Fibroblast Growth Factor-2 Stimulation of p42/44MAPK Phosphorylation and IκB Degradation Is Regulated by Heparan Sulfate/Heparin in Rat Mammary Fibroblasts

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Fibroblast growth factor-2 (FGF-2) interacts with a dual receptor system consisting of tyrosine kinase receptors and heparan sulfate proteoglycans (HSPGs). In rat mammary fibroblasts, FGF-2 stimulated DNA synthesis and induced a sustained phosphorylation of p42/44MAPK and of its downstream target, p90RSK. Moreover, FGF-2 also stimulated the transient degradation of IκBa and IκBβ. PD098059, a specific inhibitor of p42/44MAPK phosphorylation, inhibited FGF-2-stimulated DNA synthesis, phosphorylation of p42/44MAPK and p90RSK, and degradation of IκBβ. In contrast, in chlorate-treated and hence sulfated glycosaminoglycan-deficient cells, FGF-2 was unable to stimulate DNA synthesis. However, FGF-2 was able to trigger a transient phosphorylation of both p42/44MAPK and p90RSK, which peaked at 15 min and returned to control levels at 30 min. In these sulfated glycosaminoglycan-deficient cells, no degradation of IκBa and IκBβ was observed after FGF-2 addition. However, in chlorate-treated cells, the addition of heparin or purified HSPGs simultaneously with FGF-2 restored DNA synthesis, the sustained phosphorylation of p42/44MAPK and p90RSK and the degradation of IκBa and IκBβ. These results suggest that the HSPG receptor for FGF-2 not only influences the outcome of FGF-2 signaling, e.g. cell proliferation, but importantly regulates the immediate-early signals generated by this growth factor.

Fibroblast growth factor-2 (FGF-2,1 basic FGF) possesses a dual receptor system consisting of tyrosine kinase receptors (FGF receptors (FGFRs)) (1) and heparan sulfate (HS) proteoglycans (HSPGs) (2). The interaction of FGF-2 with HS is required for the stimulation of cell proliferation (3, 4) and depends on specific patterns of sulfation of the polysaccharide (5). The interaction of FGF-2 with the FGFR is thought to induce receptor dimerization and activation of the FGFR tyrosine kinase (6–9), which then phosphorylates a series of targets. The latter activate downstream signaling pathways, including those of the mitogen-activated protein kinases (MAPKs). The activation of two members of the MAPK family, ERK1 and ERK2, also called p44 and p42, respectively, occurs via phosphorylation by a cytoplasmic dual-specificity MAPK kinase, MEK1, and is often associated with the stimulation of cell proliferation (10–12). The activated p42/44MAPK will, in turn, phosphorylate an array of cellular substrates, including downstream Ser/Thr effector kinases such as p90RSK (protein of 90 kDa from the ribosomal subunit S6 kinase gene), also known as RSK1 (12–15), p42/44MAPK and p90RSK also phosphorylate and activate nuclear transcription factors, e.g. c-Fos (12, 16), and enable the translocation of cytoplasmic transcription factors such as NF-κB from the cytoplasm to the nucleus (12, 17). NF-κB was first identified as a family of transcription factors that are activated by various stimuli such as cytokines (18, 19) or growth factors, including FGF-2 (20, 21). In unstimulated cells, inactive NF-κB dimers are retained in the cytoplasm by interaction with the IκB inhibitory proteins. Following cell stimulation, IκB proteins are phosphorylated, ubiquitinated, and degraded by the 26 S proteasome (18, 22). Consequently, liberated NF-κB dimers are translocated to the nucleus, where they will regulate the transcription of specific target genes (12, 18, 23). Kinases responsible for the phosphorylation of the N terminus of IκB proteins include the IκB kinase complex (24–26), casein kinase II (27, 28), and p90RSK (29, 30). Recently, the activation of p90RSK was shown to mediate the inactivation of IκB and the subsequent activation of NF-κB in response to phorbol ester (30) and p53-induced cell death (17).

