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The GAP Related Domain of Neurofibromin Attenuates Proliferation and Downregulates N- and K-Ras Activation in *Nf1*-Negative AML Cells

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Abstract

Inactivation of the *NF1* tumor suppressor causes myeloproliferative diseases. *NF1* encodes a GTPase activating protein (GAP) for Ras. Myeloid cells with loss of NF1 have high levels of Ras-GTP, functionally equivalent to the effects of *RAS* oncogenes. We investigated the effects of the NF1 GAP-related domain (GRD) in proliferation, apoptosis and Ras-GTP levels in *Nf1*-negative acute myeloid leukemia (AML) cells. In AML cells, with cooperating mutations, the expression of the neurofibromin GRD causes significant reductions of N- and K-Ras-GTP levels, which is not incompatible with AML cell survival, but which is strongly selected against due to suppression of proliferation.

Keywords

GAP related domain; Neurofibromin; Myeloid Leukemia; Ras

INTRODUCTION

Neurofibromatosis Type 1 (NF1) syndrome is one of the most common single gene inherited diseases, affecting one in 3,500 individuals worldwide. Even though NF1 syndrome is primarily characterized as a neural crest derived tissue disease, it includes a variety of manifestations affecting multiple systems. Children with NF1 under the age of 10 have an increased malignant tumor incidence in part associated with myeloid leukemia (1). It has been shown that as high as 30% of children with juvenile myelomonocytic leukemia (JMML), a rare myeloproliferative disease (MPD), also have *NF1* mutations (2), indicating that NF1 syndrome increases risk for myeloid malignancies 200-500 fold (3). Unfortunately, a diagnosis of JMML

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CONTRIBUTIONS KJM wrote the manuscript, designed experiments and performed research. MAR designed experiments and performed research. SMW designed experiments and performed research. BVN designed experiments and revised manuscript. DAL wrote the manuscript and designed experiments.

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tends to have a poor prognosis due to a significant portion of cases relapsing, with treatment, including bone marrow transplant (4,5).

Loss of the *Nf1* gene in mouse hematopoietic cells is also associated with myeloid cell hyperproliferation and leukemia. BXH-2 strain mice spontaneously develop AML due to chronic infection with a Murine Leukemia Virus (MuLV) which is passed from mother to offspring in this strain (6). Roughly 15% of these AML harbor inactivating proviral insertions into the *Nf1* gene. In these tumors, both *Nf1* alleles are disrupted, either by two independent proviral insertions or proviral insertion into one allele and mutation of the other (7). Aged *Nf1* +/- heterozygous mice develop AML that show loss of the wild type *Nf1* allele (8). Transplantation of *Nf1*-/- fetal liver-derived hematopoietic stem cells or hematopoietic cell deletion of a conditional *Nf1* "floxed" allele leads to development of an MPD similar to JMML (9,10).

Neurofibromin, the NF1 gene product, is a 2818 amino acid, tumor suppressor protein with only one characterized functional domain, its GTPase-activating-protein (GAP) related domain (GRD) (11,12). The GRD of neurofibromin strongly stimulated GTPase activity with both yeast *RAS2* and human HRAS proteins (11). Subsequent experiments found that the GRD in neurofibromin acts as a negative regulator of N-, H-, K- and R-ras *in vitro* (13,14).

Most evidence suggests that the GRD of neurofibromin is essential for its role in tumor suppression and that Ras-GTP directed therapies could be of benefit in malignancy associated with NF1 syndrome (15). However, it has been suggested that Ras-dependent growth can be inhibited independent of neurofibromin's GTPase activity. Melanoma and neuroblastoma cell lines from NF1 patients with undetectable to little neurofibromin present appeared to have Ras-GTP levels appropriately regulated (16). Although studies have indicated that neurofibromin contributes only one third of the total GAP activity in melanocytes, the loss of this activity may result in subtle changes of Ras-GTP levels not easily detected (17). Also the NF1-GRD is sufficient to restore normal cell morphology of Ras transformed fibroblasts (18) and growth characteristics in *Nf1*-/- mast cells (19). In addition, it has been shown that exogenous NF1-GRD expression is capable of reducing Raf-1 kinase activity (20), suggesting that oncogenic signaling from Ras is suppressed. Although these experiments suggest a pivotal role for the GRD in neurofibromin function, studies have yet to focus on the GRD for growth suppression or cell survival in leukemia, particularly in myeloid leukemia cells carrying cooperating mutations.

