THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 39, Issue of September 29, pp. 30009–30018, 2000

Printed in U.S.A.

The Proliferative and Migratory Activities of Breast Cancer Cells Can Be Differentially Regulated by Heparan Sulfates*

Received for publication, April 11, 2000, and in revised form, June 2, 2000
Published, JBC Papers in Press, June 20, 2000, DOI 10.1074/jbc.M003038200

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To explore how heparan sulfate (HS) controls the responsiveness of the breast cancer cell lines MCF-7 and MDA-MB-231 to fibroblast growth factors (FGFs), we have exposed them to HS preparations known to have specificity for FGF-1 (HS glycosaminoglycan (HSGAG A)) or FGF-2 (HSGAGB). Proliferation assays confirmed that MCF-7 cells were highly responsive to FGF-2 complexed with GAGB, whereas migration assays indicated that FGF-1/HSGAGA combinations were stimulatory for the highly invasive MDA-MB-231 cells. Quantitative polymerase chain reaction for the levels of FGF receptor (FGFR) isoforms revealed that MCF-7 cells have greater levels of FGFR1 and that MDA-MB-231 cells have greater relative levels of FGFR2. Cross-linking demonstrated that FGF-2/HSGAGB primarily activated FGFR1, which in turn up-regulated the activity of mitogen-activated protein kinase; in contrast, FGF-1/HSGAGA led to the phosphorylation of equal proportions of both FGFR1 and FGFR2, which in turn led to the up-regulation of Src and $p125^{FAK}$. MDA-MB-231 cells were particularly responsive to vitronectin substrates in the presence of FGF-1/HSGAGA, and blocking antibodies established that they used the $\alpha_v\beta_3$ integrin to bind to it. These results suggest that the clustering of particular FGFR configurations on breast cancer cells induced by different HS chains leads to distinct phenotypic behaviors.

Members of the fibroblast growth factor (FGF)[1] family have been shown to be intimately involved in such diverse processes as germ layer formation, neurogenesis, angiogenesis, and wound healing (1–4). They are also involved in many forms of cancer cell growth and metastasis (5, 6). There have been at least 17 different isoforms of this factor so far described, and one of the major themes of FGF biology has been the ongoing effort to discover how each factor delivers a biologically relevant signal in ways that are tissue-specific. This problem is compounded by the fact that there are four different major forms of FGF receptor (FGFR) tyrosine kinase, each of which can be spliced by differential exon usage into multiple species (7, 8). Exactly which form of FGF binds to which FGFR to drive a particular biological outcome in any tissue remains unclear.

It is known that FGFs use an accessory form of receptor to bind to their cognate cell-surface receptors more efficiently. These are heparan sulfate (HS) glycosaminoglycan (HSGAG) sugars, which are usually found as part of transmembrane or cell-associated proteoglycan complexes (9). Exactly how HS interacts with the FGFs so that they can bind to their high affinity receptors remains controversial (7); it may simply be the case that they passively dimerize the FGFs, or FGFRs, so that signal transduction can occur (10). Recently, more interesting hypotheses have asserted that domains within HS chains are able to cross-link FGFs with FGFRs to form activated ternary complexes (11–17), perhaps with some measure of specificity (18–21). This is coincident with the variable expression of FGFRs as either long three-Ig loop forms or shorter two-loop forms seen more often in malignant cells (22); the shorter forms of receptor bind HS more avidly and are thus activated more easily than the native longer forms.

FGFs, particularly the archetypal forms FGF-1 and FGF-2, have been detected within human breast tumors as well as normal breast epithelial and myoepithelial cells (23–26). They have been identified within developing mammary glands both in rodents and humans (27). FGF-2 is mitogenic for breast epithelial cell lines such as MCF-7 and T-47D, although hormone-independent MDA-MB-231 cells do not become sensitive to FGFs until inhibitory HS is removed from their surfaces by chlorate treatment (28). Biochemical purification of HS from both MCF-7 and MDA-MB-231 cells reveals very different HS profiles, lending support to the idea that the behavior of breast tumor cells in an FGF-rich environment is contingent upon these accessory sites (28). This hypothesis has recently received important support from biosensor assays (29), where it was found that FGF-2-binding HS occurred with two separable activities: one strongly held FGF-2 away from cognate cell receptors, and the other, of lower affinity, appeared to be permissive for FGF-2 and its receptors.

We have previously purified forms of HS from a neuroepithelial cell line that have affinities for either FGF-1 or FGF-2, depending on the phenotypic state of the cells (19, 20, 30). When the cells are maintained in proliferative phase, their HS chains remain relatively short and moderately sulfated; as they begin to differentiate, the chains become longer and more complex, with greater degrees of sulfation. These chains have been purified and shown to have FGF-potentiating activity on a wide variety of other cell types, including amphibian cells (31).

* This work was supported by grants from the Ligue contre le Cancer, the Région Nord-Pas de Calais, the French Ministry of Education, the Rhone Poulenc Rohrer Corp., the Traveling Fellowship of the Australian Academy of Science, and the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HS, heparan sulfate; GAG, glycosaminoglycan; HSGAG, heparan sulfate glycosaminoglycan; RT-PCR, reverse transcription-polymerase chain reaction; MAPK, mitogen-activated protein kinase; FAK, focal adhesion kinase; DSS, disuccinimidyl suberate; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase.

The aim of this study was to explore the differences in response to FGFs that could be induced in breast cancer cells by HS preparations with known specificities. Proliferation and migration assays were carried out on MCF-7 and MDA-MB-231 cells in the presence of GAGA, which potentiates the actions of FGF-1, or GAGB, which potentiates FGF-2 (20). FGFR profiles for each cell type were obtained by RT-PCR, and the activation of the receptors by FGF/GAG combinations was monitored by cross-linking, immunoprecipitation, and phosphotyrosine assays. The results indicate that the different HS preparations activate different combinations of FGFRs, which in turn leads to differential activation of MAPK, Src, FAK, and integrins and thus rates of breast cancer proliferation and migration. We conclude that HSGAGB interacts with FGF-2 to drive proliferation through FGFR1 and that HSGAGA interacts with FGF-1 to drive a mixed proliferation and migration signal through FGFR1/FGFR2 association. We conclude that extracellular HS can help direct the phenotypic behavior of breast cancer cells through the differential activation of FGFRs.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FGF-1 and FGF-2 were from either Sigma or Amgen. Minimal essential medium, fetal calf serum, HEPES, nonessential amino acids, penicillin, streptomycin, and trypsin/EDTA solutions were from Commonwealth Serum Laboratories (Melbourne) or eurobio (Paris). The Titan One-Step PCR system was from Roche Molecular Biochemicals. The digital image analysis system was from Zolltec (Iowa City). Anti-FGFR antibodies were from Santa Cruz Biotechnology, and anti-phosphotyrosine 4G10 antibodies were from Upstate Biotechnology, Inc. The isolation of HS chains with specificities for FGF-1 and FGF-2 has been fully described previously (18–20); ~2 mg of each HS species was required for this study. Briefly, 8×10^7 cells from 40–60 mice embryos of the appropriate ages were cultured in serum-free medium for harvesting and fractionation. The conditioned medium from either the embryonic day 10 or 12 cells was chromatographed on DEAE-Sephacel, and fractions were treated with neuraminidase, chondroitin ABC lyase, and Pronase. HS chains were removed from the core protein with $NaBH_4$, and samples were run on Sepharose CL-6B columns for sizing of the released HS chains. [^{3}H]Thymidine and [^{35}S]cysteine/methionine were from ICN. Heparitinase I (heparin-sulfate lyase, EC 4.2.2.8) was obtained from Seikagaku Kogyo Co. (Tokyo). Western blot bands were visualized with ECL$^+$ reagents (Amersham Pharmacia Biotech) and quantitated on a Storm Fluorimager (Molecular Dynamics, Inc.) using NIH ImageQuant software. Disuccinimidyl suberate (DSS) cross-linker was from Pierce. Integrin anti-α and anti-β subunit antibodies were from Upstate Biotechnology, Inc. and Life Technologies, Inc. Heparin (low molecular mass) was from Sigma. Matrigel was from Calbiochem.