Although the dependence of FGF-2-stimulated cell proliferation on particular structures in HS is well established (2, 5), it has become apparent that FGF-2 can interact with the FGFR in the absence of HS (31–34). A question raised by these results is whether, in the absence of its HS receptor, FGF-2 is able to stimulate all immediate-early signaling pathways or whether there are quantitative and qualitative differences in the early signals generated by FGF-2 in the absence and presence of its HS receptor. To address this question, we have used chlorate (35–37) to inhibit the sulfation of HSPGs in rat mammary (Rama-27) fibroblasts. In control cells, FGF-2 induced a sustained phosphorylation of p42/44MAPK and p90RSK, and at least the former was associated with the subsequent stimulation of DNA synthesis. Interestingly, FGF-2 also stimulated the MEK1-dependent degradation of IκBa and IκBβ. In marked contrast, in chlorate-treated and hence sulfated glycosaminoglycan-defi-
cient cells, FGF-2 could stimulate a transient early phosphorylation of p42/44MAPK and p90RSK. However, in chlorate-treated cells in the presence of HS or heparin, FGF-2 induced the sustained phosphorylation of p42/44MAPK and p90RSK and the degradation of IxBa and IxBβ and stimulated DNA synthesis. These results suggest that the HS receptor for FGF-2 not only influences the eventual outcome of FGF-2 signaling, e.g. cell proliferation, but importantly can regulate the immediate-early signals generated by the growth factor.

EXPERIMENTAL PROCEDURES

Materials and Cells—FGF-2 was obtained from R&D Systems (Abingdon, United Kingdom), and mouse epidermal growth factor was obtained from Pepsys (Liverpool, UK). Reagents for electrophoresis were purchased from Bio-Rad. Cell culture reagents, including sulfate-free Dulbecco’s modified Eagle’s medium, were obtained from Life Technologies, Inc. Antibodies against dually phosphorylated p42/44MAPK (Thr(P)183/202/Tyr(P)185/204), against phosphorylated p90RSK, and against IxBα were purchased from New England Biolabs (Hitchin, UK). The antibody against IxBβ was from Santa Cruz Biotechnology (Colne, UK). Porcine intestinal mucosal heparin was from Sigma. The Rama-27 cells are rat fibroblasts derived from normal rat mammary gland (38). Cell Culture and DNA Synthesis—Rama-27 fibroblasts were cultured as described previously (38). For DNA synthesis assays (39), fibroblasts were seeded in 24-well plates and allowed to attach for 24 h. After rinsing twice with phosphate-buffered saline (PBS), they were grown in serum-free medium containing 0.2% (w/v) bovine serum albumin for 24 h before adding growth factors. [3H]-thymidine (40 μCi/ml; ICN, Basingstoke, UK) was added directly to the culture medium 18 h later. After a 3-h incubation, DNA was precipitated with 5% (v/v) trichloroacetic acid, and the incorporated radioactivity was measured by liquid scintillation counting. Sulfated glycosaminoglycan-deficient Rama-27 cells were prepared as described previously (39–42). Briefly, cells were incubated for 4 h in sulfate-free Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) dialyzed fetal calf serum and 15 mM NaClO₃. Following trypsinization, the cells were seeded in 24-well plates as described above, except that sulfate-free Dulbecco’s modified Eagle’s medium supplemented with 15 mM NaClO₃ was used throughout. Heparin and purified HSPGs were added directly to the cell culture medium as indicated.