In JMML, most patients demonstrate biallelic inactivation of the *NF1* gene (2) and frequently in other cases activating point mutations in the *PTPN11*, *JAK2*, *KRAS* or *NRAS* genes are observed (21). The mutually exclusive nature of these mutations suggests overlapping biochemical effects – namely increased Ras-GTP signaling. In models of *Ras* oncogene-driven cancer in mice, continued expression of the *Ras* oncogene is required for tumor cell survival. It is unclear if malignancy that develops after loss of the *NF1* gene similarly requires high levels of Ras-GTP. We therefore sought to determine if GRD expression and Ras-GTP suppression were compatible with the survival of AML cells that had biallelic *Nf1* gene inactivation. In order to accomplish this goal, we reintroduced the NF1-GRD in *Nf1*-negative AML cell lines and measured growth rates, survival, levels of activated Ras, and leukemogenicity.

MATERIALS AND METHODS

Cloning

The NF1-GRD was cloned from a cDNA library and cloned into pcDNA3.1 (K4800-01; Invitrogen, Carlsbad, CA) to create pcDNA3.1-NF1-GRD in which the GRD is V5 epitope-

tagged and driven by a cytomegalovirus (CMV) early promoter. Primers flanking NF1-GRD were used to amplify the GAP related domain. Sequence verification of NF1-GRD construct was performed.

Cell culture and viral production

BXH-2 cell lines were maintained as previously described (7). Murine stem cell virus (MSCV) NF1-GRD Ψ2 packaging cells were previously described (19). Virus from these cells was used to generate amphotrophic virus by infection of the PT67 packaging cell line (Clontech, Carlsbad, CA) and selection in puromycin. BXH-2 cells were transduced with viral supernatant twice overnight in the presence of 25 mM polybrene in ASM media (7). Cells were maintained under antibiotic selection in 4 ug/ml puromycin. B114 cells were transfected with the pcDNA3.1-NF1-GRD expression vector using the DEAE-dextran method as described by the manufacturers (27-9268-01; CellPect, Amersham Pharmacia Biotech, Piscataway, NJ) and selected/maintained in 1 mg/ml G418 to make stable transfectants.

Western Blotting

Proteins were detected via blotting as previously described (22) Briefly, lysates were electrophoresed in 10% SDS PAGE. All antibodies were incubated at manufacturer's recommended concentrations, anti-KT3 (MMS-125R; Covance, Richmond, CA), anti-V5 (R960-25; Invitrogen, Carlsbad, CA). Membranes were developed using chemiluminescent reagents (34080; SuperSignal, Pierce, Rockford, IL).

Growth and Viability Assays

Cell lines were plated at the same concentration in antibiotic containing complete media (10% serum), 1% FBS or no serum. Each successive day trypan blue exclusion was used to assess viability. Graphical and statistical analysis was performed using StatView (SAS Institute, Cary, NC) software. Significant difference was determined by ANOVA with Fisher's post-hoc tests and unpaired t-tests.

DNA Laddering

Cell lines were plated at the same concentration in media lacking serum. Each successive day an aliquot of cells was taken for genomic DNA isolation. One microgram of genomic DNA from each sample was run on a 1% agarose gel and visualized by ethidium bromide staining.

Ras-GTP Pull-down Assay

Ras-GTP levels were determined using the glutathione S-transferase-Ras binding domain (GST-RBD) assay performed as previously described (23). Briefly, *E. coli* lysates containing glutathione S-transferase fusion proteins were purified with glutathione bound to agarose beads. Beads were washed for use in the Ras pull-down assay. BXH-2 cell lines expressing MSCV NF1-GRD or control virus passaged in 10% serum conditions were lysed to obtain Ras containing supernatants. Equal concentrations of protein in cleared lysates were incubated with GST-RBD beads. Beads were washed and proteins were eluted with SDS-PAGE sample buffer. Signal reduction was measured as the mean luminosity for each band.

Tumorigenicity Assay

Single cell suspensions of NF1-GRD B114 cells or parental B114 cells were injected into mice. Recipients (SCID/NCr weanling; NCI-Frederick Animal Production Area, Frederick, Maryland) that had been sublethally irradiated, 200 Rads, in a J.L. Shepard Mark I Cs-137 irradiator were injected with 1×10^5 cells intravenously. Mice were housed in the University of Minnesota Research Animal Resources facilities and all experimental procedures approved by

Institutional Animal Care and Use Committee (IACUC). Weights were monitored daily, as an additional measure of animals' health.