Cell Culture—The human breast tumor cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection and routinely grown as monolayer cultures. They were maintained in improved modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 20 mM HEPES, 2 g/liter sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 50 μg/ml gentamycin sulfate, 1% nonessential amino acids, and 5 μg/ml insulin. Cells were maintained in 5% CO_2 in a humidified atmosphere at 37 °C. Cultures were washed three times in modified Eagle's medium and replaced with medium containing the appropriate ligands. Cells in exponential phase growth were washed twice with phosphate-buffered saline and starved for 24 h in fresh serum-free medium containing transferrin (30 μg/ml). Either FGF-1 or FGF-2 was then added at 5 ng/ml with nominated amounts of HS, and the cells were incubated for 24 h in 24-well plates. By this point S phase synthesis had reached a maximum, and 1.5 μM [^{3}H]thymidine was added to the cultures for a further 1 h; cells were then prepared for scintillation counting (19). In some experiments, MDA-MB-231 cells were pretreated with heparitinase I (0.05 IU/ml) for 3 h prior to the assay, with the same dose added upon addition of the FGF/HS combinations and every 8 h through the 24-h assay period.

Cell Migration Assays—Cells (2×10^5) were plated onto Matrigel-coated substrates (32) in 35-mm plastic tissue culture dishes and allowed to settle and adhere for 30 min within an incubator. They were then removed to a heated stage within a CO_2-controlled environment surrounding an Olympus IM35 inverted phase microscope. Cells were monitored over 150 min by time-lapse video recording (Panasonic TL260 set at 1 frame/6 s capture rate). Rates of cell movement were assessed by the digital image analysis system, which converted the video into the Quicktime format. The movement of 50 cells per treatment was assessed, and the whole experiment was repeated twice.

To assess which integrin receptor class might be involved in migration, anti-integrin blocking antibodies (100 μg/ml) were added to the medium as the cells were placed in the controlled environment surrounding the microscope stage. Movement was assessed as described above. To confirm specific blocking by the antibodies, cells were cultured on murine vitronectin, laminin, thrombospondin, or collagen IV (Sigma; all applied at a coating concentration of 10 μg/ml in phosphate-buffered saline for 2 h at room temperature). These were coated over a control layer of poly-L-ornithine (32) in the presence of soluble FGF/HS ligand combinations.

FGFR Isoform Quantitation—Amounts of each of the FGFR IIIb and IIIc isoforms were assessed by quantitative RT-PCR using the same primers as described previously (19). In addition, an extra primer spanning an intron (5′-AAGTCTCAGTAATCCTCTCAATCG-3′), directed against the N terminus of FGFR1, was used to assess the amounts of long three-Ig loop FGFR1 forms as opposed to the shorter two-Ig loop forms. Briefly, MDA-MB-231 and MCF-7 cells (5×10^7) were maintained in serum-free medium in 10-cm plastic dishes for 24 h, and their RNAs were extracted with TriPure Isolation reagent (Roche Molecular Biochemicals). RT-PCR (Titan One-Tube, Roche Molecular Biochemicals) was then performed with the appropriate receptor isoform primers. The same RNA batch preparation was used for all the FGFR determinations within a series. Each receptor isoform RNA isolate was then independently assessed for optimal cycling conditions for amplification, both by varying total RNA concentration and the number of amplification cycles according to the manufacturer's instructions. The cycling conditions for amplification were 2 min at 94 °C; 10 cycles at 94 °C for 30 s; annealing at 52 °C for 30 s; elongation at 68 °C for 45 s, followed by prolonged elongation for 7 min for 68 °C. For FGFR1, for a set 0.5 μg of total RNA, signals were in the linear range for 17–28 cycles; for FGFR2, 22–33 cycles; for FGFR3, 21–28 cycles; and for FGFR4, 35–45 cycles. Determination of the levels of PCR product was by capillary electrophoresis through 0.2-μm polyacrylamide-coated capillaries in a P/ACE System 2100 (Packard Instrument Co.) using laser-induced fluorescence (argon laser at 488 nm, with the emission collected through a band-pass filter at 520 nm using the exact method in the manufacturer's instructions (Roche Molecular Biochemicals)). Injection of samples was over 60 s, with separation at 175 V/cm. Levels of expression were quantified by integrating peak areas in relative fluorescence units for the primers (*i.e.* the PCR product as compared with the total fluorescence).

Receptor Activation—Binding of FGF/HSGAG combinations was carried out essentially according to the methods of Spivak-Kroizman *et al.* (33). Briefly, 5×10^6 cells, pre-exposed to [^{35}S]cysteine/methionine for 18 h, were washed and resuspended in serum-free medium and exposed to different FGF/HSGAG combinations as described below. Some preparations of MDA-MB-231 cells were treated with heparitinase I as detailed above to remove inhibitory HS species that might interfere with the binding results. After 60 min in the presence of the cross-linker DSS (0.3 mM in Me_2SO), the cells were pelleted (800 rpm for 5 min), lysed on ice (150 mM NaCl, 50 mM Tris, 0.1% SDS, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 2 mM benzamide, 2 mM *N*-ethylmaleimide, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and centrifuged for 5 min at 4 °C. The protein concentrations in the clarified lysates were determined with the BCA protein assay reagent (Bio-Rad). Lysates were then immunoprecipitated with anti-FGFR1, anti-FGFR2, anti-FGFR3, or anti-FGFR4 antibodies according to the methods of Lin *et al.* (34); boiled (5 min); and subjected to 5% SDS-PAGE. Gels were electroblotted onto nitrocellulose, and receptor complexes were revealed by autoradiography using Kodak XAR film. Bands were analyzed with NIH ImageQuant software.

Some cells in suspension were exposed to various FGF/HS combinations for a period of 5–30 min and lysed as described above. Lysates were clarified by the addition of protein A-Sepharose 4B (Pansorbin, Calbiochem), followed by centrifugation and protein concentration determination. Aliquots of the supernatant of each sample (1 mg) were incubated with anti-FGFR antibodies for 1 h on ice. Pansorbin was added for an additional 30 min, and the pellets were collected by centrifugation, washed, boiled in sample buffer, and subjected to SDS-PAGE. Gels were electroblotted onto nitrocellulose, and the membranes were probed with anti-phosphotyrosine monoclonal antibody 4G10 overnight at 4 °C, rinsed, and incubated with a horseradish peroxidase-conjugated anti-mouse IgG (Sigma) for 2 h at room temperature. Membranes were washed and visualized as described above with the ECL$^+$ reagent.

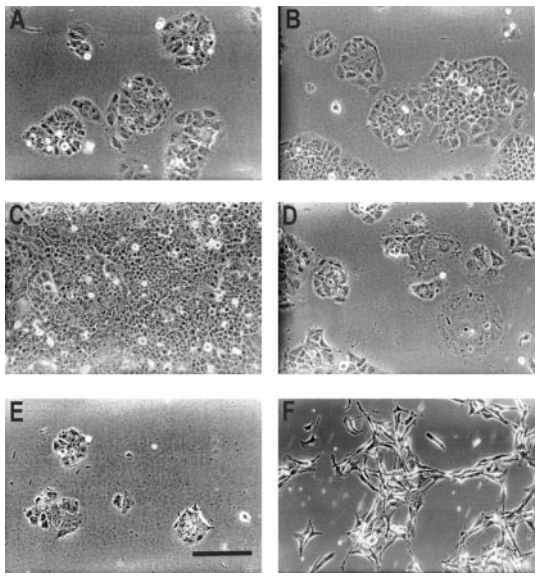

FIG. 1. **Morphologies of MCF-7 cells plated onto poly-L-ornithine substrates for 24 h in the presence of different FGF/HS combinations as revealed by phase-contrast microscopy.** *A*, control MCF-7 cells; *B*, MCF-7 cells plated in the presence of the incorrect combination FGF-2/HSGAGA; *C*, MCF-7 cells plated in the presence of the appropriate combination FGF-2/HSGAGB; *D*, MCF-7 cells plated in the presence of FGF-1 alone; *E*, MCF-7 cells plated in the presence of the incorrect combination FGF-1/HSGAGB; *F*, cells plated in the presence of the appropriate combination FGF-1/HSGAGA (note the elongated, stellate forms). In all cases where the incorrect combination of FGF-2/HSGAGA or FGF-1/HSGAGB was used, no effects were seen above controls. *Bar* = 50 μm.

