Repression of Myogenic Differentiation by aFGF, bFGF, and K-FGF Is Dependent on Cellular Heparan Sulfate

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Abstract. We have proposed a model in which fibroblast growth factor (FGF) signalling requires the interaction of FGF with at least two FGF receptors, a heparan sulfate proteoglycan (HSPG) and a tyrosine kinase. Since FGF may be a key mediator of skeletal muscle differentiation, we examined the synthesis of glycosaminoglycans in MM14 skeletal muscle myoblasts and their participation in FGF signalling. Proliferating and differentiated MM14 cells exhibit similar levels of HSPG, while differentiated cells exhibit reduced levels of chondroitin sulfate proteoglycans and heparan sulfate chains. HSPGs, including syndecan, present in proliferating cells bind bFGF, while the majority of chondroitin sulfate and heparan sulfate chains do not. Treatment of skeletal muscle cells with chlorate, a reversible inhibitor of glycosaminoglycan sulfation, was used to examine the requirement of sulfated proteoglycans for FGF signalling. Chlorate treatment reduced glycosaminoglycan sulfation by 90% and binding of FGF to high affinity sites by 80%. Chlorate treatment of MM14 myoblasts abrogated the biological activity of acidic, basic, and Kaposi’s sarcoma FGFs resulting in terminal differentiation. Chlorate inhibition of FGF signalling was reversed by the simultaneous addition of sodium sulfate or heparin. Further support for a direct role of heparan sulfate proteoglycans in fibroblast growth factor signal transduction was demonstrated by the ability of heparitinase to inhibit basic FGF binding and biological activity. These results suggest that activation of FGF receptors by acidic, basic or Kaposi’s sarcoma FGF requires simultaneous binding to a HSPG and the tyrosine kinase receptor. Skeletal muscle differentiation in vivo may be dependent on FGFs, FGF tyrosine kinase receptors, and HSPGs. The regulation of these molecules may then be expected to have important implications for skeletal muscle development and regeneration.

FIBROBLAST growth factors (FGF), a family of seven related members, exhibit pleiotropic biological activities and affinity for heparin (Burgess and Maciag, 1989; Olwin, 1989; Klagsbrun and D’Amore, 1991). Their affinity for heparin suggests that heparan sulfate proteoglycans (HSPG) interact with FGF in vivo. Several studies have demonstrated that FGF binds to cell surface-derived and extracellular matrix-derived heparan sulfate proteoglycans (Gospodarowicz and Cheng, 1986; Saksela et al., 1988; Flaumenhaft et al., 1989; Flaumenhaft et al., 1990; Saksela and Rifkin, 1990; Vlodavsky et al., 1991a,b). HSPGs have been proposed to serve as storage reservoirs for FGF and to protect FGF from proteolytic degradation (Gospodarowicz and Chang, 1986; Flaumenhaft et al., 1989; Flaumenhaft et al., 1990; Vlodavsky et al., 1991; Vlodavsky et al., 1991a,b). We and others have recently proposed that HSPGs are essential for basic FGF (bFGF) activity and high affinity binding (Bernard et al., 1991; Rapraeger et al., 1991; Yayon et al., 1991).

A number of cell surface HSPGs have been described. One HSPG that may be involved in FGF action is syndecan, a mixed heparan and chondroitin sulfate proteoglycan. Syndecan is an integral membrane protein present on the cell surface (Rapraeger et al., 1985; Saunders et al., 1989). A soluble form is thought to arise by cleavage of syndecan at an extracellular site near the membrane-spanning region (Rapraeger and Bernfield, 1985). Expression cloning of FGF-binding proteins identified syndecan as a low affinity bFGF binding site (Kiefer et al., 1990). Of additional interest is the observation that the glycosaminoglycan content of syndecan can be regulated by growth factors in epithelial cells (Rapraeger, 1989). Thus, the availability of syndecan for FGF binding could be regulated in a number of ways.