RESULTS

NF1-GRD Expression

Characterization of three AML cell lines isolated from BXH-2 strain mice, B106, B114 and B117, has shown proviral insertion into the *Nf1* gene and lost or altered neurofibromin expression by northern and western blotting (7). We created *Nf1*-negative AML cell lines harboring MSCV NF1-GRD or empty vector constructs and analyzed for expression. Viral vectors containing a puromycin selection cassette under the control of a phosphoglycerate kinase (PGK) promoter and murine stem cell virus (MSCV) LTRs driving expression of NF1-GRD with a KT3 carboxy terminal tag (Figure 1A) (19) were selected for expression in B106 and B117 cell lines. B117 MSCV NF1-GRD cell lines did show initial NF1-GRD expression as in Figure 1A, but detectable protein expression was lost in later passage cells (data not shown). Similar results were obtained with B106 (data not shown). Alternate vectors for NF1-GRD expression were used in some experiments. Vectors containing a cytomegalovirus immediate early (CMV) promoter to drive expression of NF1-GRD with a C-terminal V5 epitope tag were used to transfect B114 cells, illustrated in Figure 1B. Expression of the V5-tagged NF1-GRD was detected by western blot in early passage cells. However, late passage B114 cells also lost NF1-GRD expression (data not shown). These data indicate that expression of the NF1-GRD is strongly selected against in *Nf1*-negative AML cells.

Growth of GRD Expressing Cells

Since it has been shown that full-length NF1 can inhibit growth of melanoma cell lines (24) and NF1-GRD can restore normal growth to *Nf1*^{-/-} primary mast cells (19) we wanted to determine the effects of NF1 GAP activity on leukemia cell growth. Growth of tumor cells is generally more aggressive than non-tumorigenic cells, is sustained when exposed to serum deprivation (25), and tumor cells can typically withstand unfavorable conditions longer, resulting in increased survival. Therefore, tests were done to determine if NF1-GRD expression alters BXH-2 *Nf1*-negative leukemia cell growth as compared to controls.

Two of the BXH-2 AML cell lines, B106 and B117, were transduced with MSCV NF1-GRD retroviral vectors shown in Figure 1A to test NF1-GRD's effect on cell growth. Initial passage of the cell lines demonstrated a difference in growth rates between control virus and NF1-GRD expressing cell lines immediately after transduction. Statistical significance was determined through ANOVA analysis of replicate experiments. Figures 2A and 2B illustrate that at days 2 and 3 in culture both cell lines had a delay in growth rate. These growth differences were apparent only in early passage B106 and B117 MSCV NF1-GRD transduced cells (data not shown). However the difference in growth rate does not seem to be caused by increased cell death of the MSCV NF1-GRD expressing cells, since the viability of the cells was similar among all the groups (Figures 2A and 2B). Of the three cell lines, only B114 cells were capable of CMV NF1 GRD expression by transfection. Similar to the results with B106 and B117 cells, there was a significant difference in growth rates between B114 stable CMV NF1-GRD transfectants and parental or empty vector expressing B114 cells in 10% serum in multiple growth assays (Figure 3 and data not shown).

In order to examine sensitivity to apoptosis, we exposed NF1-GRD⁺ and control cells to no serum or 1% serum for prolonged periods. B114 CMV NF1-GRD or empty vector cell lines exhibited little or no growth when media lacked serum with similar viability ratios (Figure 3, upper right hand graph). However there was a significant difference in proliferation between B114 CMV NF1-GRD and empty vector cell lines when plated in either 1% or 10% serum

(Figure 3). B114 CMV NF1-GRD cells grew at a much slower pace than B114 empty vector cells in both 1% and 10% serum conditions measured at 24 and 48 hours. However, no difference was observed in the amount of DNA laddering (Supplementary Figure 1) or the propidium iodide staining properties (data not shown), assays to detect the downstream effects of apoptosis, between NF1-GRD expressing and control cells upon serum withdrawal.

GRD Effect on Ras Activation

Since a major function of NF1 is regulating Ras activation, we wanted to determine if GRD expression in *Nf1*-negative cells altered levels of activated Ras. Ras regulation by the NF1-GRD in hematopoietic cells has not been shown, therefore experiments using the *Nf1*-negative BXH-2 cell lines expressing the MSCV NF1-GRD were analyzed for an effect on Ras-GTP levels. Using an activated Ras pull-down assay (23), cellular lysates from puromycin selected B117 cells expressing MSCV NF1-GRD were analyzed for differential levels of activated Ras by incubating with the Ras binding domain of Raf-1 (RBD) fused to glutathione S-transferase (GST) bound to glutathione beads. The RBD bound beads selectively bound activated Ras molecules from the lysates and the bound proteins were visualized by western blot. Figure 4 shows that levels of activated N- and K-Ras were reduced by 80% and 75%, respectively, as measured by loss of mean luminosity, in B117 cells expressing MSCV NF1-GRD as opposed to B117 control virus cells, given that similar levels of total N- and K-ras were present. Similar results were obtained with the B106 cell line (data not shown). In addition, we found that there was little to no H-ras present within these *Nf1*-negative AML cell lines (data not shown).

