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Down-regulation of Sprouty2 via p38 MAPK plays a key role in the induction of cellular apoptosis by tumor necrosis factor- α

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Abstract

Mammalian Sprouty2 (Spry2) is a key regulator of the receptor tyrosine kinase/ERK signaling pathway and involved in many biological processes, including cell growth, migration and tumor suppression. Here, we demonstrated that the intracellular protein level of Spry2 was significantly down-regulated by tumor necrosis factor- α (TNF- α) in both murine Swiss 3T3 fibroblasts and MLE15 lung epithelial cells. Although TNF- α activates multiple signaling cascades, only the inhibitor of p38 MAPK pathway blocked TNF- α -induced Spry2 down-regulation. Moreover, since both the mRNA level and protein half-life of Spry2 were unaltered by TNF- α treatment, this indicated the possible involvement of a translational mechanism in mediating the inhibitory effect of TNF- α . Importantly, rescue of the TNF- α -induced down-regulation of Spry2 by gene over-expression led to reverse of the apoptotic effect of TNF- α in Swiss 3T3 cells. To our knowledge, this study is the first that reported the association of Spry2 with TNF- α signaling pathway.

Introduction

Sprouty2 (Spry2) belongs to the Sprouty family of proteins, which are evolutionarily conserved modulators of receptor tyrosine kinases (RTKs) signaling. As the first member of this family, *Drosophila* Sprouty (*dSpry*) was originally discovered in a genetic screen for novel genes implicated in early tracheal branching [1], and was subsequently demonstrated to be a general inhibitor of RTKs. Accordingly, it antagonizes mitogen-activated protein kinase (MAPK) activation induced by insect FGF receptor, EGF receptor (EGFR), Torso and Sevenless [2–4]. Four orthologs of *dSpry* (*Spry1–4*) have been identified in mammals [1], with *Spry2* exhibiting the highest level of homology to the ancestral gene and being the focus of the majority of studies. Similar to *dSpry*, mammalian Spry2 protein plays a key role in the developmental processes of many branching tissues including lung, kidney, prostate, blood vessels, testis and breast ducts [5].

Nevertheless, the functions of mammalian Spry2 protein seem to be more complicated than its insect ortholog. For example, Spry2 can prolong EGF-stimulated MAPK signaling in some cell types by attenuating EGFR ubiquitination and endocytosis [6]. In addition, overexpression of *Spry2* inhibits cell proliferation and migration in multiple tumor cell lines [7,8]. Importantly, this inhibitory effect is suggested to be independent of MAPK activation and attributed to a possible tumor suppressor activity of Spry2 [9,10]. As further evidence supporting the link between Spry2 and cancer, down-regulated expression of Spry2 has been separately reported

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to occur in various cancers, such as lung, liver, breast and prostate [11]. So far, the mechanism responsible for the deregulation of Spry2 in cancer remains unclear.

Prior studies have demonstrated that modulation of Spry2 activity occurs on multiple levels including cytoplasmic localization, tyrosine/serine phosphorylation, protein degradation and gene expression [12]. For example, the transcriptional expression of *Spry2* is induced by a number of ligands of RTKs, including EGF, PDGF, HGF and members of the FGF family [7]. In contrast, we have recently discovered that the Spry2 level is down-regulated by TGF β through mechanisms involving not only repression of *Spry2* transcription but also enhancement of Spry2 protein degradation [13]. All these emphasize that a tight and balanced control of the intracellular Spry2 abundance is necessary for its proper functions in a wide variety of biological processes.

