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Oncogenic Ras-induced Proliferation Requires Autocrine Fibroblast Growth Factor 2 Signaling in Skeletal Muscle Cells

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Abstract. Constitutively activated Ras proteins are associated with a large number of human cancers, including those originating from skeletal muscle tissue. In this study, we show that ectopic expression of oncogenic Ras stimulates proliferation of the MM14 skeletal muscle satellite cell line in the absence of exogenously added fibroblast growth factors (FGFs). MM14 cells express FGF-1, -2, -6, and -7 and produce FGF protein, yet they are dependent on exogenously supplied FGFs to both maintain proliferation and repress terminal differentiation. Thus, the FGFs produced by these cells are either inaccessible or inactive, since the endogenous FGFs elicit no detectable biological response. Oncogenic Ras-induced proliferation is abolished by addition of an anti–FGF-2 blocking antibody, suramin, or treatment with either sodium chlorate or heparitinase, demonstrating an autocrine requirement for FGF-2. Oncogenic Ras does not appear to alter cellular export rates of FGF-2, which does not possess an NH_2-terminal or internal signal peptide. However, oncogenic Ras does appear to be involved in releasing or activating inactive, extracellularly sequestered FGF-2. Surprisingly, inhibiting the autocrine FGF-2 required for proliferation has no effect on oncogenic Ras-mediated repression of muscle-specific gene expression. We conclude that oncogenic Ras-induced proliferation of skeletal muscle cells is mediated via a unique and novel mechanism that is distinct from Ras-induced repression of terminal differentiation and involves activation of extracellularly localized, inactive FGF-2.

Key words: mutant • Ras • myoblasts • FGF-2 • proliferation

Introduction

Ras is a multifunctional signaling molecule acting as an essential component of signal transduction pathways that regulate cellular physiology (Campbell et al., 1998). Members of the Ras family of proteins that are constitutively activated by point mutations play a major role in the onset of a large number of human cancers, including those originating from skeletal muscle tissue (Yoo et al., 1999). Rhabdomyosarcomas are the most common soft tissue sarcomas in children and adolescents. These skeletal muscle tumors are incapable of differentiation and thus do not withdraw from the cell cycle. Up to 35% of these tumors contain activating Ras point mutations, suggesting a major involvement of Ras in rhabdomyosarcomas (Stratton et al., 1989).

Constitutively active Ras mutants stimulate secretion of growth and angiogenic factors (Rak et al., 1995), potentially allowing neoplastic cells to overcome growth restrictions in their normal tissue environment. In skeletal muscle cells, activated Ras mutants have been shown to promote secretion of an unidentified factor that can repress myogenic differentiation and may participate in the development of rhabdomyosarcomas (Weyman and Wolfman, 1997). Of particular interest is the observation that cultured human embryonal rhabdomyosarcoma cells express the *fgf-2* gene and produce biologically active FGF-2 (Schweigerer et al., 1987). Release of FGF-2 may stimulate the growth and neovascularization of human rhabdomyosarcomas and contribute to tumor development. Although ectopic expression of oncogenic Ha-Ras in myogenic cell lines represses terminal differentiation, it is not reported to elicit a proliferative response (Olson et al., 1987; Konieczny et al., 1989; Weyman and Wolfman, 1997). From these studies, it has been concluded that Ras inhibits muscle differentiation without affecting proliferative response pathways. FGFs are likely candidates for such factors since they play critical roles in regulation of skeletal muscle differentiation in cultured cells (Linkhart et al., 1981; Allen et

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al., 1985; Kardami et al., 1985; Seed and Hauschka, 1988; Rando and Blau, 1994; Flanagan-Steet et al., 2000), in skeletal muscle development in vivo (Flanagan-Steet et al., 2000), and in skeletal muscle regeneration (Anderson et al., 1995; Floss et al., 1997).