Among the biological activities described for FGFs is the capacity to repress the terminal differentiation of skeletal muscle cells (Gospodarowicz et al., 1975; Linkhart et al., 1981; Lathrop et al., 1985; Clegg et al., 1987). Our previous work has been directed at understanding the mechanisms involved in FGF-mediated repression of skeletal muscle differentiation using a mouse skeletal muscle satellite cell line (MM14) as a model. Rather than directly regulating proliferation, FGF acts to maintain cell growth by repressing...
terminal differentiation (Linkhart et al., 1981; Clegg et al., 1987). Since this activity cannot be replaced by any other known growth factors, these cells provide a unique system for studying the mechanisms involved in FGF signaling. Deprivation of FGF, even in the continued presence of serum, induces a terminal differentiation program, in which the first observable phenotypic change is an irreversible withdrawal from the cell cycle. This permanent loss of FGF response is accompanied by a loss of FGF binding capacity (Olwin and Hauschka, 1986, 1988).

Recently, we have shown that heparan sulfate is required for the action of bFGF on Swiss 3T3 cells. This suggested a model in which bFGF-mediated signaling in these cells is dependent on simultaneous binding of bFGF to cell surface HSPG and a tyrosine kinase. In this study, we demonstrate that chlorate treatment causes a reduction in sulfation of proteoglycans that correlates with the loss of FGF binding and FGF action. Furthermore, we find that all FGFs active in MM14 cells including acidic FGF (aFGF), bFGF, and Kapost et al.'s Sarcoma FGF (K-FGF) (Dell-Bovi et al., 1987) require heparan sulfate, suggesting a universal requirement of heparan sulfate for FGF binding and signaling.

Materials and Methods

Human bFGF was purified from yeast expressing an artificially constructed human bFGF gene as previously described. Bovine acidic FGF was purified from bovine brain as previously described (Olwin and Hauschka, 1986). K-FGF was kindly provided by Claudio Basllico (Columbia University, New York). Choramin P for iodinations was purchased from Eastman Kodak Co. (Rochester, NY) and Enzymobeads were from BioRad Laboratories (Richmond, CA). Na125I was purchased from DuPont Instruments Co., Downers Grove, IL). For treatments, the cells were plated and incubated for 14 h, then 2 ~Ci of [3H]thymidine were added and the cells were incubated for an additional 8-10 h. [3H]thymidine incorporation was determined using a TriCarb scintillation counter (Packard Instruments Co., Downers Grove, IL). FGF Binding Assay

Binding of radiolabeled FGF was performed by passing cells with trypsin and plating at 5 x 10^4 to 1 x 10^5 cells/35-mm dish in the presence or absence of sodium chlorate and/or sodium sulfate. This passage is necessary for the action of bFGF on Swiss 3T3 cells. This suggested a model in which bFGF-mediated signaling in these cells is dependent on simultaneous binding of bFGF to cell surface HSPG and a tyrosine kinase. In this study, we demonstrate that chlorate treatment causes a reduction in sulfation of proteoglycans that correlates with the loss of FGF binding and FGF action. Furthermore, we find that all FGFs active in MM14 cells including acidic FGF (aFGF), bFGF, and Kapost et al.'s Sarcoma FGF (K-FGF) (Dell-Bovi et al., 1987) require heparan sulfate, suggesting a universal requirement of heparan sulfate for FGF binding and signaling.

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The expression of proteoglycans changes upon differentiation of MM14 cells. Significant reductions in both chondroitin sulfate proteoglycan (CSPG) and heparan sulfate glycosaminoglycan fragments are seen. However, the total amount of HSPG appears relatively unchanged. Nonetheless, examination of syndecan, a specific HSPG, demonstrates that it is expressed on proliferating but not differentiated MM14 cells (Kudla, A., A. Lopes, A. Rapraeger, and B. B. Olwin, manuscript in preparation).