In the present study, we aimed at examining whether the intracellular level of Spry2 could be regulated by tumor necrosis factor- α (TNF- α), a well-known proinflammatory cytokine that has been implicated in many pathological conditions, most notably autoimmune diseases (such as rheumatoid arthritis and inflammatory bowel disease), asthma, septic shock, diabetes and cancer [14]. We found that TNF- α also had repressive effects on Spry2 levels as does TGF- β , but through a distinct mechanism. Mammalian TNF- α exerts its biological effects by binding to two cell surface receptors, both belonging to the TNF receptor (TNFR) family. These receptors subsequently activate several intracellular signaling pathways, which include the I- κ B kinase/ NF- κ B, c-Jun N-terminal kinase (JNK), p38 and p42/44 MAPK cascades [15]. To determine which signal transduction pathways regulated Spry2 levels, we utilized various inhibitors for protein kinases and our results indicated the involvement of p38 MAPK signaling cascade in mediating the repressive effect of TNF- α on Spry2 levels. Since TNF- α is capable of triggering both proliferation and apoptosis in Swiss 3T3 cells [16], we further examined whether these two functions of TNF- α could be affected by change of Spry2 levels. We found that overexpression of Spry2 protected Swiss 3T3 cells from TNF- α -induced apoptosis but had no impact on cell proliferation, thus suggesting a novel role of Spry2 in directly regulating the cellular apoptotic effect of TNF- α .

Materials and methods

Materials

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St Louis, MO). EGF and anti-Spry2 antibody were purchased from Upstate Biotechnology (Lake Placid, NY); TNF- α was obtained from R & D Systems (Minneapolis, Minnesota); SB-431542 was purchased from Tocris Biosciences (Ellisville, MO); PD98059, SP600125, SB203580 and BAY 11-7028 were from Calbiochem (San Diego, CA).

Cell Culture

Swiss 3T3 cells were obtained from the American Type Culture Collection (Manassas, VA) and were routinely cultured in Dulbecco's modified minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS). Cultures were maintained at 37°C in a humidified incubator containing 95% room air and 5% CO₂ atmosphere.

Cell Lysis and immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1: 100 dilution of protease inhibitor cocktail set III (Calbiochem). Cell lysates representing equal amount of total protein were subject to immunoblotting, which was performed essentially as previously described [13].

Plasmids and Transient Transfection

The expression vector that contains full-length mouse *Spry2* cDNA with a HA-epitope sequence fused to the COOH terminus has been described previously [13]. Transfections were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Cell Apoptosis Assay

Cells transfected with different expression vectors were plated at 3×10^4 cells/cm² on a 48-well plate. After attachment, cells were serum-starved in DMEM for 24 h and subsequently treated with or without 10 ng/ml TNF- α for additional 16 h. Thereafter, DNA fragmentation was assessed using the cell death detection ELISA kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Absorbance at a wavelength of 405 nm was measured with a VICTOR 3V multilabel plate counter (Perkin Elmer, Shelton, CT). Absorbance readings were normalized against control wells with medium alone.

Statistical Analysis of Data

Data were expressed as the mean $\pm$ SD where $n = 3$. A Student's *t*-test was used to assess the difference between two groups, and criterion for Statistical significance was set at $P < 0.05$.

Results

TNF- α down-regulates the protein level of Spry2

To study whether the expression of Spry2 can be regulated by TNF- α , cultured Swiss 3T3 cells were treated with different concentrations of TNF- α for 24 h. Samples were then harvested, and total protein was prepared for immunoblot analysis. As shown in Fig. 1A, Spry2 protein levels declined in a dose-dependent manner in response to treatment with 0.02–10 ng/ml TNF- α . The lower limit for detection of the TNF- α effect was 0.2 ng/ml, and the maximum suppression of Spry2 was attained at a TNF- α concentration of 10 ng/ml in the medium. To detect the maximum effect, 10 ng/ml TNF- α was used for all subsequent experiments.

To further examine the time course effect of TNF- α on Spry2 protein abundance, Swiss 3T3 cells were treated with TNF- α for 1, 3, 6, 9, 15 and 24 h. A decline in Spry2 protein level was detected as early as 1 h after exposure to TNF- α . The maximum reduction was reached in 9 h and remained at this level thereafter for up to 24 h (Fig. 1B). Similar effects of TNF- α were also observed in mouse lung epithelial MLE15 cells (data not shown).