MM14 myoblasts express FGF-1, -2, -6, and -7 but are absolutely dependent on exogenously supplied FGFs to repress myogenesis and promote cell proliferation (Clegg et al., 1987; Hannon et al., 1996; Fedorov et al., 1998; Kudla et al., 1998). FGF-2 is one of four FGFs that do not possess signal peptides and do not use the classical ER/Golgi-dependent secretory pathways for export from the cell (Florkiewicz et al., 1995). Since ectopically expressed Ha-Ras can repress differentiation of MM14 cells (Fedorov et al., 1998), we asked if Ha-Ras was capable of stimulating proliferation in MM14 cells. Here we report that constitutively active Ras stimulates MM14 myoblast proliferation via a novel mechanism that is dependent on export of endogenously produced FGF-2 and subsequent release or activation of the exported FGF-2. Moreover, we also found that the signaling pathways used by oncogenic Ras to stimulate proliferation and repress differentiation in myogenic cells are distinct and mediated independently.

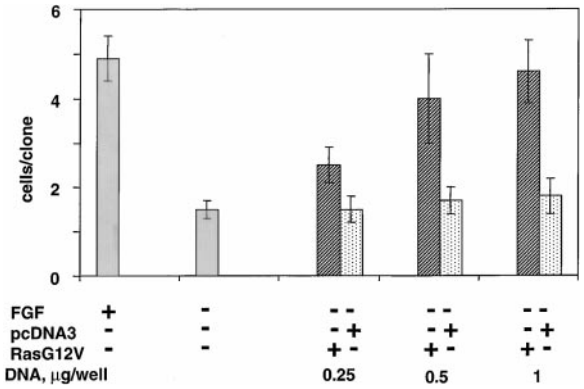

Figure 1. Ras-G12V induces MM14 cell proliferation in the absence of FGF. MM14 cells were cotransfected with plasmids encoding Ras-G12V (▨), pcDNA3 (▩), or pBSSK+ (□), and 5 μg of CMV-LacZ as described in Materials and Methods. Cells were replated in clonal density 1 h after transfection, incubated with or without FGF-2 (300 pM), and then fixed and scored 36 h after transfection for LacZ-positive cells/clone. Confidence intervals (P = 0.05) of three independent experiments, each conducted in triplicate, are shown. No less than 100 clones were counted per point.

Materials and Methods

Cell Culture

Mouse MM14 cells (Lim and Hauschka, 1984) were cultured as described previously (Kudla et al., 1995). BaF3/FR1 cells, a BaF3 cell clone stably expressing FGF receptor (FGFR)[1]-1, was cultured as described by Ornitz et al. (1992). WEHI3 cells were purchased from the American Type Culture Collection and BaF3/FR1 cells (Ornitz et al., 1992) were a gift from Dr. Dave Ornitz (Washington University Medical School, St. Louis, MO). Human recombinant FGF-2 was purified from a yeast strain expressing this growth factor (Rapraeger et al., 1994). Heparin, NaCl, and $NaClO_3$ were purchased from Sigma-Aldrich. A monoclonal anti–FGF-2 antibody specific for FGF-2 (Savage et al., 1993) and anticysteine-rich FGFR control antibody (Zuber et al., 1997) were used as described previously (Hannon et al., 1996).

DNA Transfection

DNA was transiently transfected into MM14 cells by a calcium phosphate DNA precipitation method as described previously (Kudla et al., 1995). Equivalent DNA concentrations were maintained by the addition of a pBSSK+ (Stratagene) plasmid. The pDCR-H-Ras (G12V) expression vector encoding a constitutively active mutant of human Ha-Ras, RasG12V (White et al., 1995), was provided by Dr. Channing J. Der (University of North Carolina, Chapel Hill, NC).

Clonal Growth Assay

MM14 cells were grown on 6-well plates to a density of 5×10^4 and transfected with the indicated expression vectors or control plasmids. Cells were trypsinized (0.05% trypsin, 0.53 mM EDTA) and replated at clonal density (1,000 cells per 10-cm plate) 1 h after transfection. The cells were maintained in the presence or absence of FGF-2 (0.3 nM unless otherwise indicated), cultured for 36 h, then processed for β-galactosidase histochemistry as described elsewhere (Sanes et al., 1986). The number of cells in β-galactosidase–positive clones was quantified.

Muscle-specific Promoter Assay

A differentiation-sensitive muscle-specific reporter activity assay was used to determine the extent of MM14 differentiation after transient transfection. The reporter contained the firefly luciferase gene driven by a muscle-specific promoter (MSP; human anti-cardiac actin promoter) (Kudla et al., 1995). MM14 cells were assayed for luciferase activity as described previously (Fedorov et al., 1998).