HSPG on Proliferating MM14 Cells Binds FGF. Sulfate-labeled glycosaminoglycans from proliferating MM14 cells were applied to a bFGF affinity column and binding of the three distinct populations was examined (Fig. 2). Greater than 90% of the applied material bound to the column in the presence of 0.15 M NaCl was eluted from the column at a salt concentration of <0.5 M NaCl. The material requiring higher concentrations of NaCl for elution included the majority of the HSPG, ~30% of the CSPG and 20% of the heparan sulfate fragments (Fig. 2). Syndecan, identified by immunochemical methods, is retained on the bFGF affinity column and co-elutes with the HSPG, demonstrating that it is a FGF-binding HSPG present in MM14 cells.

Chlorate Reduces Sulfation of Newly Synthesized Glycosaminoglycans in MM14 Myoblasts. Chlorate ion is a competitive inhibitor of the ATP-sulfurylase responsible for
the sulfation of glycosaminoglycan chains during their biosynthesis (Farley et al., 1978; Keller and Keller, 1987; Keller et al., 1989). To determine the effectiveness of chlorate treatment on reducing proteoglycan sulfation in MM14 cells, we examined the reduction in charge density of glycosaminoglycans derived from proliferating cells cultured in the presence or absence of 10 mM sodium chlorate. Radiosulfate incorporation into all newly synthesized GAGs was reduced by 89%. Furthermore, DEAE-Sephacel chromatography demonstrated that whereas HSPG and CSPG elute at 0.45 and 0.7 M NaCl, respectively, labeled glycosaminoglycans from chlorate-treated cells eluted at ~0.35 M NaCl, approaching that of hyaluronic acid, a nonsulfated GAG (Fig. 3).

Chlorate Reduces Low and High Affinity Binding of FGFs

We analyzed low and high affinity binding sites for aFGF and bFGF on MM14 cells as described in Materials and Methods. For bFGF, low affinity sites were defined as those from which bFGF is released by a 2.0 M NaCl wash at neutral pH, whereas high affinity sites are those from which bFGF is released by a subsequent 2.0 M NaCl wash at pH 4.0. For aFGF, the NaCl wash was 0.75 M, as this factor exhibits a lower affinity for heparin than bFGF. Although previous work has suggested that binding to low affinity sites is to HSPG and high affinity sites are tyrosine kinases, recently published data identify high affinity binding sites as a complex of HSPG and FGF receptor (Rapraeger et al., 1991; Yayon et al., 1991). As expected, chlorate treatment reduces low affinity binding of $^{125}$I-aFGF and $^{125}$I-bFGF (Fig. 4 a). Chlorate inhibition of FGF binding to low affinity sites for both FGFs is reversed by the simultaneous addition of sodium sulfate, which restores GAG sulfation (Fig. 4 a). Approximately 10-fold more bFGF binds to low affinity sites than aFGF. This may be due either to a higher affinity of bFGF for HSPG or bFGF recognition of a wider range of binding sites on the HSPGs. Binding of both aFGF and bFGF to high affinity sites is also significantly reduced by chlorate treatment and restored by the simultaneous addition of sulfate (Fig. 4 b). Despite the differences in low affinity binding, aFGF and bFGF bind to a similar number of high affinity sites.

Chlorate Blocks the Effect of bFGF on Myogenic Differentiation

MM14 skeletal muscle myoblasts are dependent on FGF for repression of myogenesis. Withdrawal of FGF results in the initiation of an irreversible differentiation program, including expression of muscle-specific genes and fusion into multinucleated myotubes. Treatment of MM14 cells with chlorate caused no reduction in cell viability based on counting cells. The number of nuclei in chlorate treated cultures is virtually identical to the number of nuclei in untreated cultures grown to the same density or treated with both chlorate and sulfate (Fig. 5). However, in cells treated with chlorate the majority of cells have fused as documented by the number of nuclei present in myotubes, indicating that the cells are terminally differentiated. Thus, chlorate treatment induces terminal differentiation and does not cause a reduction in the number of cells before or following differentiation.