Kinase Assays—MAPK activity was determined by immunoprecipitation of lysates with anti-ERK1 and anti-ERK2 antibodies as described above and revealed on gels with an *in vitro* assay using myelin basic protein (MBP) as a substrate, where the phosphorylated myelin basic protein was visualized by autoradiography (35). Src kinase activity was determined by immunoprecipitation with anti-Src monoclonal antibody 327 (35). The antibody complexes were washed three times with lysis buffer and once with kinase buffer (30 mM Tris (pH 7.4) and 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 of acid-denatured enolase (Sigma) at room temperature for 10 min. The phosphorylated proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. Subsequently, immunoblot analysis was performed using the anti-Src-2 antibody (35). The activity of FAK was assessed by immunoprecipitation of cell lysates with rabbit anti-mouse FAK antibody (Sigma) and immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10.

RESULTS

Effect of HS on Breast Epithelial Cell Proliferation—The effects of various concentrations of HSGAGs on the response of cells to FGF-1 or FGF-2 in serum-free medium were assessed by both microscopic examination (Fig. 1) and thymidine incorporation after 24 h of exposure. The stimulatory effect of the HSGAGs was dose-dependent, although they had a much greater effect on the MCF-7 cells than on the MDA-MB-231 cells (Fig. 2). The greatest potentiating effect of HSGAGB on MCF-7 cells was reached at 5 μg/ml with an FGF-2 concentration of 5 ng/ml; at higher levels, its potentiating effects began to decline, consistent with it now interfering with the access of the growth factor to the cell surface. In all cases, where the incorrect combination of FGF-2/HSGAGA or FGF-1/HSGAGB was used, no effects were seen above controls. The stimulatory effects of HSGAGA and FGF-1 on MCF-7 cells were not as dramatic as those of HSGAGB, but were greater than controls. The effects of HSGAGs and FGFs on native MDA-MB-321 cells were very much less than those on MCF-7 cells, indeed almost negligible, consistent with previous findings that demonstrated an endogenous, FGF-inhibitory HS species associated with these cells (28). Although pretreating the cells with heparitinase I reduced the inhibitory effects of endogenous HS on the cells, an effect noted by Rahmoune *et al.* (29) after sodium chlorate treatment, the HSGAG potentiation was less than for the MCF-7 cells. We used low concentration heparitinase I treatment here as a milder and more specific form of removal of these endogenous inhibitory HS sequences and maintained it in the medium during treatment with the FGF/HSGAG combinations. Although it is likely that the maintained heparitinase I treatment affects the exogenous experimental HSGAGs, we have previously shown it to liberate active, FGF-binding GAG subdomains from HS chains (18–20). Nonetheless, it was clear with these cells that the FGF-2/HSGAGB combination provided a greater drive for proliferation than did the FGF-1/HSGAGA combination.

Cell Migration Assays—The addition of FGF-1/GAGA to substrates led to noticeable changes in the cell morphologies of both the MCF-7 and MDA-MB-231 cells; the cells became much more stellate, with greater proportions of lamellipodia and filopodia and trailing edges (Fig. 1). As this influence seemed to be a stimulus for cells to move, we quantitated rates of cell migration on different FGF/HS combinations with time-lapse photomicrography. Dispersed cells were plated onto either designated FGF/HS-coated substrates or Matrigel and allowed to adhere, and their movements were recorded over the following 150 min. These tapes were then processed through the digital image analysis system. The cells showed by far the greatest migratory behavior in medium containing FGF-1/HSGAGA. Although this effect was greatest for the highly motile MDA-MB-231 cells, it could also be seen in the more quiescent MCF-7 cells (Fig. 3). Increases in motility were seen on HSGAGA or FGF-1 alone, but the effects of their combination were additive and led to noticeably greater migratory speeds. Interestingly, these speeds were not maintained over the 150 min, but plateaued out after ~60 min. In contrast, the motility observed when either MCF-7 or MDA-MB-231 cells were exposed to FGF-2, either alone or in combination with HSGAGB, was barely above control levels. In all cases where the incorrect combination of FGF-2/HSGAGA or FGF-1/HSGAGB was used, no effects were seen above controls.

Receptor Profiles—An RT-PCR assay designed specifically to detect the expression of the IIIb and IIIc variant forms of the FGFRs was used to analyze the expression of these isoforms in the breast cells essentially as described previously (19), except that the quantitation was accomplished by laser-induced fluorescence after capillary electrophoresis. Although differential splicing within the FGFR genes generates many variants within a single receptor type, splicing events that particularly influence receptor affinity for a specific FGF involve the C-terminal half of the Ig-like loop III in the extracellular domain. Studies on the genomic organization of FGFR1, FGFR2, and FGFR3 have shown that each of these genes contains two exons, b and c, which lie in close proximity to each other and can be alternatively spliced into the C-terminal half of loop III to generate an FGFR IIIb or IIIc variant (36–39). RT-PCR was performed for quiescent non-confluent cells with different combinations of primer pairs (double determinations; data not shown), and calibration curves for each FGFR isoform were established with varying numbers of cycles for each primer combination (data not shown). Measured points from the different cycle combinations deviated very little, proved quite robust, and confirmed the fidelity and accuracy of the system.

The levels of FGFR IIIb and IIIc loop expression for each of the receptor forms proved to be distinctive for both MCF-7 and MDA-MB-231 cells (Fig. 4). MCF-7 cells expressed much higher

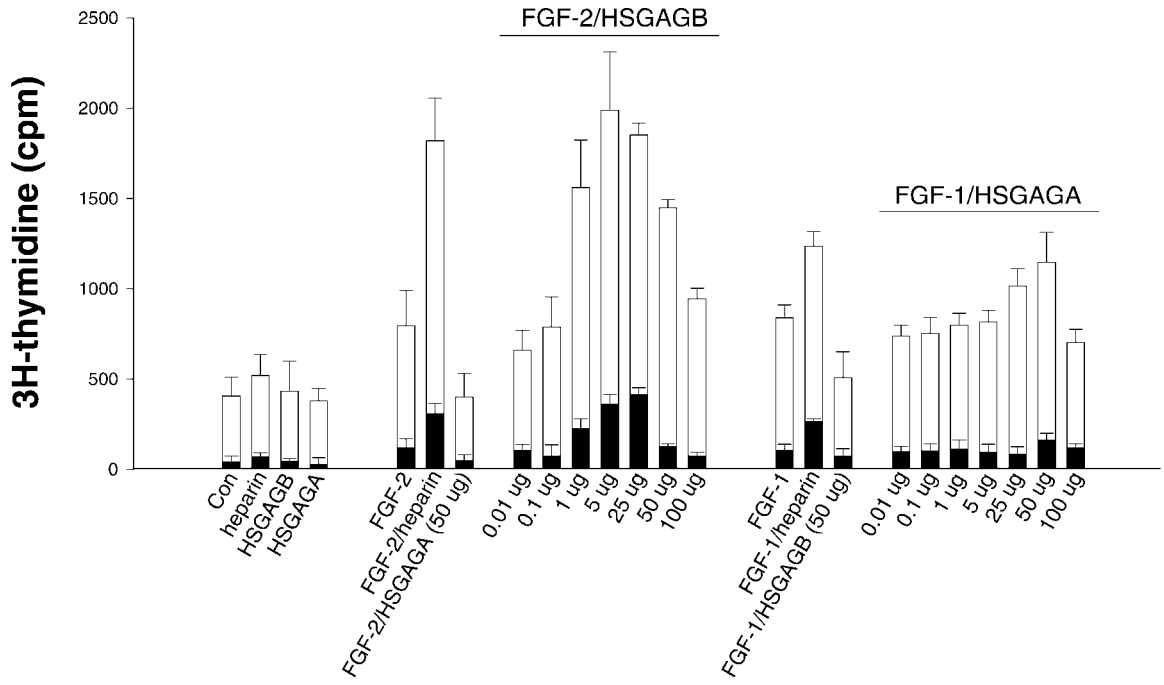

FIG. 2. **Dynamics of DNA synthesis in MCF-7 or MDA-MB-231 cells maintained in FGF/HS ligand combinations for 24 h and tritiated thymidine for the last hour.** Native MCF-7 cells (*black bars*) or heparitinase I-pretreated MDA-MB-231 cells (*white bars*) were left alone (control (*Con*)) or exposed to porcine heparin alone (5 μg/ml), to HSGAGB or HSGAGA alone (5 μg/ml), to FGF-2 alone (5 ng/ml), to FGF-2 and nonspecific heparin (5 ng/ml to 5 μg/ml), to FGF-2 with the incorrect HSGAGA (50 μg/ml), or to increasing levels of HSGAGB in the presence of a fixed FGF-2 concentration (5 ng/ml). Analogous experiments were carried out on the two cell types with FGF-1. The pretreatment was used to try to nullify the effects of endogenous inhibitory HS species on this cell type.