FGF-2 Export Assay

To determine their FGF-2 export capabilities, transfected cells were plated at 10^5 cells per well in 6-well plates and incubated for 36 h in growth media without FGF. Cells were then washed once with PBS (pH 7.2) and incubated for 1 h at room temperature in 1 ml of BaF3/FR1 growth medium supplemented with 50 μg/ml of heparin. The medium was then collected and filtered through a 0.2-μm filter. BaF3/FR1 cells (10^4 cells per well in 24-well plates) were incubated in the collected medium for 72 h. The number of living cells in each well was quantified by counting the number of cells that exclude trypan blue.

Results and Discussion

MM14 cells are absolutely dependent on exogenously supplied FGFs to repress myogenesis and promote proliferation, yet they express several FGFs (Hannon et al., 1996), suggesting that the endogenously produced FGFs are inaccessible to FGFR-1. In addition, we have established that distinct FGFR-1 signaling pathways mediate the proliferative and differentiation inhibitory responses in MM14 cells (Kudla et al., 1998; Jones et al., 2000). To test the involvement of Ras in both FGF-dependent pathways, MM14 cells were transiently transfected with the oncogenic Ras, RasG12V. Ectopic Ha-Ras expression stimulated proliferation and repressed differentiation of MM14 cells in the absence of exogenous FGF (Fig. 1). Activated Ras appears to replace only FGF-dependent signaling events since MM14 cells transfected with oncogenic Ras were unable to proliferate in growth medium with reduced (2.5%) serum (data not shown).

We hypothesized that Ha-Ras may induce FGF export or secretion of endogenously produced FGFs and therefore treated MM14 cells expressing RasG12V with agents that block FGF signaling. Addition of suramin, a negatively charged polysulfonated binaphthyl urea used as a

[1]*Abbreviations used in this paper:* FGFR, FGF receptor; HSPG, heparan sulfate proteoglycan.

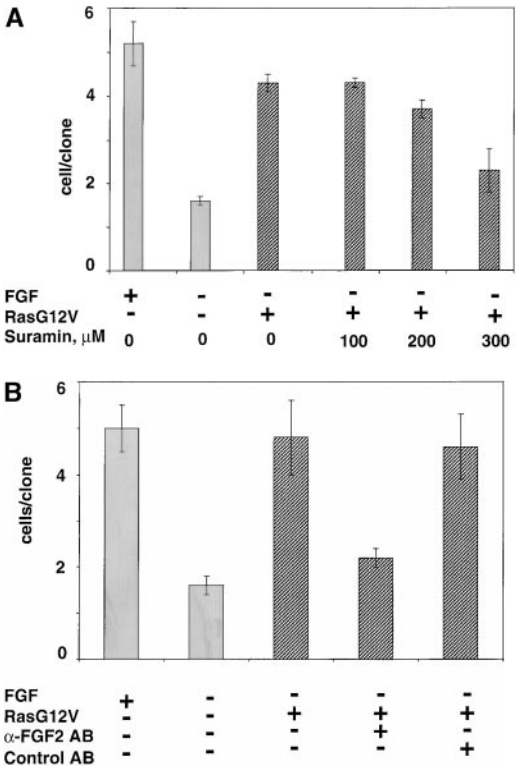

Figure 2. Inhibition of FGF-2 signaling by suramin or an anti–FGF-2 antibody blocks Ras-G12V–stimulated proliferation. MM14 cells were cotransfected with the indicated expression or control vectors, fixed, and scored as described in the legend to Fig. 1 (1 μg Ras-G12V or pBSSK+ per well). Suramin (A), an anti–FGF-2 antibody, and control antibody (B) were added to Ras-G12V–transfected cells 1 h after replating. Data and confidence intervals ($P = 0.05$) of three (A) or four (B) independent experiments, each conducted in triplicate, are shown. No less than 100 clones were counted per each point.