The repressive effect of TNF- α on Spry2 is mediated through a TGF- β -independent but p38 MAPK-dependent signal transduction pathway

It has been reported that TNF- α is capable of inducing TGF- β 1 expression in Swiss 3T3 fibroblasts [17]. This suggests a possible involvement of TGF- β 1 in mediating the TNF- α -induced down-regulation of Spry2, since we have previously demonstrated a strong repressive effect of TGF- β 1 on Spry2 expression [13]. Therefore, we pretreated cells with a potent and specific inhibitor of TGF- β type I receptor kinase [13], SB-431542 for 1 h prior to TNF- α or TGF- β 1 exposure. As illustrated in Fig. 2A, pretreatment of cells with SB-431542 abolished down-regulation of Spry2 by TGF- β 1 but did not influence the response to TNF- α . Thus, our result excluded the participation of TGF- β 1 in mediating the TNF- α -induced Spry2 down-regulation.

The binding of TNF to TNF receptors leads to activation of several signal transduction pathways, which include NF- κ B and three MAPK cascades (including Erk1/2, p38, and JNK). Therefore, to determine which signal transduction pathways are necessary for down-regulation

of Spry2 by TNF- α , Swiss 3T3 cells were respectively pretreated with a series of selective chemical inhibitors for 1 h and then incubated with or without TNF- α for 24 h. As shown in Fig. 2B, we tested PD98059 (a specific inhibitor of MEK1, which is an activator of Erk1), SP600125 (a selective inhibitor of JNK), as well as BAY 11-7028, a pharmacological compound which is known to irreversibly inhibit NF- κ B activation [18]. Blockage of all these three signaling cascades had no effect on down-regulation of Spry2, indicating they are not mediators of the TNF- α -induced Spry2 down-regulation. However, as shown in Fig. 2C, SB203580 (a selective inhibitor of p38 MAPK) blocked the TNF- α -induced Spry2 repression in a concentration-dependent manner. Taken together, our data suggested that the TNF- α -induced repression of Spry2 expression might be mediated through activation of p38 MAPK.

The effects of TNF- α on Spry2 mRNA abundance and protein stability

To determine whether the decrease in Spry2 protein abundance was due to repression of its transcription, we measured *Spry2* mRNA level using real-time quantitative PCR. According to our results, the levels of Spry2 mRNA did not significantly change with TNF- α treatment (data not shown). Since TNF- α did not repress *Spry2* at transcriptional level, this suggested the involvement of post-transcriptional mechanisms in the process.

Accordingly, two reasons are most likely to be responsible for the decrease in Spry2 protein abundance: either at the translational level, the rate of protein synthesis was inhibited, or at the post-translational level, the rate of protein degradation was increased. Initially, to assess whether TNF- α affected the protein stability of Spry2, Swiss 3T3 cells were treated with or without TNF- α for 16h, after which cycloheximide was added to block *de novo* protein synthesis, thus the reduction in Spry2 levels would predominantly reflect the degradation process of Spry2 protein. As shown in Fig. 3A, the rates of Spry2 turnover were similar in the absence and presence of TNF- α . This suggested that the rate of Spry2 protein degradation was not enhanced by TNF- α .

On the other hand, when cells were pretreated with cycloheximide to inhibit mRNA translation before the addition of TNF- α , the ability of TNF- α to decrease the protein level of Spry2 was efficiently abolished under this condition (Fig. 3B), thus indicating that the observed down-regulation of Spry2 by TNF- α was likely to be regulated at the translational level.