GRD Effect on Tumorigenicity

Given the ability of the NF1-GRD to revert the growth of Ras transformed fibroblasts (18) and restore normal growth characteristics in *Nf1*^{-/-} mast cells (19), we wanted to test the effect it's expression would have on tumorigenicity of *Nf1*-negative AML cell lines *in vivo*. B114 empty vector transfected and NF1-GRD transfected cell populations were injected intravenously at 1×10^5 cells/mouse into SCID/NCr mice and the latency to leukemia was determined. An average latency of approximately 100 days did not differ significantly between the two groups (data not shown). Analysis of bone marrow leukemic cells displayed antibiotic resistance and lack of NF1-GRD expression (data not shown). We suspect that NF1-GRD expression was rapidly silenced in the B114 transfected population *in vivo*.

DISCUSSION

In this report, utilizing *Nf1*-negative acute myeloid leukemia cells, we demonstrate that reintroduction of the GRD of NF1 attenuates Ras activation and inhibits proliferation. Antibiotic selection for two different expression vectors led to a very strong bias against NF1-GRD expression. Early passage NF1-GRD expressing cells experienced two-thirds reduction in activated K- and N-Ras, shown here for the first time to be specific targets of the NF1-GRD *in vivo*. Surprisingly, NF1-GRD expression had no effect on sensitivity to apoptosis, but did dramatically reduce proliferation rates in AML cells. These results have therapeutic implications since agents designed to reduce Ras-GTP levels in *NF1*-deficient myeloid leukemia may slow growth, but not eliminate the malignant clone.

While mouse models of *Ras* oncogene-driven cancer have been shown to require its continued expression for tumor cell maintenance, similar experiments had not determined if NF1-deficient AML cells require that NF1-GRD function remain off for leukemia cell maintenance until this work. When a *RAS* oncogene is turned off in mouse models, using the Tet-regulated transgenes, the tumor cells undergo apoptosis (26). This is true for melanomas induced by *p16Ink4a* deletion plus expression of an *HRAS* oncogene (17) and for lung cancer induced by a *Kras* oncogene (27). Results like these have led to the model of "oncogene" addiction (28).

These results have profound implications for cancer therapy since these models are known to carry, or were generated in the presence of, cooperating mutations. Nevertheless, the *Ras* oncogene is critical for tumor cell survival and the tumors are addicted to high levels of Ras-GTP. We find in contrast that the NF1-GRD expression and subsequent Ras-GTP down-regulation profoundly slows cellular proliferation without causing apoptosis. Thus, NF1-negative AML is not “addicted” to high levels of Ras-GTP in the way *Ras* oncogene-driven cancers have been found to be. This difference could be due to differences in the cell type: lung cancer and melanoma cells versus AML cells. Alternatively, the difference could be due to the fact that we have used established cell lines, which have been selected for resistance to apoptosis, a phenomenon described for mouse lymphoma cell lines (29). We favor the hypothesis that high levels of Ras-GTP, due to loss of a negative regulator like *NF1* or alterations in upstream receptors, has a different role in tumor cell biology compared to high levels of Ras-GTP due to expression of a *RAS* oncogene. Specifically, the former are not “addicted” to high levels of Ras-GTP as are the latter. We therefore, suspect that NF1 syndrome-associated malignancy will need to be treated with agents that target Ras-GTP to cause cytostasis, but which also can induce death of the malignant clone by another means.

By using BXH-2 cell lines, we were able to stably reintroduce the NF1-GRD into *Nf1*-negative cell lines. Two different constructs were capable of NF1-GRD expression as illustrated in their corresponding western blots (Figure 1). However, expression was eventually lost in stable cell lines after successive passaging. This suggests that NF1-GRD expression has a negative effect on cells that is selected against as cells continuously grow. Since NF1-GRD cells were derived from pools of transduced or transfected cells, clones expressing no/low levels of the NF1-GRD may have a growth advantage over higher expressing cells. However attempts to make single clones from retroviral transduction or DNA transfection repeatedly failed (data not shown) suggesting that NF1-GRD cells utilize mechanisms, possibly methylation, to suppress NF1 GRD expression from the expression cassette. This may indicate it will be difficult to use gene therapy approaches to introduce NF1-GRD expression vectors into neoplastic cells *in vivo* in NF1 patients to achieve a long-term therapeutic effect.