levels of FGFR1 IIIb and IIIc than the FGFR2 or FGFR3 forms. In contrast, the MDA-MB-231 cells had greater levels of FGFR2 than FGFR1, especially of the FGFR2 IIIc form; the differences in expression levels were not nearly so marked as for the FGFR IIIb forms. To examine whether full-length FGFR1 or shorter forms were being expressed, we designed an extra primer corresponding to the bases coding for the first eight N-terminal amino acids of the full-length receptor (primer 11) and used it in PCR (40 amplification cycles) with primer 6. The signal was compared with that generated for the IIIb and IIIc signals, and the relative levels of short and long forms were calculated as proportions of 100%. The results show that, for FGFR1, the proliferative MCF-7 cells have much greater proportions of long three-Ig-like loops rather than shorter two-loop forms; in contrast, the slowly proliferating, but highly invasive MDA-MB-231 cells have a much greater proportion of shorter forms of FGFR1.

Receptor Clustering Induced by Different HS Preparations—To examine the FGFRs being recruited and stimulated by the FGF/HS combinations, immunoprecipitations were performed after cross-linking the ligand combinations to cell surfaces with DSS (Fig. 5). Cells were maintained in [^{35}S]cysteine/methionine for 18 h and then exposed to ligand concentrations for 60 min and cross-linked; the cells were lysed, and the receptors were immunoprecipitated with specific antibodies. The results demonstrated that for native MCF-7 cells, the FGF-2/HSGAGB combination led to the immunoprecipitation of FGFR1 alone; this was seen, albeit at much lower levels, even if HSGAGB was used by itself without exogenous FGF-2. Similarly, the addition of DSS by itself as control, without either FGF or HSGAG, did not lead to receptor patterns any different from those brought down in response to FGF alone, although band intensity was up to 8-fold less as quantitated by image analysis. A similar, although less intense result was also seen if untreated MDA-MB-231 cells were used (data not shown). Receptor monomers were also precipitated by the antibodies in these experiments; in contrast to the cross-linked dimers, which ran at just under 300 kDa on gels, the monomers ran at under 200 kDa. Monomer bands were never seen on blots probed with anti-phosphotyrosine antibodies.

The results were distinctly different when cells were exposed to FGF-1/HSGAGA combinations. This combination led to the immunoprecipitation of approximately equal amounts of FGFR1 and FGFR2. A very much weaker (11-fold), but substantially similar signal was seen if just HSGAGA was used (data not shown). When incorrect FGF and HS pairs were used, such as FGF-1/HSGAGB, no receptor cross-linking could be detected; a minor amount of FGFR1 could be detected in response to the unspecific FGF-1/HSGAGB pairing if the blot was exposed to film for >3 weeks.

To confirm that the cross-linked receptors were active, we examined the influence of HS on the ability of FGF-1 and FGF-2 to stimulate the phosphorylation of FGFR1 and FGFR2 (Fig. 5*B*). Cell lysates were prepared as described above from unlabeled cells that had been exposed to different FGF/HS combinations, immunoprecipitated with anti-FGFR antibodies, subjected to SDS-PAGE, and electroblotted; and the gels were probed with anti-phosphotyrosine antibodies. The results demonstrated that the FGFR1 that cross-linked to FGF-2/HSGAGB was phosphorylated as a result; in contrast, both FGFR1 and FGFR2 were phosphorylated in the receptor clusters cross-linked to FGF-1/HSGAGA. The phosphorylation results therefore closely mirrored the receptor coupling results. Despite the apparent disparities between FGFR1 and FGFR2 densities for both cell types, HSGAGA appeared to result in approximately equal amounts of phosphorylation for FGFR1 and FGFR2. Substantially similar results were seen with the MDA-MB-231 cells. Again, in cases where the incorrect combination of FGF-2/HSGAGA or FGF-1/HSGAGB was used in either assay, no selectivity different from growth factor treatment alone was seen in either the binding or activation assays (data not shown).

