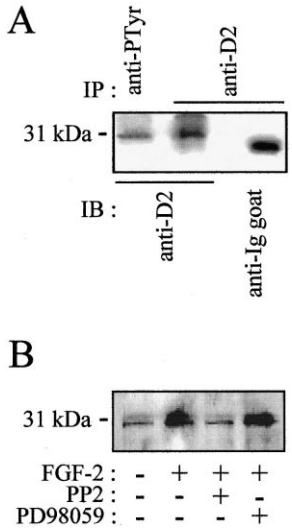

Fig. 6. A: Immunoblotting with anti-cyclin D2. MCF-7 cells were stimulated by FGF-2 (10 ng/ml) for 10 min and cell lysates immunoprecipitated with anti-phosphotyrosine or anti-D2 antibodies. After SDS–PAGE, immunoblotting was performed with anti-D2 antibodies using anti-goat antibodies as control. B: Tyrosine phosphorylation of cyclin D2. MCF-7 cells were exposed to FGF-2 (10 ng/ml) for 10 min with or without PD98059 (20 μM) or PP2 (50 nM). Cell lysates were immunoprecipitated with anti-D2 antibodies and immunoblotting performed with anti-phosphotyrosine antibodies.

confirmed using specific antibodies. As shown in Fig. 6A, when we performed immunoprecipitation with either anti-phosphotyrosine or anti-cyclin D2 antibodies, followed by immunoblotting for cyclin D2, we detected the same band at 30 kDa. In addition, immunoprecipitation of cyclin D2 followed by immunoblotting with anti-phosphotyrosine antibodies confirmed that tyrosine phosphorylation of cyclin D2 is induced by FGF-2 and that this phosphorylation is inhibited by PP2 but not by PD98059 (Fig. 6B).

4. Discussion

In this study, we have examined the intracellular signaling pathway involved in the FGF-2 mitogenic stimulation of the breast cancer cell line MCF-7. Immunoblot analysis of phosphotyrosyl proteins from MCF-7 cells revealed changes in the tyrosine phosphorylation of several bands. Specifically, FGF-2 induced an increase in the activation of FGFR, FRS2, Src and MAP kinase. We further detected a 30-kDa protein whose tyrosine phosphorylation was induced by FGF-2; this we identified with mass spectrometry and immunoblotting as cyclin D2.

The FGFR complement was rapidly tyrosine phosphorylated after FGF-2 stimulation of MCF-7 cells. To date, four distinct members of the FGFR family have been described (flg or FGFR-1, bek or FGFR-2, FGFR-3 and FGFR-4) and are known to be expressed in breast cancer cells [6]. Our experiments were performed with an antibody that recognized FGFR-1, but due to the structural similarities between FGFR family members, we cannot exclude other receptor isotypes. FGFR activation by tyrosine phosphorylation leads to the direct or indirect binding of SH2 domain-containing proteins which are crucial for mitogenic intracellular signaling pathways [2]. A role for Src in FGF-2 mitogenic signaling has been demonstrated [27] and members of the Src family participate in the control of a variety of cellular functions, including response to growth factors [28]. We have observed a small but significant increase of Src tyrosine phosphorylation after FGF-2 stimulation (data not shown). However, Src protein can be phosphorylated on tyrosine in an inactive as well as active state [25], so the increase of tyrosine phosphorylation that we observed after FGF-2 stimulation cannot be linked to the activation of Src tyrosine kinase activity. Using an in vitro kinase assay in the manner of LaVallee and coworkers [21], we demonstrated that Src kinase activity is indeed induced in MCF-7 cells by FGF-2. In addition, the pharmacological inhibition of Src that we report here using herbimycin and PP2 indicates that the tyrosine kinase activity of Src is required for the mitogenic stimulation by FGF-2 in breast cancer cells. In contrast, pharmacological inhibition of PI3 kinase activity by wortmannin caused only a slight decrease in the FGF-2 mitogenesis and had no effect on the phosphorylation of glycogen synthase kinase 3 (GSK3; data not shown), which is known to be a direct target of this enzyme [29]. Therefore PI3 kinase does not seem to play a significant role in the FGF-2-induced proliferation of MCF-7 cells.