Western Blotting—Rama-27 fibroblasts were seeded at equal densities in 10-cm diameter culture dishes and then treated as for the DNA synthesis assay up to and including the addition of growth factor. The times indicated, Rama-27 cells were washed twice with ice-cold PBS; lysed in 300 μl of 250 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 0.006% (w/v) bromphenol blue, 2% (v/v) β-mercaptoethanol, and protease inhibitor mixture (Roche Molecular Biochemicals, Lewes, UK); scraped with a rubber policeman; and collected in 1.5-ml tubes. Identical amounts of cell pellets were analyzed by SDS-PAGE and transferred to nitrocellulose membrane and probed with the primary antibody, which was detected by means of secondary peroxidase-conjugated polyclonal antibodies and visualized with the SuperSignal chemiluminescent system (Pierce and Warriner, Chester, UK). Antigen-antibody complexes were developed using 2:300 volume of solution containing 1% (v/v) H₂O₂ and 0.5% (v/v) H₂O₂ and 0.5% (v/v) Triton X-100. Proteoglycans were purified by applying these samples onto a 20-ml DEAE-Sephrose Fast Flow column (Amersham Pharmacia Biotech). Strongly anionic molecules, including proteoglycans, were eluted with PBS containing 2 M NaCl. The 2 M NaCl fractions were desalted on a 75-ml Sephadex G-25 column (Amersham Pharmacia Biotech) equilibrated in 100 mM NH₄HCO₃. After freeze-drying, the powders were weighed, and the resulting HSPGs were concentrated on a 1-ml DEAE Fast Flow column, and the 2 M NaCl fraction was desalted using a 10-ml Sephadex G-25 column as described above. After freeze-drying, the powders were weighed, and stock solutions were made at 1 mg/ml HSPGs in PBS. Purified HSPGs were always boiled 5 min before adding to the culture medium (39, 42).

RESULTS

Effect of FGF-2 on DNA synthesis and phosphorylation of p42/44MAPK and p90RSK in Rama-27 fibroblasts. A, effect of PD098059 on FGF-2-induced proliferation. Rama-27 cells were cultured for 24 h in serum-free medium and then incubated with 10 ng/ml FGF-2 as described under “Experimental Procedures.” PD098059 was added where shown 15 min before FGF-2 addition. Results are the mean ± S.E. of three different experiments. B, effect of PD098059 on p42/44MAPK and p90RSK phosphorylation induced by 10 ng/ml FGF-2 after 15 min. p42/44MAPK and p90RSK phosphorylation were detected by Western blotting as described under “Experimental Procedures.” C, time course of p42/44MAPK and p90RSK phosphorylation following FGF-2 addition. FGF-2 (10 ng/ml) was added to Rama-27 fibroblasts for 0–60 min as indicated, and phosphorylated proteins were detected by Western blotting using phospho-specific antibodies (see “Experimental Procedures”).
MAPK (Fig. 1) inhibits MEK1 and consequently the phosphorylation of p42/44 at least 60 min (Fig. 2). PD098059, which was added where shown 15 min before FGF-2 addition. Proteins were extracted 30 min after the addition of FGF-2.

The antibody to phosphorylated p90RSK, a downstream target of p42/44 MAPK (12–15), to the level observed in unstimulated cells (Fig. 1B). The kinetics of phosphorylation of p42/44MAPK and p90RSK were then examined (Fig. 1C). In Rama-27 fibroblasts, the dual phosphorylation of p42/44MAPK was stimulated 5 min after the addition of 10 ng/ml FGF-2 and reached a maximum level at 10 min. Thirty minutes after the addition of FGF-2, the dual phosphorylation of p42/44MAPK had decreased to near half-maximal levels, and this level of phosphorylation was maintained until at least 60 min (Fig. 1C). The antibody to phosphorylated p90RSK recognized a major band at 90 kDa (Fig. 1, B and C), and after a long exposure of polypeptides separated on 10% (v/v) polyacrylamide gels, two weaker bands of lower molecular mass were also apparent (Fig. 1C) (11, 46). Phosphorylation of p90RSK was observed within 5 min of addition of FGF-2 and increased slowly to reach a maximum after 15 min. After that time, the level of phosphorylation decreased to a lower level, which was also maintained to the end of the experiment.