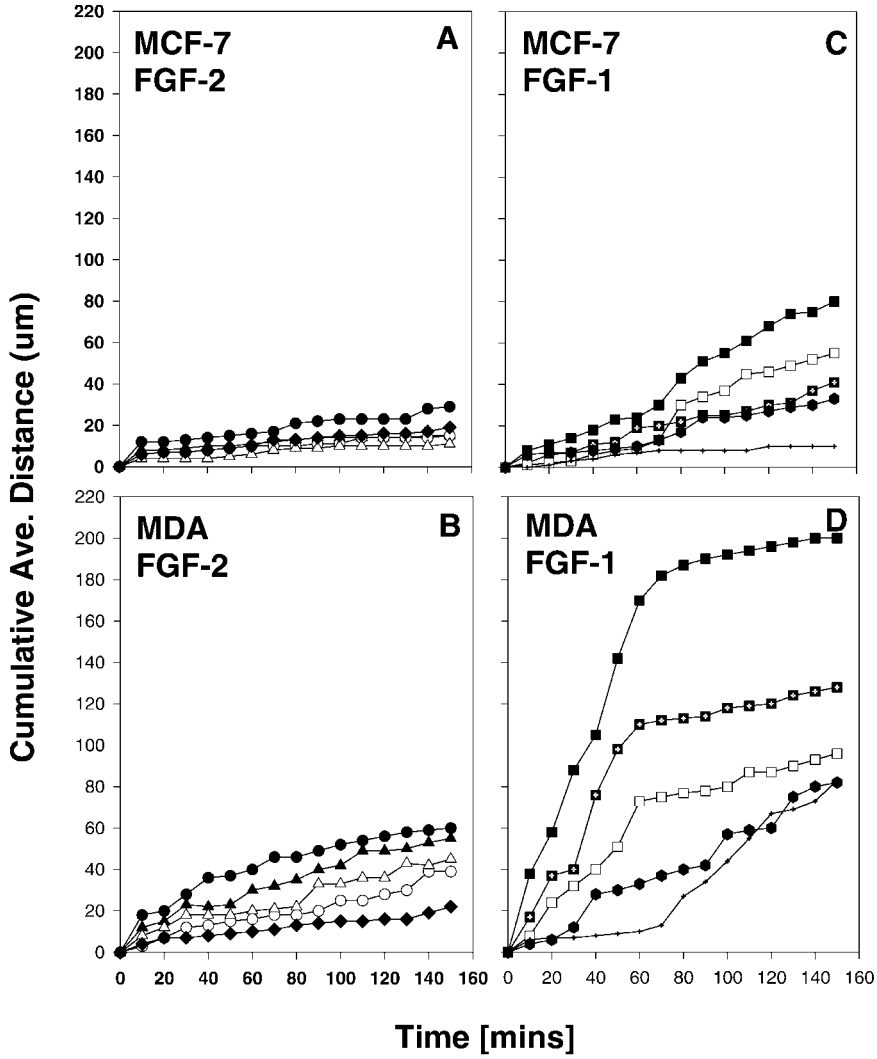

FIG. 3. **Migration assays for MCF-7 and MDA-MB-231 cells plated onto Matrigel substrates for 150 min in the presence of different FGF/HS combinations.** FGFs were present at 5 ng/ml, and HS was present at 5 μg/ml. Movement rates of cells were quantitated after time-lapse video photomicrography, digital conversion, and processing through image analysis. *A*, MCF-7 cells exposed to FGF-2/HSGAGB (●), HSGAGB alone (▲), FGF-2 alone (○), the incorrect combination FGF-2/HSGAGA (♦), or no additives (△); *B*, MDA-MB-231 cells exposed to FGF-2/HSGAGB (●), HSGAGB alone (▲), FGF-2 alone (○), the incorrect combination FGF-2/HSGAGA (♦), or no additives (△); *C*, MCF-7 cells exposed to FGF-1/HSGAGA (■), HSGAGA alone (*black squares with plus signs*), FGF-1 alone (□), the incorrect combination FGF-1/HSGAGB (•), or no additives (+); *D*, MDA-MB-231 cells exposed to FGF-1/HSGAGA (■), HSGAGA alone (*black squares with plus signs*), FGF-1 alone (□), the incorrect combination FGF-1/HSGAGB (•), or no additives (+). Each point is the average (*Ave.*) of 50 determinations, and the S.D. values on each point never exceeded 4% of the mean. The entire experiment for each condition was repeated at least twice.

Kinase Regulation by Different HS Preparations—FGF-1 and FGF-2 are able to induce the tyrosine phosphorylation of many intracellular second messenger candidates. For example, it has previously been demonstrated that the interaction of FGF-1 with FGFR1 up-regulates MAPK activation through both the Ras and phospholipase Cγ pathways (40). To investigate the effects of the FGF/HS ligand pairs on activation of $p44^{mapk}$ and $p42^{mapk}$, we examined their levels of tyrosine phosphorylation after immunoblotting (Fig. 6). Exposure of cells to FGF-2/HSGAGB resulted in substantially greater levels of phosphorylation of both $p44^{mapk}$ and $p42^{mapk}$,for MCF-7 cells and either heparitinase I-treated or native MDA-MB-231 cells (although the first two showed by far the greater proportional increases). The levels were greater than cells exposed to the FGF-2/heparin combination and markedly greater than for either FGF-2 or HSGAGB alone. The levels of $p44^{mapk}$ and $p42^{mapk}$ triggered by FGF-1/HSGAGA were greater than those triggered by control conditions, but still only about half those provided by FGF-2/HSGAGB. This result was also seen with the heparitinase I-treated MDA-MB-231 cells.

FGFs are also known to induce the tyrosine phosphorylation of Src, which then feeds into a number of pathways, including the control of extracellular adhesion through the FAK pathway (41). Because the Src protein can be phosphorylated on tyrosine in an inactive as well as an active state (42), we used an *in vitro* kinase assay in the manner of LaVallee *et al.* (35) to determine the effects of FGF/HS combinations on Src activity. In contrast to the results seen with MAPK, the FGF-1/HSGAGA combination was much more effective in increasing Src than the FGF-2/HSGAGB combination both for the MCF-7 cells as well as the heparitinase I-treated and native MDA-MB-231 cells (Fig. 7).

The results for Src activation as well as the higher migration rates of cells on FGF-1/HSGAGA substrates led us to examine the regulation of the intermediary protein species FAK (Fig. 8). It is known to be involved in the formation of focal adhesion plaques where cortical actin has been brought into register with the activated intracellular cytoplasmic domains of members of the integrin receptor superfamily (41). Consistent with the specificity of FGF-1/HSGAGA effects in preferentially up-regulating Src over MAPK pathways, we found a very marked increase in the levels of phosphorylated FAK; this was particularly so in the MDA-MB-231 cells left untreated by heparitinase I. These latter cells are noticeable for their lack of response to stimuli including FGF-2, in part due to their endogenous complement of inhibitory HS (28, 29). There were no major changes in the general FAK pool under any of the conditions tested.

Integrin Receptor Blockade—As the FAK pathway is a key regulatory element in the intracellular adhesion triggered by integrin receptors, we next attempted to determine whether there was any specificity in the adhesion signals triggered by the increase in Src and FAK in response to FGF-1/HSGAGA or FGF-2/HSGAGB. The FGF/HS combinations were applied to the cells in soluble rather than substrate-fixed form. The cells

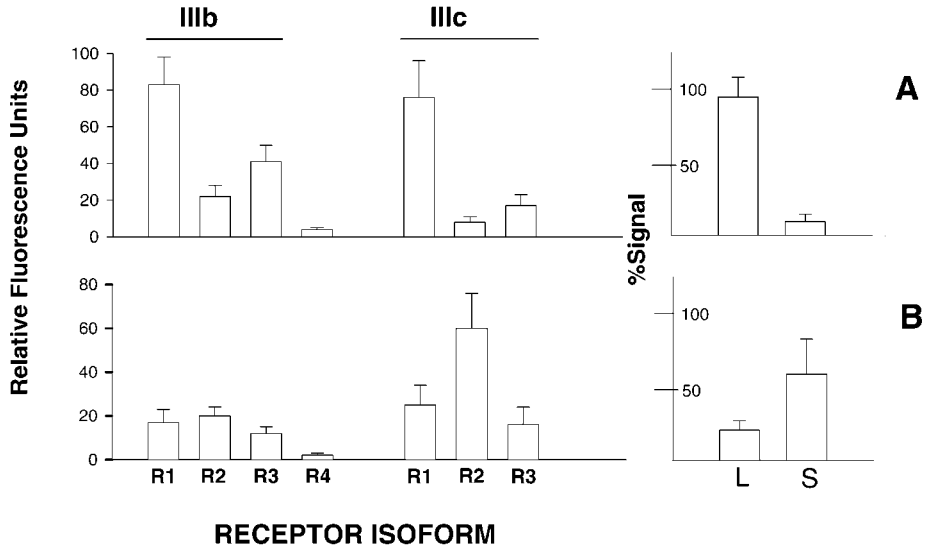

FIG. 4. **Determination of both the expression levels of FGFR isoforms as determined by RT-PCR/capillary electrophoresis/laser-induced fluorescence and the proportions appearing as short (two-Ig loop) and long (three-Ig loop) forms.** Note that FGFR4 (*R4*) has only one isoform. RT-PCR products were quantified (in relative fluorescent units) and calibrated for each receptor isoform primer pair with capillary electrophoresis/laser-induced fluorescence. Determination of PCR products by capillary electrophoresis was carried out in a P/ACE System 2100 with laser-induced fluorescence detection by argon ion laser. Total RNA was extracted from quiescent non-confluent cells. *A*, receptor levels for MCF-7 cells and the proportion of FGFR1 types appearing as short (*S*) or long (*L*) forms; *B*, receptor levels for MDA-MB-231 cells.

