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Merlin inhibits growth hormone-regulated Raf–ERKs pathways by binding to Grb2 protein

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Abstract

Numerous studies have suggested that the NF2 protein merlin is involved in the regulation of abnormal cell growth and proliferation. In this study, to better understand the merlin's mechanisms that contribute to the inhibition of tumorigenesis, we examined the potential action of merlin on the cell proliferative signaling pathways in response to growth hormone (GH). Merlin effectively attenuated the GH-induced serum response element (SRE) and Elk-1-mediated transcriptional activation, as well as the endogenous SRE-regulated gene c-fos expression in NIH3T3 cells. In addition, merlin prevented the Raf-1 complex activation process, which resulted in the suppression of MAP kinase/ERK, extracellular signal-regulated kinase (ERKs), and Elk-1 phosphorylation, which are the downstream signals of Raf-1. Moreover, it was shown that merlin interacted with endogenous growth factor receptor bound 2 (Grb2) protein and inhibited its expression. These results suggest that merlin contributes, via its protein-to-protein interaction with Grb2 and consequent inhibition of the MAPK pathways, to the regulation of the abnormal cell proliferation, and this provides a further mechanism underlying the tumor suppressor function of merlin.

Keywords: Neurofibromatosis type 2; Merlin; Raf-1 activation; Growth factor receptor bound 2; Proliferative signals

The development of cancer has been associated with a number of alterations in the related genes [1]. A new tumor suppressor, the neurofibromatosis type 2 (NF2) gene, has recently been characterized. NF2 is a familial cancer syndrome that features the development of tumors of the nervous system, and particularly schwannomas and meningiomas [2]. The disease is caused by an inherited mutation of the NF2 gene; the sporadically occurring tumors of these types frequently exhibit the inactivation of both NF2 alleles. The NF2 gene encodes for a 595 amino acid protein that is termed merlin or schwannomin, and this is highly related to the ERM proteins ezrin, radixin, and moesin [3]. However, merlin has distinct properties and functions for regulating cell growth and proliferation that are not ascribed to the ERM proteins [4–6]. Merlin represents the first identified structural protein that is thought to suppress tumor development [3]. The results of experiments in which NF2 protein merlin was overexpressed support the idea that the merlin functions as a tumor suppressor by inhibiting the proliferation of fibroblast cells [7], schwannoma cells [6], and meningioma cells [4].

It has been demonstrated that the overexpression of merlin was able to reverse some aspects of the Ras protein-induced malignant phenotype such as anchorage-independent growth in soft agar, and it restores contact inhibition of cell growth [8]. Further, we have reported that merlin inhibited the Ras-mediated activator protein-1 (AP-1) activity and also the ERKs signals that are strongly related to cell transformation and tumor development [9,10]. In addition, merlin was shown to inhibit the p21-activated kinase, Pak1, which is a common downstream target of both Rac and Cdc42 [11]. Moreover, it was recently observed that merlin inhibited the Ral guanine nucleotide

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dissociation stimulator (RalGDS), a downstream molecule of Ras, via direct interaction [12]. So far, great progress has been made in understanding how the pathways are controlled by NF2 protein, but the merlin's exact mechanisms that contribute to the inhibition of tumorigenesis remain to be elucidated. For a better understanding of merlin's role, we investigated the antiproliferative action of merlin in response to growth hormone (GH).

GH is a major regulator of normal body growth and metabolism, and it also regulates cellular gene expression [13,14]. Transcription of the proto-oncogene c-fos is rapidly stimulated by GH [13,15], and the serum response element (SRE), an enhancer sequence upstream of the c-fos gene, can mediate the transcriptional activation [16,17]. The binding of GH to its receptor results in the activation of JAK2 [18], and this initiates several signaling cascades, including one in which a SHC–Grb2–SOS complex activates Ras [19]. These events correlate in time with the GH-induced activation of mitogen-activated protein (MAP) kinases, an important mediator of signal transduction and a key regulator of many cellular processes such as cell growth, proliferation, and differentiation [20,21].

Therefore, it is possible that merlin functions as a tumor suppressor by regulating the cell proliferative signals in response to GH. To examine this possibility, this study investigated merlin's antiproliferative action and also investigated its mechanism that contributes to proliferative inhibition.

Materials and methods

Plasmids. The expression plasmid for the merlin (pcDNA-NF2) was kindly provided by Dr. David Gutmann (Washington University, USA). The expression plasmid for the GAL4 DBD-fused transactivating domain of Elk (pFA2-Elk1), and the luciferase reporters with the GAL4 DNA binding sites (pFR-Luc) were purchased from Stratagene, and the SRE-Luc reporter gene was purchased from Promega. The pCMV-Raf-1 vector was purchased from Clontech.