To quantitatively analyze the effects of chlorate and
Figure 5. Chlorate treatment results in terminal differentiation of MM14 cells. MM14 myoblasts were plated as described for Fig. 4 at $5 \times 10^4$ for control and chlorate plus sulfate treatments and $1 \times 10^5$ cells per dish for chlorate, respectively, so that the final cell numbers were identical for all treatments 24 h later at which time they were fixed. The number of nuclei present in myotubes and in single cells were scored. Nuclei present in myotubes (a), and nuclei in single cells (b).

heparitinase treatments, we monitored cell cycle exit, which is the first phenotypic change that occurs upon initiation of differentiation (Olwin and Hauschka, 1986). Treatment with sodium chlorate resulted in a dose-dependent loss in the bFGF response with a complete loss occurring at 30 mM sodium chlorate (Fig. 6 a). This is reversed in a dose-dependent manner by the simultaneous addition of sodium sulfate; complete reversal occurs at 3 to 10 mM sodium sulfate (Fig. 6 b). The loss of FGF responsiveness is not due to changes in the ionic strength of the medium and is specific for chlorate as addition of 40 mM sodium chloride had no effect on the proliferation or differentiation of MM14 skeletal muscle cells (data not shown). The effects of chlorate on bFGF high affinity binding are consistent with the hypothesis that high affinity bFGF binding requires sulfated glycosaminoglycans.

**HSPG Is Required for aFGF and K-FGF Repression of MM14 Myoblast Differentiation**

We have tested several additional members of the FGF family, aFGF, K-FGF, FGF-5 (Zhan et al., 1988), and KGF (keratinocyte growth factor) (Rubin et al., 1989), for prevention of MM14 cell cycle exit. Although FGF-5 and KGF were not active on these cells (data not shown), aFGF and K-FGF repressed differentiation and maintained cell growth in a manner analogous to bFGF (Fig. 7). Heparan sulfate is also required for the action of aFGF and K-FGF on MM14 myoblasts (Fig. 7). Simultaneous addition of sodium sulfate reversed the chlorate inhibition for all three growth factors.

**Addition of Heparin to Chlorate-treated MM14 Myoblasts Restores the Action of aFGF, bFGF, and K-FGF**

Addition of heparin in the presence of maximally stimulating concentrations of aFGF, bFGF, and K-FGF restores the biological action of all three factors in chlorate-treated cells (Fig. 8). In this experiment [3H]thymidine incorporation is normalized to the response elicited by the same concentrations of factors in untreated cells. Chlorate treatment abolishes the action of all three growth factors, but low heparin concentrations restore it. The EC_{50} for heparin stimulation of K-FGF, bFGF, and aFGF activities were 0.1, 0.1, and 1.0 $\mu$g/ml, respectively. Addition of higher heparin concentrations results in a greater FGF response for aFGF and K-FGF than that seen in untreated cells. The restoration of FGF action by the addition of heparin demonstrates that the FGF signalling pathway for these three FGFs is unaffected by treatment of the cells with sodium chlorate. These data also provide additional evidence for the requirement of heparan
Chlorate inhibition and sulfate recovery of FGF-mediated repression of myogenesis. MM14 myoblast cultures were passaged and replated in 24-well dishes containing 200 pM bFGF in the presence of increasing concentrations of sodium chlorate (A, a), 30 mM sodium chlorate and increasing concentrations of sodium sulfate (B, b). The [3H]thymidine incorporation in each treatment was normalized to that of untreated cells (maximum of 3,595 cpm). The background was not subtracted and averaged 200 cpm or 5.6% of maximum values, similar to the values obtained in the presence of 30 mM chlorate. Data points represent the mean and SD of triplicate points. The experiment was repeated three times with similar results.