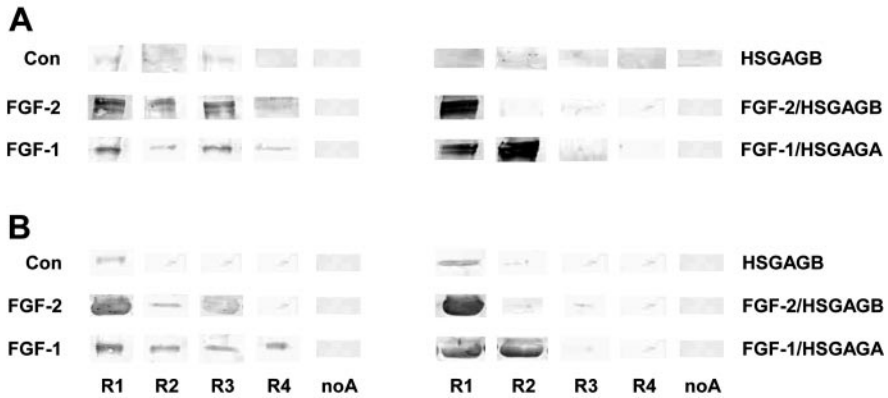

FIG. 5. **Differential FGFR clustering by different ligand combinations.** MCF-7 cells were exposed to different FGF/HSGAG combinations for 60 min in the presence of the cross-linker DSS; lysed; and then immunoprecipitated with anti-FGFR1, anti-FGFR2, anti-FGFR3, or anti-FGFR4 antibodies against a control (*Con*) of no antibodies (*noA*) and then subjected to 5% SDS-PAGE. Gels were electroblotted and autoradiographed, and the bands were analyzed. The bands are complexes of FGF, FGFR, and HS and ran just under 300 kDa. *A*, radiolabeled cells were exposed to no growth factor (*i.e.* DSS alone), FGF-2 alone, or FGF-1 alone (*left panel*) or to HSGAGB alone, FGF-2/HSGAGB, or FGF-1/HSGAGA (*right panel*). Note how the presence of sugar restricted the receptor coupling. *B*, non-radiolabeled MCF-7 cells in suspension were exposed to the FGF/HS combinations for 60 min and lysed as described above. Lysates were incubated with anti-FGFR antibodies, immunoprecipitated, and subjected to SDS-PAGE. Gels were electroblotted, and the membranes were probed with anti-phosphotyrosine monoclonal antibody 4G10 prior to fluorimaging. *R1*, FGFR1.

were plated onto extracellular matrix-rich Matrigel and monitored with time-lapse photomicrography for 150 min in the presence of specific integrin-blocking antibodies (Fig. 9). The results demonstrated that FGFR1/FGFR1-utilizing cells use different integrin combinations than FGFR1/FGFR2-utilizing cells to bind to Matrigel in the presence of FGF-1/HSGAGA. Cells utilizing FGFR1/FGFR1 used $\alpha_6\beta_1$ (Fig. 9*A*), thought to be a receptor for laminin (43), and cells utilizing FGFR1/FGFR2 signaling used primarily $\alpha_v\beta_3$ dimer combinations (Fig. 9*B*), thought to be one of the receptors for vitronectin (44). To explore this more fully, cells were plated onto substrates coated with increasing concentrations of either laminin or vitronectin (Fig. 9, *C* and *D*). The results demonstrated that movements for the FGFR1/FGFR2-utilizing cells were up-regulated on vitronectin substrates to a much greater degree than on laminin in the presence of FGF-1/HSGAGA, but that the opposite was true for FGFR1/FGFR1 cells. Interestingly, there was no particular substrate preference when either cell type was monitored in the presence of HSGAGB or FGF-2.

DISCUSSION

The results of this study demonstrate that breast cancer epithelial cells will up-regulate their responses to FGFs when they are presented in combinations with activating HS chains. Furthermore, defined HS chains that potentiate the activities of FGF-2 through FGFR1 lead to proliferation by up-regulating the activities of the MAPK pathway. In contrast, defined HS chains that up-regulate the activities of FGF-1 do so through the signaling activities of FGFR1 and FGFR2 combinations, which as well as up-regulating MAPK, up-regulate the activity of Src and its downstream target FAK. This latter activity correlates with an increase in the initial rates of cellular migration, which in turn correlates with the activity of particular integrins, including the $\alpha_v\beta_3$ receptor in the case of MDA-MB-

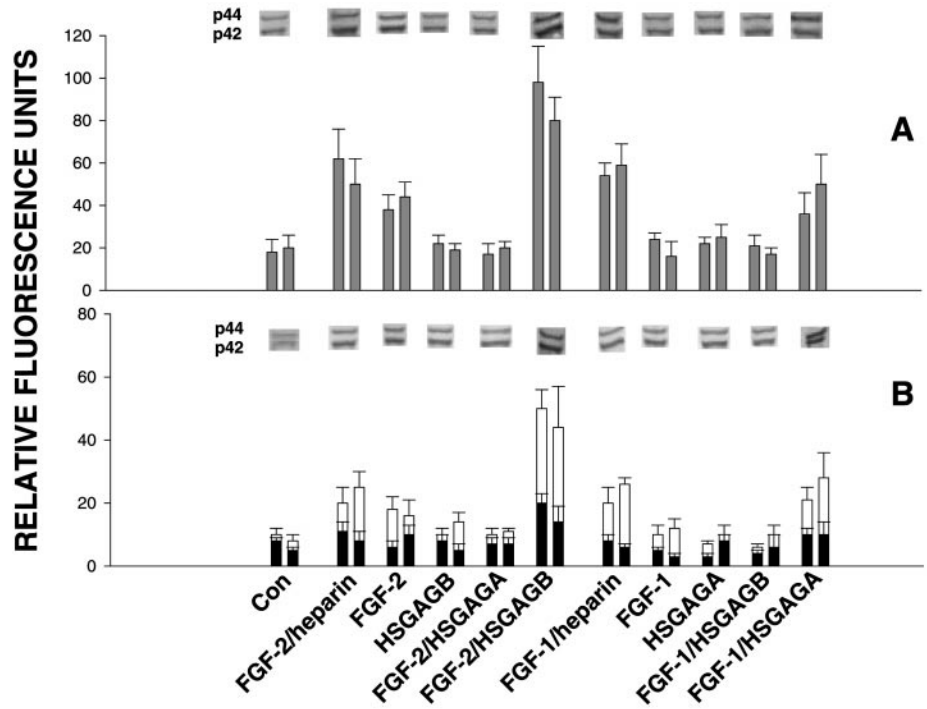

FIG. 6. **Quantitation of *in vitro* MAPK tyrosine phosphorylation in MCF-7 and MDA-MB-231 cells exposed to different FGF/HS combinations.** MAPK activity was determined by immunoprecipitation of lysates with anti-ERK1 and anti-ERK2 antibodies and revealed on gels with anti-phosphotyrosine monoclonal antibody 4G10. The phosphorylated ERK bands were visualized by fluorimaging, and the density of the bands was quantitated; each treatment thus resulted in a double band. *A*, MCF-7 cells (with one of the three sets of gels that were quantitated above); *B*, native MDA-MB-231 cells (*black bars*) and heparitinase I-treated MDA-MB-231 cells (*white bars*; with one of the three sets of gels that were quantitated above). Each *bar* represents the mean ± S.D. of three replicate cell preparations. *Con*, control.

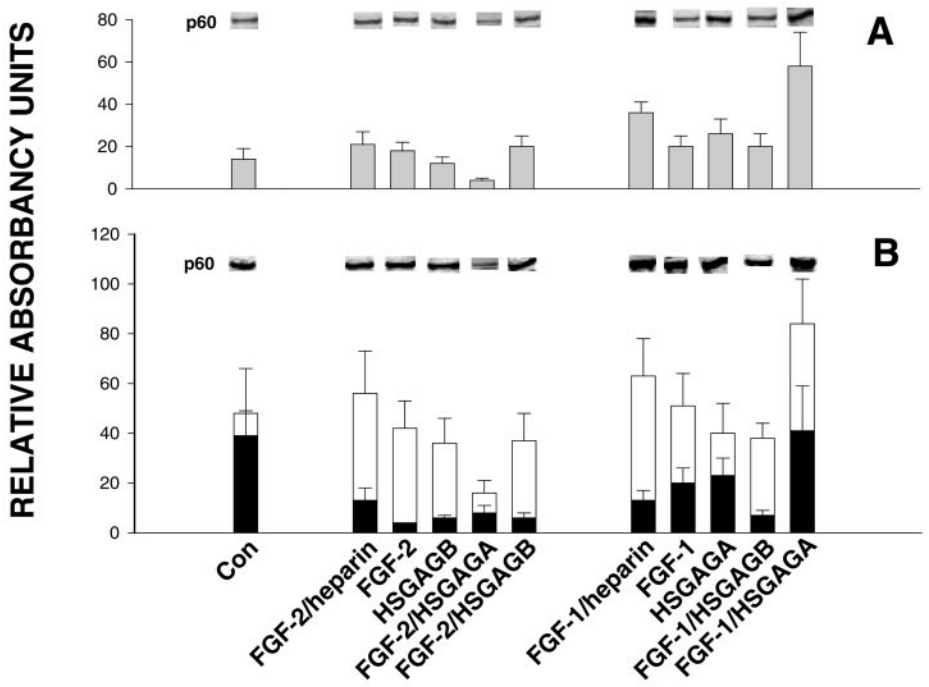

FIG. 7. **Quantitation of *in vitro* Src kinase activity in MCF-7 and MDA-MB-231 cells exposed to different FGF/HS combinations for 4 h.** Src kinase activity was determined by immunoprecipitation with anti-Src monoclonal antibody 327, and *in vitro* kinase assays were performed using enolase as a substrate (34). The density of the enolase bands was then quantitated using calibrated densitometry. One of the three sets of gels that were quantitated is shown above each *histogram*. *A*, MCF-7 cells; *B*, native MDA-MB-231 cells (*black bars*) and heparitinase I-treated MDA-MB-231 cells (*white bars*). Each *bar* represents the mean ± S.D. of three replicate cell preparations. *Con*, control.