**Effect of FGF-2 on Degradation of IκB Proteins**—In various systems, after phosphorylation mediated by a variety of kinases, including IκB kinases, casein kinase II, and p90RSK (28–30), IκB proteins are recognized and degraded by the 26S proteasome (18, 23). In Rama-27 fibroblasts, the level of IκB protein began to decrease at 30 min after FGF-2 addition and reached a minimum at 45 min, before returning to basal levels at 60 min (Fig. 2A). After FGF-2 addition, the degradation of IκBβ was almost maximal at 30 min and, with IκBα, returned to basal levels at 60 min (Fig. 2A). PD098059, which inhibits MEK1 and consequently the phosphorylation of p42/44MAPK (Fig. 1B) (44, 45), also inhibited the degradation of IκBβ (Fig. 2B). This result suggests that FGF-2 stimulates the degradation of IκB proteins by a MEK1-dependent mechanism.

**Signaling Events Induced by FGF-2 on Chlorate-treated and Sulfated Glycosaminoglycan-deficient Rama-27 Fibroblasts**

Effect of FGF-2 on DNA Synthesis and Phosphorylation of p42/44MAPK and p90RSK in Chlorate-Treated Cells—As previously observed, FGF-2 strongly stimulated DNA synthesis in Rama-27 cells (Figs. 1A and 3). In contrast, FGF-2 did not stimulate DNA synthesis when the cells were grown in the presence of 15 mM sodium chloride (Fig. 3), even at a dose as high as 60 ng/ml (data not shown). Chlorate itself did not affect the intrinsic ability of the cells to mount a growth stimulatory response since epidermal growth factor, a growth factor that does not bind to HSPGs, stimulated DNA synthesis in chlorate-treated Rama-27 cells (Fig. 3). Moreover, the addition of 7.5 mM Na₂SO₄, which will relieve the inhibition of PAPS synthesis by 15 mM chlorate (35–37) and hence enable the synthesis of sulfated glycosaminoglycan chains by the cells, restored the growth stimulatory effect of FGF-2 (Fig. 3). The addition of soluble heparin simultaneously with FGF-2 also restored the growth stimulatory effects of FGF-2 in chlorate-treated fibroblasts (Fig. 3). Heparin alone had no effect on DNA synthesis. Similarly, the addition of Rama-27 fibroblast-derived HSPGs, which were boiled for 5 min before addition to the cell culture medium, restored the growth stimulatory activity of FGF-2 in a dose-dependent manner (Fig. 4).

In chlorate-treated and hence sulfated glycosaminoglycan-deficient Rama-27 cells, stimulation of the dual phosphorylation of p42/44MAPK was nevertheless apparent 15 min after the addition of FGF-2. Moreover, 30 min after the addition of FGF-2, the level of dually phosphorylated p42/44MAPK declined, and by 60 min, it had returned to the basal levels observed in unstimulated cells (Fig. 5A). Thus, in the absence of sulfated glycosaminoglycans, although FGF-2 could not stimulate DNA synthesis (Figs. 3 and 4), it could elicit a transient stimulation of the dual phosphorylation of p42/44MAPK (Fig. 5). When heparin was added simultaneously with FGF-2 to chlorate-treated Rama-27 cells, stimulation of the dual phosphorylation of p42/44MAPK was apparent at 10 min and reached a maximum at 15 min. The level of dually phosphorylated p42/44MAPK subsequently declined to the half-maximal level at 20 min, a level that was then sustained for 120 min. Similarly, in the presence of HSPGs and FGF-2, stimulation of the dual phosphorylation of p42/44MAPK was maximal at 15 min and then declined to half of this maximal level by 30 min. This level was maintained to the end of the experiment. Interestingly, when heparin was added alone to chlorate-treated Rama-27 fibroblasts, a transient dual phosphorylation of p42/44MAPK was also induced. Dually phosphorylated p42/44MAPK was apparent 10 min after...
the addition of heparin and reached maximal levels after 15 min, before declining to basal levels by 30 min. This effect of heparin is presumably due to its interaction with heparin receptors on the cells (47, 48).