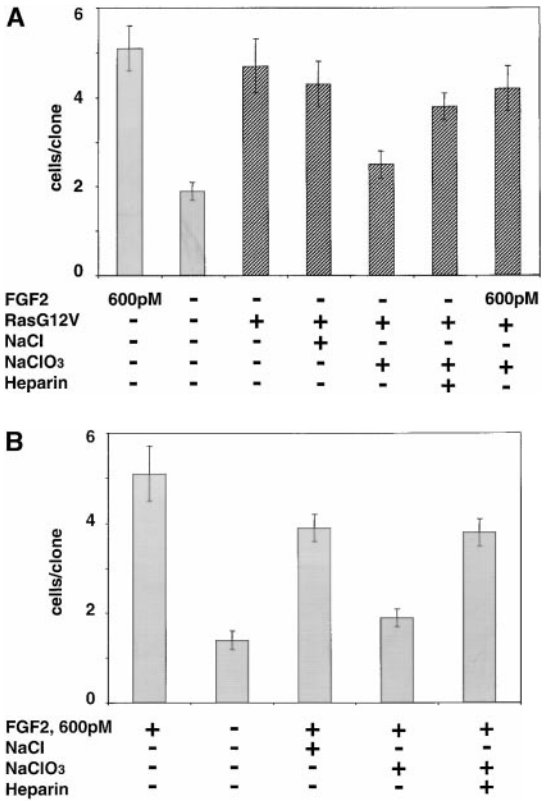

Figure 3. Heparan sulfate proteoglycans are required for Ras-G12V–stimulated proliferation. MM14 cells were cotransfected with the indicated expression or control vectors, fixed, and scored for β-galactosidase–positive clones as described in the legend to Fig. 1. NaCl (30 mM), $NaClO_3$ (30 mM), or $NaClO_3$ and heparin (50 μg/ml), in the presence or absence of FGF-2, were added to transfected cells 1 h after replating. Data and confidence intervals ($P = 0.05$) of four (A) and two (B) independent experiments, each conducted in triplicate, are shown for both A and B. No less than 100 clones were counted for each point.

general heparin-binding growth factor antagonist (Lozano et al., 1998), to cells ectopically expressing oncogenic Ras inhibited proliferation in a dose-dependent manner (Fig. 2 A). To test the involvement of specific FGFs, MM14 cells transiently expressing RasG12V were incubated with a neutralizing monoclonal anti–FGF-2 antibody specific for FGF-2 (Savage et al., 1993). Treatment with the anti–FGF-2 antibody completely abolished the capacity of Ha-Ras to stimulate proliferation (Fig. 2 A), whereas addition of a control monoclonal antibody had no effect (Fig. 2 B). Unexpectedly, we found that the ability of Ha-Ras to stimulate proliferation appears dependent on extracellularly supplied FGF-2.

FGF signaling is dependent on heparan sulfate, which involves the interaction of heparan sulfate with both FGF and the FGFR tyrosine kinases (Rapraeger et al., 1991; Yayon et al., 1991; Plotnikov et al., 1999). In addition, heparan sulfate proteoglycans (HSPGs) participate in FGF storage, sequestration, and release (Rifkin and Moscatelli, 1989). Treatment of MM14 cells with sodium chlorate, a reversible inhibitor of intracellular sulfation, prevents FGF binding and induces terminal differentiation (Rapraeger et al., 1991; Olwin and Rapraeger, 1992). Incubation of oncogenic Ras-transfected MM14 cells with heparitinase (not shown) or sodium chlorate significantly decreases cell proliferation (Fig. 3 A). Both heparitinase (not shown) and chlorate-induced inhibition of MM14 cell proliferation was rescued by addition of heparin (50 μg/ml), indicating that the effect is heparan sulfate specific (Fig. 3 A). Surprisingly, addition of 600 pM FGF-2 to chlorate-treated Ha-Ras–transfected cells promoted proliferation, ameliorating the inhibitory chlorate effect (Fig. 3 A). This was unexpected since addition of FGF-2 had no effect on chlorate-treated parental MM14 cells or MM14 cells transfected with a pcDNA3 vector control (Fig. 3 B). The requirement for HSPGs and the ability to overcome this requirement with high concentrations of exogenously added FGF-2 suggests a more complicated role for HSPGs in addition to their known requirement for signaling. Taken together, our data demonstrate that RasG12V induces proliferation of skeletal muscle cells and that induction of proliferation requires an autocrine FGF-2 response.