231 cells. It is important to note that the highly motile MDA-MB-231 cells had to be pretreated with heparitinase I to remove endogenous inhibitory HS sequences that would otherwise depress the responses to our exogenous HS challenge, consistent with previous results (28).

The proliferation assays demonstrated that FGF-2 with HSGAGB proved to be a better mitogenic stimulus than FGF-1/HSGAGA for all cell types. The growth of breast cancer cells can be regulated by a large variety of peptide factors, including transforming growth factors β and nerve growth factor, as well as by retinoic acid and sodium butyrate (45, 46). HS is abundant within breast tissue, as are the FGFs, although it is highly probable that other heparin-binding growth factor classes contribute to the mitogenic environment (47, 48). Clearly, the balance between these highly complex systems will dictate the phenotypic behavior of these cells.

These results cannot be explained by differential receptor expression, as both MCF-7 and MDA-MB-231 cells express forms of each of FGFR1, FGFR2, FGFR3, and FGFR4 (49), albeit at different levels. The major differences were seen between the relative message levels of FGFR1 and FGFR2 on the MCF-7 and MDA-MB-231 cells. For the MCF-7 cells, the receptor profiles were substantially similar for both the IIIb and IIIc forms, whereas the MDA-MB-231 cells expressed markedly more of the FGFR2 IIIc form than the FGFR2 IIIb form. It remains hard to understand exactly what this means, however,

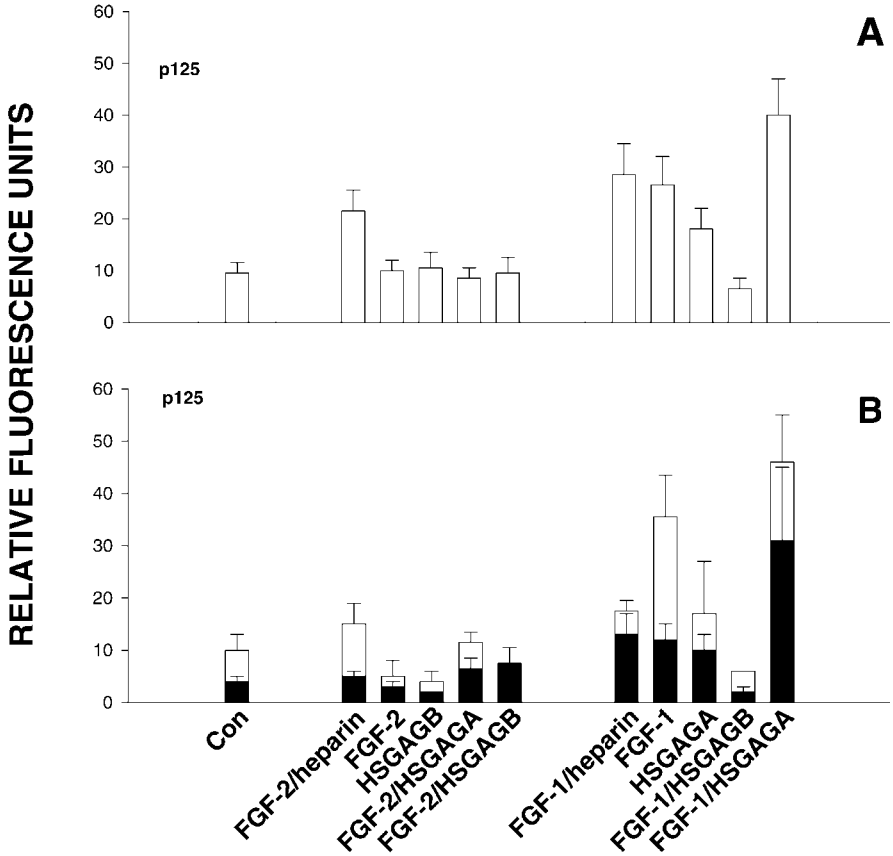

FIG. 8. **Immunoblot analysis of FAK tyrosine phosphorylation in MCF-7 and MDA-MB-231 cells exposed to different FGF/HS combinations for 4 h.** The activity of FAK was assessed by immunoprecipitation and immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10. The phosphorylated FAK was visualized by fluorimaging, and the density of the bands was quantitated. One of the three sets of gels that were quantitated is shown above each *histogram*. *A*, MCF-7 cells; *B*, native MDA-MB-231 cells (*black bars*) and heparitinase I-treated MDA-MB-231 cells (*white bars*). Each *bar* represents the mean ± S.D. of three replicate cell preparations. *Con*, control.

as it has not yet been possible to correlate isoforms definitively with phenotypic outcomes. The problem is compounded by the fact that cells need only use a small fraction of their high affinity receptors on their surfaces to activate signaling cascades that profoundly influence their subsequent behaviors (50).

Another difference between the motile and proliferative cell types was that MCF-7 cells had a far greater proportion of its FGFR1 expressed in shorter two-Ig loop β forms than full-length three-Ig loop α forms. Although the two immunoglobulin loops that compose the β isoform appear to be sufficient for ligand binding (where the ligand consists of FGF/HS), the N-terminal loop of the three-loop α isoform can interact with the two-loop ligand-binding site (51, 52). We have not yet detected such heterodimeric interactions in our assays. Our assay did not distinguish between receptor variants that might have foreshortened tyrosine kinase domains at their intracellular C-terminal ends, such as has been seen for liver cells lacking a complete catalytic domain and the two major intracellular tyrosine autophosphorylation sites Tyr-653 and Tyr-766 (51). Dimerization of monomers arising from regulated combinatorial splicing of coding sequences for the extracellular domains and the intracellular kinase domains from a single FGFR gene is thought to explain the concentration-dependent effects of a single FGF ligand on growth within a single cell type (35). Our results demonstrate a correlation between shorter forms of FGFR and growth responses through the association of HSGAGB and FGF-2. This result is consistent with those that showed that the loss of expression of the α exon of the FGFR2 gene in normal rat prostate epithelial cells also correlates with malignancy due to the increased binding of potentiating forms of HS to the shorter receptors (53). We do not believe that we have a spurious association of HS and FGFR where, for example, HSGAGB randomly binds more of the FGFR1 in MCF-7 cells because there is simply more of this receptor species than any other. The same was true for HSGAGB and MDA-MB-231 cells, in which the FGFR2 form predominated.

Our receptor activation studies demonstrate that the two different HSGAGs bring different combinations of FGFRs into activating complexes. Oligomerization and, most important, dimerization of the monomeric protein subunits (22) of tyrosine kinase transmembrane receptors facilitate transphosphorylation of tyrosines within the substrate-binding and catalytic intracellular domains. This is obligatory for the subsequent activation of second messenger substrates of the SH2, Grb2, Ras, and phospholipase Cγ domain classes (54). Our results confirm the idea that the enforced clustering of different proportions of FGFRs by specific HS sequences leads to different phenotypic outcomes. From these results, we would predict that HSGAGB-triggered FGFR1 homodimerization or higher order oligomerization favors the activation of the MAPK pathway. In contrast, we would predict that the clustering of FGFR1 and FGFR2 oligomers leads to quantitatively different transphosphorylation events that favor not only proliferation, but also the activation of Src and its downstream target FAK.