Figure 6. Chlorate inhibition and sulfate recovery of FGF-mediated repression of myogenesis. MM14 myoblast cultures were passaged and replated in 24-well dishes containing 200 pM bFGF in the presence of increasing concentrations of sodium chlorate (A, a), 30 mM sodium chlorate and increasing concentrations of sodium sulfate (B, b). The [3H]thymidine incorporation in each treatment was normalized to that of untreated cells (maximum of 3,595 cpm). The background was not subtracted and averaged 200 cpm or 5.6% of maximum values, similar to the values obtained in the presence of 30 mM chlorate. Data points represent the mean and SD of triplicate points. The experiment was repeated three times with similar results.

Heparitinase Reduces bFGF Binding and bFGF Activity

To confirm that FGF action is dependent on the presence of heparan sulfate, proliferating MM14 cells were treated with heparitinase before and during incubation with bFGF. This treatment reduced the low and high affinity binding of bFGF by 70% (Fig. 9 a), similar to the finding with chlorate treatment. In addition, [3H]thymidine incorporation was diminished in the cell cycle exit assay (Fig. 9 b), demonstrating...

Figure 7. Repression of MM14 myoblast differentiation by aFGF and K-FGF requires HSPG. MM14 myoblasts were cultured in 50 pM aFGF, bFGF, or K-FGF containing either no additions (a), 30 mM sodium chlorate (b), or 30 mM sodium chlorate and 10 mM sodium sulfate (c). The cells were processed and harvested for DNA synthesis as described in Fig. 6. The mean and SD are from triplicate determinations of two independent experiments. Maximum [3H]thymidine incorporation was normalized to the [3H]thymidine incorporation obtained for the same concentrations of each respective FGF in untreated cells. Background incorporation in the absence of FGFs was subtracted.
Heparin restores the action of aFGF, bFGF, and K-FGF in chlorate-treated MM14 myoblast cultures. MM14 cells were cultured in 30 mM sodium chlorate and 50 μM aFGF (○), bFGF (●), or K-FGF (▲) in the presence of increasing concentrations of heparin. Cell cycle exit assays were performed as described in Fig. 6. Relative [³H]thymidine incorporation in treated cells was normalized to the [³H]thymidine incorporations observed with identical concentrations of the respective FGFs in untreated cells. Incorporation in the absence of FGF ranged between 100 and 200 cpm and was subtracted. The data represent the mean and SD of triplicate points. The experiment was repeated two times with similar results.

Discussion

Withdrawal of FGF from G1-phase MM14 skeletal muscle cells for 2-3 h initiates a terminal differentiation program that begins with an irreversible exit from the cell cycle. We determined that aFGF, bFGF, and K-FGF are capable of repressing differentiation, whereas FGF-5 and KGF are not. Treatment of MM14 myoblasts either with chlorate or heparitinase initiates a terminal differentiation program that is indistinguishable from that induced by FGF withdrawal. These observations are in agreement with a model in which simultaneous binding of FGF to a HSPG and a tyrosine kinase is required for FGF signalling (Rapraeger et al., 1991).

Proliferating and differentiated MM14 cells contain chondroitin sulfate proteoglycans, heparan sulfate proteoglycans, and free heparan sulfate chains. Several differences between the proteoglycan content of proliferating and differentiated cells are seen. These include a dramatic reduction in chondroitin sulfate proteoglycans and loss of syndecan but little change in total HSPGs. In addition, a reduction in the free heparan sulfate chains is observed. These chains are likely to result from rapid breakdown of HSPGs. Rapid HSPG turnover is also suggested by the fact that the chlorate effect on MM14 cells is observed within 2 to 4 h of its addition, suggesting that within this short time period the native
HSPGs at the cell surface have been replaced by their non-sulfated counterparts. A reduction in the synthesis of sulfated GAGs during differentiation of chick primary skeletal muscle cultures (Angello and Hauschka, 1979) demonstrates that the changes observed in the MM14 cell line also occur in primary culture.