The phosphorylation of p90RSK, a downstream target of p42/44MAPK activation following FGF-2 addition. Rama-27 fibroblasts were stimulated with FGF-2 (10 ng/ml), purified HSPGs (3 μg/ml), or heparin (30 ng/ml) for 0–120 min as indicated, and activated p42/44MAPK was detected by Western blotting with a phospho-specific antibody directed against dually phosphorylated p42/44MAPK (Thr202/Tyr204) (see “Experimental Procedures”). B, time course of p90RSK phosphorylation following exposure to FGF-2 at 10 ng/ml, to FGF-2 and heparin at 30 ng/ml, and to heparin alone at 30 ng/ml. Phosphorylated p90RSK was detected by Western blotting as described under “Experimental Procedures.”

**DISCUSSION**

To stimulate cell proliferation, FGF-2 must interact with a dual receptor system consisting of tyrosine kinase receptors (FGFRs) and a polysaccharide receptor, HS (3, 4). The original explanation for the dependence of the proliferative activity of FGF-2 on HS was that the HS was required for a high affinity interaction between FGF-2 and the FGFR. However, a body of evidence has accumulated that indicates that FGF-2 is able to interact with high affinity with the FGFR (39, 43) and the phosphorylation of p90RSK detectable at 15 min after the addition of heparin, but which declined to basal levels by 30 min (Fig. 5B). Effect of FGF-2 on Degradation of IκB Proteins in Chlorate-Treated Cells—In chlorate-treated cells, FGF-2 did not cause any change in the level of IκBo or IκBβ (Fig. 6A and B). In contrast, after the addition of FGF-2 and heparin to chlorate-treated Rama-27 fibroblasts, the level of IκBo decreased at 30 min before returning to basal levels at 60 min. The changes in the level of IκBβ were more marked, as observed in control cells (Fig. 2A). Thus, 15 min after the addition of FGF-2 and heparin to chlorate-treated cells, IκBβ declined to a low level (Fig. 6B).

The low level of IκBβ was maintained at 30 min, but by 60 min, the level of IκBβ, like that of IκBo, had returned to basal levels (Fig. 6B).
which is well established in many cellular systems (3, 4, 41, 49, 50).

In chlorate-treated Rama-27 cells, FGF-2 stimulated the early peak of p42/44MAPK dual phosphorylation, but failed to maintain the subsequent sustained plateau of phosphorylation of p42/44MAPK (Fig. 2). As in control cells (Fig. 1C), the dual phosphorylation of p42/44MAPK was faithfully mirrored by the phosphorylation of p90RSK (Fig. 5B). This result agrees with those that show an early (7–10 min) stimulation of p42/44MAPK dual phosphorylation by FGF-2 in cells lacking sulfated HS (34, 48). Therefore, the early peaks of dual phosphorylation of p42/44MAPK and the consequent phosphorylation of p90RSK are not dependent on HS. In the presence of endogenous HSPGs, FGF-2 elicits an early peak of phosphorylation of p42/44MAPK and p90RSK, and the phosphorylation of these kinases is subsequently maintained at half-maximal levels. Moreover, we observed that PD098059 inhibits DNA synthesis and the phosphorylation of p42/44MAPK (Fig. 2). As in control cells (Fig. 1), the dual phosphorylation of p42/44 MAPK, which is corrected by the addition of exogenous HSPGs or heparin. Thus, HS would appear to regulate the kinetics of phosphorylation of the MAPKs by FGF-2.

In the absence of HS, the interaction between FGF-2 and the FGFR is of high affinity (31–33), which explains why FGF-2 is able to stimulate the transient dual phosphorylation of p42/44MAPK. One mechanism whereby HS could elicit its effects would be to reduce the deactivation of the ligand-bound FGFR and thus prolong the signaling capacity of the receptor-ligand complex. Two studies have suggested that the rate of dissociation of FGF-2 from the FGFR is considerably slower in the presence of HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedly dependent on HS (32, 54). Other evidence suggests that although the endocytic rates of FGF-2 are not markedl...


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