MAPKs are activated rapidly in response to a great variety of extracellular stimuli, including mitogens, heat shock, neurotransmitters, and phorbol esters (55). Our work has a complicated relationship with some previously described. Removal of FGF-1 from the culture medium of fibroblasts is sufficient to reverse the tyrosine phosphorylation of FGFR1 and the MAPK pathway, events that correlate with reductions in FGF-1-induced DNA synthesis (35). A transient exposure of just a few hours for these cells results in a sustained activation of the FGF-1-induced Src pathway, leading to changes in the cytoskeleton that maintain migratory potential. These experiments are not altogether easy to interpret, however, as FGF-1

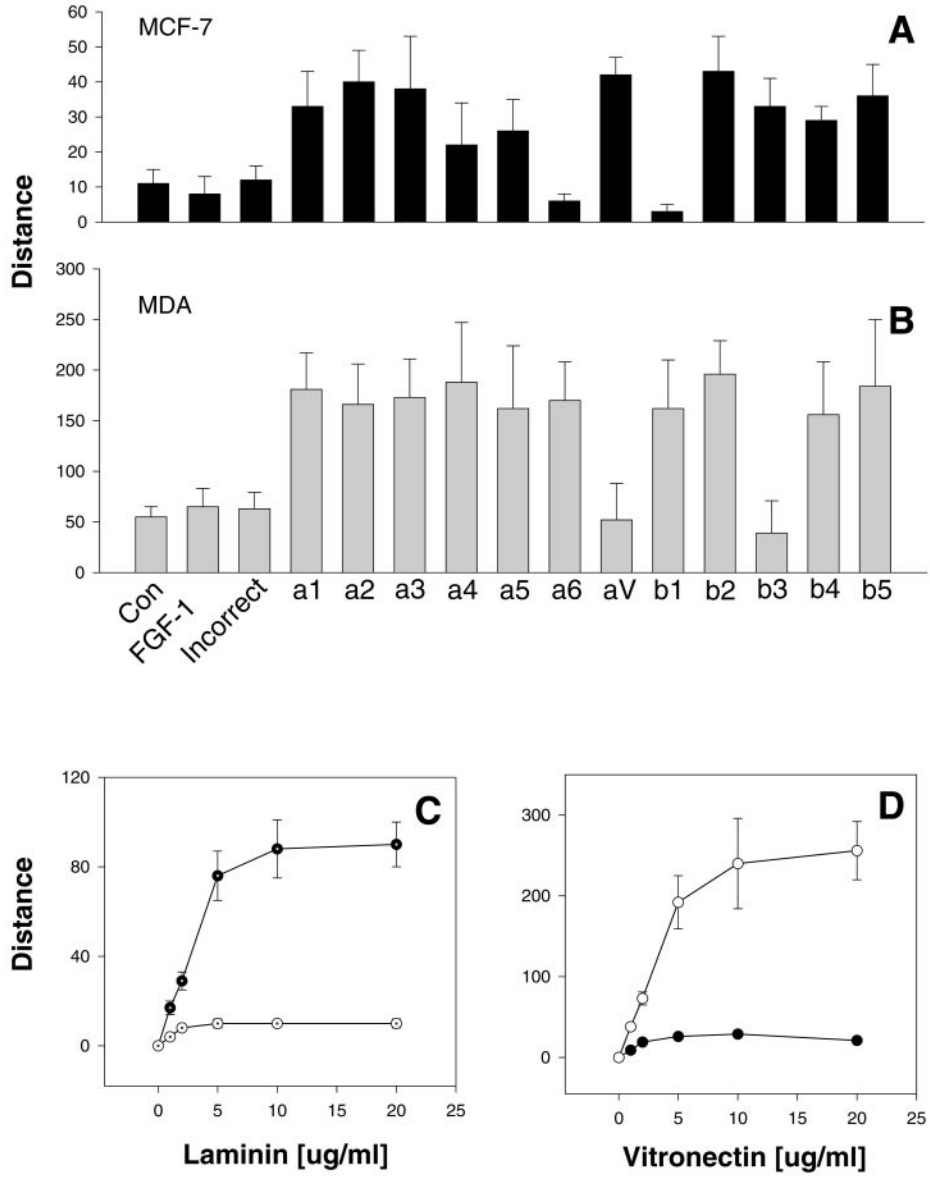

FIG. 9. **Specificity in the adhesion signals triggered by FGF-1/HSGAGA.** MCF-7 and MDA-MB-231 cells were plated onto Matrigel substrates and exposed to soluble FGF/HSGAGA (5 ng/ml FGF-1 with 5 μg/ml HSGAGA) and specific integrin-blocking antibodies (100 μg/ml) for 150 min while their movements were monitored by time-lapse photomicrography. *A*, distance traveled by MCF-7 cells. The controls consisted of no additives (*Con*), FGF-1 (5 ng/ml) by itself, and the incorrect pairing FGF-1/HSGAGB. *B*, distance traveled by MDA-MB-231 cells. *C*, effect of FGF-1/HSGAGA on the migration of MCF-7 cells (●) and MDA-MB-231 cells (○) on various concentrations of laminin. Each point represents the mean ± S.D. of three replicate wells and at least 150 cells. *D*, the same experiment using vitronectin as the substrate. Note the change of scale.

was presented in association with heparin, which may cross-link a vast series of physiologically irrelevant heparin-binding molecules. In particular, it can carry FGF-1 to every variant form of FGFR. In our case, HSGAGA seems to mimic the effects of transient FGF-1 exposure, perhaps by controlling the rate of access of FGF-1 to its receptor.

The receptor tyrosine kinase-mediated Ras/Raf/MAPK pathway, which eventually leads to the up-regulation of Fos (56) and Myc (57), is currently thought to be essential for cell replication. However, it is becoming clear that there are complicated relationships between particular cell types, particular mitogens, and subsequent activity in the nucleus. Epidermal growth factor-induced scattering of epithelial cells appears to be dependent only on Src, and not on Myc (58); however, when 3T3 fibroblasts are exposed to platelet-derived growth factor, Src does activate Myc (59). FGF-1 activates Src and Fos in umbilical endothelial cells, but not in senescent endothelial cells (60, 61). Src strongly associates with FAK near focal adhesion plaques (62), and cells from FAK knockout mice show poor migratory behaviors (63). Our data support the idea that HSGAGA, through differential receptor association, leads to a bifurcation of the FGF-1 signal into separable migratory and mitogenic pathways; this signaling is not seen with signaling through FGFR1 alone. It is also not immediately clear why FGF-2/HSGAG is more inhibitory for Src kinase activity in normal rather than heparitinase I-treated cells (Fig. 7). The result implies that the endogenous inhibitory and exogenous stimulatory GAGs somehow combine to disrupt a receptor-mediating pathway that results in Src regulation.

As phosphorylated FAK strongly associates with matrix receptors (62, 64), our last experiments were directed toward which integrin α and β subunit combinations might be activated by FGF-1/HSGAGA. The FGFR1/FGFR1-utilizing MCF-7 cells use a different complement of integrins to migrate than the FGFR1/FGFR2-utilizing MDA-MB-231 cells. The former use the laminin receptor $\alpha_6\beta_1$, whereas the latter primarily use the vitronectin receptor $\alpha_v\beta_3$. This result extends previous results demonstrating the avid attachment of breast cells to vitronectin substrates (44, 65). Breast cells have a large variety of integrin dimers on their surfaces for binding to matrix components (43), but primarily utilize $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ for vitronectin (66). Our results are interesting because $\alpha_v\beta_3$ activation correlates with high rather than low malignant potential (65, 67). We conclude that the different species of FGF-specific HS can couple different combinations of FGFR to drive particular phenotypic outcomes. As active HS domains

can be split into smaller fragments that can act as competitive inhibitors, this work may help lead to the discovery of HS fragments with which to control the cellular behavior of breast tumor cells.

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J. Biol. Chem. 2000, 275:30009-30018.
doi: 10.1074/jbc.M003038200 originally published online June 20, 2000