Little is known regarding the mechanisms involved in FGF-2 export since FGF-2 has no signal peptide sequence and is not secreted through the established Golgi-dependent secretory pathway (Mignatti et al., 1992). Instead, an unusual ATP-dependent pathway that includes the Na^+/K^+-ATPase

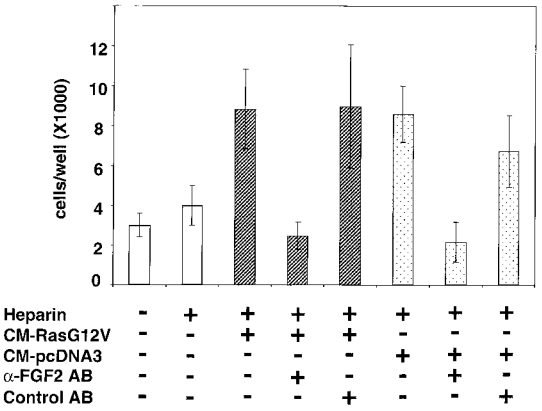

Figure 4. Transfection with Ras-G12V does not stimulate export of FGF-2 by MM14 cells. MM14 cells (10^5 cells per well in a 6-well plate) were cotransfected as described in the legend to Fig. 1 (1 μg Ras-G12V, pcDNA3, or pBSSK+ per well). Cells were washed once with BaF3/FR1 growth medium and incubated in the same medium containing 50 μg/ml heparin for 1 h at room temperature and then collected. BaF3/FR1 cells (10^4 cells per well in a 24-well plate) were grown in conditioned medium from Ras-G12V (CM-RasG12V; ▨), pcDNA3-transfected (CM-pcDNA3; ▩) MM14 cells, or in control conditions (no MM14-conditioned media; RPMI supported with 15% calf bovine serum; □) for 72 h. Viable cells were scored based on their ability to exclude trypan blue dye. Data and the standard deviation shown represent two independent experiments, each performed in triplicate.

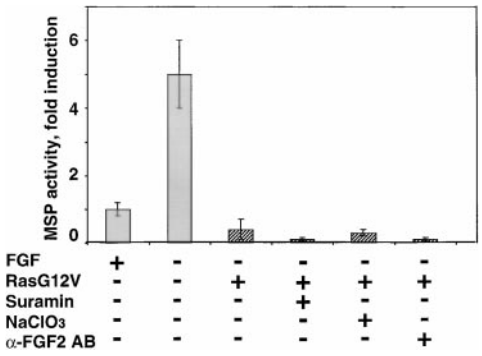

Figure 5. Blocking FGF-2 signaling by suramin, anti–FGF-2 antibody, or sodium chlorate has no effect on inhibition of differentiation by Ras-G12V. MM14 cells were cotransfected with plasmids encoding CMV-LacZ, a luciferase reporter driven by α-cardiac actin promoter (1 μg each), and either RasG12V (▨) or pBSSK+ (■) (1 μg each) plasmids. Cells were incubated in the presence or absence of FGF-2 and treated with suramin, anti–FGF-2 antibody, or sodium chlorate as indicated (all reagents were added 1 h after transfection). Cells were fixed and assayed 36 h after transfection. Data show fold induction of luciferase activity relative to cells incubated in the presence of FGF-2 (equal to 1). Each point represents three independent experiments, each conducted in triplicate, with the standard deviations indicated.