We discovered that NF1-GRD expression and suppression of Ras-GTP levels was profoundly selected against in AML cells due to a severe reduction in proliferation rates. The mechanisms by which this occurs are not clear. We suspect, based on prior literature, that G1 to S progression is blocked by limiting amounts of G1 cell cycle components. A reduction in cyclin D1 or cyclin E, known targets of Ras-GTP signaling (30), is a prime candidate for such an effect.

Previous studies have shown that the NF1-GRD has activity *in vitro* against a variety of small GTPases including N-, K-, H- and R-Ras. However, no previous publications had examined specific Ras isoforms for NF1-GRD sensitivity in living cells. In fact, previous assays performed with NF1's GRD have used H-Ras as the model Ras isoform to study NF1's GAP function (11,31). However the AML cells currently under investigation lack H-Ras expression (data not shown). The predominant Ras species, N-Ras and K-Ras, are clearly regulated by NF1 GRD expression in myeloid cells. Given the lack of H-Ras in these leukemic cells and that NF1 may play a specific role in regulating each Ras isoform, analyzing NF1 GAP activity on N- and K-ras, and their downstream effector pathways is crucial to understanding the biology of NF1-associated leukemias. Those pathways downstream of N- and K-ras-GTP most involved in myeloid leukemia remain to be determined. Among the candidates are the MAPK, the PI3K, and the Ral pathways (32). As we saw no effect on viability after GRD reintroduction the primary effect of loss of NF1 may not be to deregulate PI3K, which has been associated with apoptosis regulation (32). Instead, we saw a pronounced effect on cell proliferation rates, which might be mediated by suppression of other pathways downstream of Ras-GTP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Effects of NF1-GRD expression on proliferation in B106 and B117 cells

(A) To determine if GRD expression can suppress proliferation, control virus or MSCV NF1-GRD B106 cell lines were plated at the same concentration and the number of living and dead cells were counted every 24 hours. Within two or three days a significant difference in growth rates is already apparent. P-values for control cells to GRD cells are day 2 = 0.01, day 3 = 0.02 and day 4 = 0.003. Control virus cells have reached saturation between Day 3 and Day 4, and the decrease in viability is due to overgrowth of the culture. (B) Growth assays were performed with control virus or MSCV NF1-GRD B117 cell lines were plated at the same concentration. At day 3 a significant difference in growth rates is apparent. P-value for control cells to MSCV NF1-GRD cells is day 3 = 0.002.

Figure 3. Effects of NF1-GRD expression on proliferation in B114 cells under low serum concentrations

Growth and viability assays were performed with empty vector or CMV NF1-GRD transfected B114 cell lines in 10%, 1% or no serum. In the upper right hand graph, at days 1, 2 and 3 a significant difference in growth rates is apparent in 10% serum. P-values for empty vector cells to CMV NF1-GRD cells are day 1 = 0.001, day 2 < 0.0001 and day 3 = 0.0002. Similar results are seen in 1% serum (lower left hand graph) in which much reduced proliferation is seen in NF1-GRD transfected B114 cells. P-values for empty vector cells to CMV NF1-GRD cells are 24 hrs = 0.0156 and 48 hrs < 0.0001. Empty vector or CMV NF1-GRD B114 cell lines were plated at the same concentration in media lacking serum (upper right hand graph). No significant difference in growth rates at the timepoints analyzed, nor any significant difference in viability is apparent.

Figure 4. Ras-GTP pull-down assays

Isoforms of GTP-bound Ras were precipitated from lysates from *Nf1*-negative cell line B117 expressing either the NF1-GRD, or control virus, with GST bound to Ras-binding-domain of Raf. Activated Ras was run out on SDS-PAGE and blotted with the indicated isoform specific antibodies to specifically detect activated N-Ras, K-Ras or total N-Ras and K-Ras levels. Mean luminosity values were measured to determine the level of signal reduction. Activated N-Ras in control cells, mean luminosity of 97. Activated N-Ras in NF1 GRD cells, mean luminosity of 18. Activated K-Ras in control cells, mean luminosity of 187. Activated K-Ras in NF1 GRD cells, mean luminosity of 45.