FGF binding to a subset of low affinity sites is critical for FGF action; this subset is HSPG. Treatment of proliferating myoblasts with 30 mM chlorate reduces binding of 125I-

bFGF and 125I-aFGF to low affinity sites by 56 and 36%, respectively. In contrast to the reduction in low affinity FGF binding, 10 mM chlorate reduced GAG sulfation by almost 90% and 30 mM chlorate should be even more effective. If low affinity binding of 125I-

bFGF and 125I-aFGF was exclusively to HSPG then a reduction of at least 90% would be expected. This suggests that the low affinity sites are likely to be a combination of HSPG and non-GAG proteins and that the non-GAG sites are incapable of promoting high affinity binding and FGF signalling. This is clearly evident for aFGF where 64% of the low affinity binding is to non-GAG sites. These data suggest that FGF signalling requires that FGFs must be bound to HSPG and not simply be localized at the cell surface.

Restoration of FGF-mediated expression of differentiation in chlorate treated cells with FGF and heparin indicates a specific requirement of heparin or heparan sulfate glycaminoglycans for FGF action. As soluble heparin restores FGF activity in chlorate treated cells, GAG chain attachment to the core proteins is not necessary. Although the maximum response elicited by bFGF in untreated cells and in chlorate treated cells with heparin is identical, the activities of aFGF and K-FGF in chlorate treated cells plus heparin are substantially greater than in untreated cells. Either the addition of soluble heparin stabilizes these FGFs in the culture medium or the cell surface HSPGs are not functioning to yield the maximum response in untreated cells. The latter possibility is interesting as it implies that specific GAG sequences may be involved in signalling for different FGFs.

We have demonstrated that three different forms of FGF require heparan sulfate for FGF signalling, suggesting a universal requirement for heparan sulfate. This requirement may be specific for different FGF receptor and cell types. Four and possibly five distinct tyrosine kinase FGF receptor genes have been identified (Lee et al., 1989; Pasqualle and Singer, 1989; Pasqualle, 1990; Reid et al., 1990; Avivi et al., 1991; Partanen et al., 1991; Stark et al., 1991). We determined which FGF receptor protein tyrosine kinases are expressed in MM14 myoblasts by reverse transcription of mRNA isolated from proliferating cells followed by amplification using the polymerase chain reaction (Kudla, A., A. Lopez, A. Rapraeger, and B. B. Olwin, manuscript in preparation). MM14 cells express only mouse FGF receptor I or the mouse homolog of Flg (Lee et al., 1989; Dionne et al., 1990); if other FGF receptors are expressed by these cells they are below the limits of detection. Thus, the activities of aFGF, bFGF, and K-FGF in mouse MM14 myoblasts are all likely to be mediated via FGFR1. It is possible that all FGFs interacting with FGFR1 will require heparan sulfate for high affinity FGF binding and FGF action. It is not yet known whether the three remaining tyrosine kinase–containing FGF receptors will also exhibit a heparan sulfate requirement. It will also be interesting to determine if specificity resides in different HSPGs at the cell surface. The identification of two additional FGF receptors, one of which is neither a tyrosine kinase nor a HSPG may further complicate the interactions described (Burrus and Olwin, 1989; Sakaguchi et al., 1991). The contribution of these additional receptors to cell surface FGF binding and FGF signal transduction is not yet known.

Satellite cells in vivo are likely to possess FGF receptors as isolated satellite cells have been shown to respond to FGF in culture (Gospodarowicz et al., 1975; Linkhart et al., 1981; Clegg et al., 1987). The presence of bFGF in the basement membrane surrounding skeletal muscle provides additional evidence that repression of satellite cell differentiation in vivo could be mediated by FGF (DiMario et al., 1989; Joseph-Silverstein et al., 1989). Thus, differentiation of satellite cells may depend on FGF, tyrosine kinase receptors and the appropriate HSPGs. A similar process may operate during limb development as both FGF and an FGF-binding HSPG (syndecan) are localized to the proliferating mesenchyme in chick embryos (Joseph-Silverstein et al., 1989; Solursh et al., 1990). Future experiments will be required to identify the relevant HSPGs and determine whether regulation of these molecules is an important mode for regulation of skeletal muscle development and regeneration.

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