Downstream of the activated receptors, we observed the tyrosine phosphorylation of FRS2 in response to FGF-2 stimulation. FRS2, a membrane-anchored docking protein, has been shown to play a major role in mediating the signal from activated FGF receptors to the Ras/MAP kinase cascade. Ong et al. (2000) have recently shown that the PTB domain of FRS2 associates with the juxtamembrane region of FGFR-1 [30]. Moreover, FRS2 tyrosine phosphorylation seems to be essential for the FGF mitogenic signaling pathway because inhibition of FRS2 reduces activation of MAP kinase [31] and the lack of FRS2 tyrosine phosphorylation in MCF-7 Ras is correlated with an inhibition of the proliferation induced by FGF-2 [20]. Interestingly, FRS2 is also a substrate for the nerve growth factor (NGF) receptor $p140^{TrkA}$ [32]. We have recently demonstrated that NGF is also mitogenic for breast cancer cells through the tyrosine kinase activity of $p140^{TrkA}$ and the activation of the MAP kinase cascade [24]. This suggests that mitogenic stimulation of both FGF-2 and NGF requires the activation of the Ras/MAP kinase pathway in breast cancer cells. However, it should be noted that, in contrast to FGF-2, herbimycin A and PP2 do not exhibit any inhibiting activity for NGF-induced mitogenesis, whereas wortmannin does (unpublished data). These data confirm significant differences in the FGF-2 and NGF mitogenic signaling pathways despite their common requirement for the Ras/MAP kinase cascade.

The MAP kinase pathway is considered to be essential for the activation of proliferation. The MAP kinases are known to be overexpressed in human breast cancer cells and abnormalities in this signaling pathway play an important role in growth deregulation of these cells [3]. We have shown that FGF-2 mitogenic signaling does induce a transient activation of the MAP kinase cascade. A transient activation of this cascade is usually reported during mitogenic stimulation [3], although it has also been shown that constitutive MAP kinase activation (induced by Ras overexpression) can lead to an inhibition of proliferation in MCF-7 cells [33]. In our experiments, the requirement for activated MAP kinase is supported by the complete inhibition of FGF-2-induced proliferation in

the presence of the MEK inhibitor, PD98059. Activation of MAP kinase is necessary for the NGF-induced stimulation of MCF-7 cell proliferation [24] whereas mitogenic stimulation by insulin-like growth factor-1 (IGF-1) is independent of the MAP kinase cascade and instead requires PI3 kinase activation [34]. These differences between IGF-1 and FGF-2 signaling indicate that the MAP kinase cascade is not always necessary for the stimulation of proliferation and that the signaling pathways used by IGF-1 and FGF-2 in breast cancer cells are clearly distinct.

The main consequence of the activation of MAP kinases is their translocation into the nucleus and the activation of transcription factors. This leads to changes in gene expression which ultimately result in the stimulation of cell proliferation. Progression through the cell cycle is under the strict control of cyclins and their catalytic subunits, the Cdks (cyclin-dependent kinases). In most cases, mitogenic factors act during the G1 phase of the cell cycle, stimulating both the proliferation of quiescent cells arrested in G0 and the progression of cycling cells through the restriction point in late G1 [35]. One link between cell cycle progression and growth factor signaling is provided by the D-type cyclins whose genes are induced as a secondary response to mitogenic stimulation [36,37]. Although changes in cyclin D synthesis and activity have been studied during cell cycle progression [38], modifications in the tyrosine phosphorylation of these proteins have not been well described. Our data indicate that a major modification of tyrosine phosphorylation induced by FGF-2 involves a 30-kDa protein that we have identified as cyclin D2. The phosphorylation of cyclin D1 on tyrosine has already been described in osteosarcoma and lung epithelial cells [39], demonstrating that cyclins can be recognized as substrates for tyrosine kinases. The tyrosine phosphorylation of cyclin D2 that we have observed does not require MAP kinase activation as it was not affected by treatment with PD98059. In contrast, Src protein is a candidate kinase for cyclin D2 phosphorylation. Indeed, Src has been shown to phosphorylate cyclin D1 in vitro [39] and, in our experiments, herbimycin A and PP2 decreased the tyrosine phosphorylation induced by FGF-2. Further experiments are necessary to precisely define the signaling pathway leading to cyclin D2 phosphorylation and the function of this post-transcriptional modification.

In conclusion, our data lead to the following model. In MCF-7 human breast cancer cells, the signaling pathway for FGF-2 requires FGFR and FRS2 phosphorylation, as well as Src and MAP kinase activation. In addition, FGF-2 mitogenic stimulation induces the tyrosine phosphorylation of cyclin D2, possibly through a mechanism involving Src. We therefore hypothesize that cyclin D2 phosphorylation is an important signaling event for the proliferation of breast cancer cells.

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