Cell culture and transient transfection and reporter assay. The NIH3T3 cells were obtained from the American Type Culture Collection (Manassas,VA), and wild type and NF2-deficient mouse embryonic fibroblasts ($Nf2^{+/+}$ and $Nf2^{-/-}$ MEFs) were kindly provided by Dr. McClatchey. All of cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere containing 5% CO_2 at 37 °C. The Gene Porter2 transfection reagent (Gene Therapy System) was used to transfect the plasmids according to the manufacturer's instruction. For the reporter assay, 3×10^5 NIH3T3 cells were plated in a 35 mm plates. After 16 h, the cells were transfected with 0.3 μg of reporter plasmids, 0.1 μg, 0.5 μg or 1.0 μg pcDNA-NF2, and combinations of expression plasmids. Total amount of the transfected DNA was kept same between samples using the pcDNA3.1 plasmid. For GH treatment, the transfected cells were starved by culturing them in 0.1% FBS DMEM for another 18 h. The cells were then treated for 4 h with 500 ng/ml GH. The cells were washed in cold phosphate-buffered saline twice and then lysed with 100 μl of 1× lysis buffer (25 mM, Tris-phosphate, pH 7.8, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100) for 20 min, and supernatant was collected by centrifugation at 14,000*g* for 15 min. The luciferase activity was measured. An internal control for the transfection efficiency could not be used because merlin influences the expression of most of the commercial promoters. Instead, the experiments were repeated three times and the results were averaged. The standard errors are shown on the top of each bar.

Antibodies and Western blot analysis. The merlin and GFP antibodies (Santa Cruz Biotechnology), β-actin antibody (Sigma), Grb2 antibody (BD Transduction Laboratories), phosphor-Raf-1, Raf-1, and phospho-ERKs, ERKs, Elk, phospho-Elk, and Ras (New England Biolabs) antibodies were obtained from commercial sources. For Western blot analysis, the cells were rinsed with phosphate-buffered saline and lysed for 30 min on ice in RIPA-B buffer (0.5% Nonidet P-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM DTT, and 50 μg/ml PMSF). Insoluble material was removed by centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant was subjected to SDS–PAGE, and Western blot analysis performed. Blots were blocked in phosphate-buffered saline with 5% skim milk and 0.05% Tween 20, incubated with the appropriate antibodies and then incubated with the secondary antibodies conjugated to horseradish peroxidase. The blots were subsequently assayed using the ECL detection system (Amersham Pharmacia Biotech).

Immunoprecipitation and in vitro kinase assay. For the Raf-1 immunoprecipitation/kinase assays, cells were lysed in ice-cold RIPA, and the lysates were clarified by centrifugation at ~25,000*g* at 4 °C. The supernatant protein levels were assayed, 100 μg aliquots were incubated with 2 μg antibody for 90 min at 4 °C, and protein A–agarose beads were then added for an additional 30 min to immunoprecipitate the Raf-1. The pellets were washed and resuspended in 20 ml of kinase buffer (20 mM Pipes (pH 7.0), 10 mM $MnCl_2$, 20 mg/ml aprotinin, and 200 mM Na_3VO_4) that contained 1 ng of kinase-inactive MEK-1 and 5 mCi of [γ-^{32}P]ATP. Raf-activated MEK-1 was resolved on SDS–polyacrylamide gels, then dried, and autoradiographed.

Immunocytochemistry. The cells were fixed with 4% paraformaldehyde at room temperature (RT) for 1 h. After fixing, the cells were washed with phosphate-buffered saline (PBS), blocked with serum in 0.3% Triton X-100 in PBS, and next incubated with the primary antibody:monoclonal anti-Raf-1 (mouse, 1:200, Chemicon) overnight at 4 °C. After rinsing, cells were incubated for 1 h at RT with 1:100 Texas red-conjugated secondary antibodies (Vector Laboratories). Cells were visualized using a fluorescence microscope.

Results

Merlin inhibits GH-induced SRE and Elk-1-mediated transcriptional activation, as well as the endogenous c-fos expression

The well-known effects in response to GH on cell proliferation involve the increase of c-fos expression through the SRE transcriptional activation [16,22]. For insight into the merlin's inhibitory mechanisms on the GH-stimulated cell proliferative signaling pathways, we first investigated whether merlin blocks the GH-induced SRE transcriptional activation, as well as the SRE-regulated c-fos expression. The NIH3T3 cells were transfected with the SRE-Luc reporter plasmid with or without the merlin expression plasmid. As shown in Fig. 1A, GH stimulated the increase of SRE luciferase activity, but the co-transfection with higher amounts of merlin caused a dramatic reduction of this activity in a dose-dependent manner. Western blot analysis confirmed that the merlin levels were increased in a concentration-dependent manner.

We further investigated whether merlin also blocks the GH-induced and Elk-1-mediated transcriptional activation with the trans-reporter systems, the ternary complex factor