appears to be involved (Florkiewicz et al., 1998). To determine whether oncogenic Ras is directly involved in regulating FGF-2 export, we asked if MM14 cells expressing Ha-Ras exhibited increased levels of extracellular FGF-2. Although transfection of MM14 cells with FGF-2 results in export of biologically active FGF-2 (Hannon et al., 1996), this extracellular FGF-2 cannot be detected in the tissue culture media, presumably due to its strong association with membrane-bound and extracellular matrix–associated heparan sulfate. Therefore, we have designed two assays to quantify FGF-2 export. One assay utilizes heparin treatment of MM14 cells to release bound FGF-2, which is then assayed on BaF3 cells expressing FGFR-1 (BaF3/FR1). BaF3 cells are pre-B cells that undergo apoptosis after interleukin 3 withdrawal and do not express either FGFRs or HSPGs. As such, these cells are unresponsive to FGFs, unless they ectopically express FGFRs and heparin is added as an HSPG substitute (Ornitz et al., 1992). We found that both Ras-G12V and control (pcDNA3)-transfected MM14 cells release similar levels of factor(s) that support BaF3/FR1 survival and promote BaF3/FR1 proliferation (Fig. 4). These activities are neutralized by a monoclonal anti–FGF-2 antibody, demonstrating that the released material is FGF-2 (Fig. 4). A second assay involves cotransfection of MM14 cells with a construct encoding an FGF-2–luciferase fusion protein and either Ras-G12V or a control vector. The exported FGF-2 is quantified using a luciferase assay after a heparin wash. The results from this assay are indistinguishable from the BaF3/FR1 cell assay, suggesting that similar levels of FGF-2 are exported by control and Ha-Ras–transfected cells (data not shown). We conclude that oncogenic Ras does not affect the level of FGF-2 export from MM14 cells. Although MM14 cells produce FGF-2 and export FGF-2 that can be recovered in an active form, this FGF-2 is not normally available to the cells (Hannon et al., 1996). Thus, our data suggest that exported FGF-2 is normally retained in an inactive form on the cell surface. We propose that Ha-Ras "activates" this inactive extracellular pool of FGF-2 either by promoting its release from HSPGs or by providing a mechanism for FGF-2 to gain access to cell surface FGFR-1. Although the mechanisms involved are not understood, the ability of the Ha-Ras mutant to promote proliferation is dependent on exogenous FGF-2 and subsequent FGF-2–mediated signaling events.

The ability of oncogenic Ras to inhibit skeletal muscle differentiation has been well documented, but the Ras effector mediating repression of differentiation is not known (Ramocki et al., 1998). We wanted to determine if the ability of Ras to effectively inhibit MM14 differentiation was dependent on extracellular FGF-2, as is the proliferation response. Addition of suramin, $NaClO_3$, or the neutralizing anti–FGF-2 antibody did not affect the ability of Ras-G12V to repress myogenesis (Fig. 5). These data are consistent with our results published previously, which demonstrate that independent FGF signaling events mediate repression of differentiation and proliferation (Kudla et al., 1998; Jones et al., 2000). Thus, similar to FGFR, Ha-Ras appears to utilize independent signaling mechanisms to repress terminal differentiation and promote proliferation.

Although the downstream signaling events that mediate the repression of myogenesis by Ras are not understood, it is well documented that oncogenic Ras stimulates secretion of growth factors and angiogenic factors (Rak et al., 1995). Moreover, ectopic expression of activated Ras releases a factor that represses myogenic differentiation (Weyman and Wolfman, 1997). Data presented in this report argue that oncogenic Ras may be involved in the activation or release of extracellularly localized FGF-2 that is normally sequestered in a biologically inactive state. It is noteworthy that all of the extracellular activity observed is neutralized

by a specific blocking FGF-2 monoclonal antibody, since proliferating MM14 cells synthesize FGF-1, -2, -6, and -7 (Hannon et al., 1996). Although detectable, FGF-2 mRNA is present at extremely low concentrations in many adult tissues despite the presence of high levels of FGF-2 activity, suggesting a mechanism for retaining or storing FGF-2 in a biologically inert form (Baird et al., 1986). Oncogenic Ras appears to be involved in activating or releasing inactive, extracellularly localized FGF-2. The FGF-2 produced by skeletal muscle cells cannot be detected in the tissue culture medium, forming the basis for previous conclusions that the Ras-secreted myogenic inhibitory factor was not an FGF (Weyman and Wolfman, 1997). The Ras-dependent proliferation factor we identified is released by a heparin wash and its activity is abrogated by treatment with either chlorate, heparitinase, suramin, or a monoclonal anti–FGF-2 antibody, implying that the proliferation factor is FGF-2. Together with the prevalence of oncogenic Ras mutants in rhabdomyosarcomas and the involvement of FGFs in regulation of myogenesis, our data suggest that FGF-2 may be a critical factor for supporting Ras-dependent growth of rhabdomyosarcomas.

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