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 NH₂-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 (Schweiger 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; Koneczny 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
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.

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)\(^{-1}\), 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 FGF receptor 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\(^{-3}\) 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 \(\times\) 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 \(\beta\)-galactosidase histochemistry as described elsewhere (Sanes et al., 1986). The number of cells in \(\beta\)-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.

Abbreviations used in this paper: FGFR, FGF receptor; HSPG, heparan sulfate proteoglycan.
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
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 FGF-2 receptor (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 (Ramoocki 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, NaClO₃, 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 FGF-R, 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.

Figure 4. Transfection with Ras-G12V does not stimulate export of FGF-2 by MM14 cells. MM14 cells (10⁵ 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 (10⁴ cells per well in a 24-well plate) were grown in conditioned medium from Ras-G12V (CM-Ras-G12V; ), 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 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.
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.

We would like to acknowledge a gift of expression vectors made by Dr. Channing J. Der, and a gift of BaF3/FRI cells made by Dr. Dave Ornitz. This work was supported by a grant from the National Institutes of Health (AR39467) to B.B. Olwin. R.S. Rosenthal was supported by a postdoctoral training grant from the National Heart, Lung, and Blood Institute (HL07851).

Submitted: 28 September 2000
Revised: 22 December 2000
Accepted: 19 January 2001

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