Over-expression of Spry2 protect cells from the apoptotic effect of TNF- α

TNF- α is known to be a mediator of a variety of cellular responses including apoptotic death or proliferation depending on the target cell type. In the case of murine 3T3 fibroblast, both types of effects can be observed simultaneously. More specifically, when cells are arrested in G₀ phase by serum deprivation, TNF- α treatment drives only half of the population of cells to enter into the cell cycle, but leads to apoptosis in those cells that are unable to proliferate [16]. To explore whether Spry2 plays a role in mediating either effect of TNF- α , we overexpressed Spry2 in Swiss 3T3 cells by transfection with a Spry2 expression plasmid. A LacZ expression vector was used as the negative control. Both mitogenic and apoptotic effects of TNF- α were respectively measured in the transfected cells, which were serum-starved for 24 h prior to treated with or without TNF- α for additional 16 h. In the apoptosis assay shown in Fig. 4, we detected that some degree of cell apoptosis was induced by serum deprivation alone, and there was no apparent difference between the cells transfected with Spry2 plasmid and the cells transfected with the control vector. However, the cell's responsiveness to TNF- α treatment showed obvious distinction. In the cells that were transfected with the control vector, TNF- α induced a significantly higher level of cell death, whereas in the cells transfected with Spry2 expression plasmid, the apoptotic effect of TNF- α was reversed to be anti-apoptotic, and therefore much less apoptosis was observed in the presence of TNF- α . On the other hand, in the cellular proliferation assay, Spry2 transfection did not show any significant effect on the

mitogenic effect of TNF- α (data not shown). Taken together, these data suggested that Spry2 played a critical role in controlling the apoptotic action of TNF- α . Down-regulated Spry2 expression might be required for TNF- α to trigger apoptosis. In contrast, overexpressed Spry2 level might convert the function of TNF- α to be facilitating the serum-starved cells to maintain in a growth-arrested but viable state.

DISCUSSION

TGF- β is the first endogenous cytokine showing capability to down-regulate Spry2 [13], and the functional significance of Spry2 down-regulation has been associated with both cancer formation and enhancement of cellular proliferation. Herein, we further showed that Spry2 down-regulation can be induced by another cytokine, TNF- α , and plays a critical role in regulating apoptosis.

Cytokines such as TNF- α are able to regulate gene expression via multiple signal transduction pathways. Therefore, we initially studied the signaling mechanisms that mediate the TNF- α -induced Spry2 down-regulation in 3T3 cells. Although NF- κ B proteins are key proinflammatory transcription factors that mediate the TNF- α -dependent gene regulation events [19], our studies showed that the TNF- α -induced Spry2 down-regulation did not require activation of NF- κ B, since an irreversible I- κ B/NF- κ B inhibitor, BAY 11-7028, did not affect Spry2 expression in response to TNF- α . In addition to the NF- κ B-dependent pathway, at least three different subtypes of MAP kinase are known to be activated by TNF- α : p42/p44 MAPK, p38 MAPK, and JNK [15]. Here we showed that inhibition of p38 MAPK, but not JNK and p42/44 MAPK, by selective chemical inhibitors, blocked the repressive effect of TNF- α . Taken together, these data suggested that down-regulation of Spry2 in Swiss 3T3 cells by TNF- α is mediated through p38 MAPK, but not p42/44 MAPK, JNK or NF- κ B pathway.

As demonstrated in our previous report, down-regulation of Spry2 at the protein level by TGF- β is due to both transcriptional repression and enhanced posttranslational degradation [13]. In comparison, The Spry2 reduction following TNF- α treatment is likely to be mediated by different mechanisms. Initially, we did not observe any change of Spry2 mRNA abundance upon TNF- α treatment, this ruled out the participation of transcriptional mechanism. Considering possible post-transcriptional mechanisms, down-regulated protein expression could result from two possibilities: either the protein stability is decreased or the mRNA translational efficiency is inhibited. Accordingly, we further ruled out the first possibility because similar half-lives of Spry2 protein in the presence or absence of TNF- α were detected. This led us to propose that the observed Spry2 reduction was regulated at the translational level. As supporting evidence, we found that pretreatment of cells with cycloheximide to inhibit mRNA translation completely abolished the ability of TNF- α to decrease Spry2 protein. Taken together, our data suggested that the decrease in Spry2 protein level is due to decreased *de novo* Spry2 protein synthesis. However, additional experiments such as pulse-chase labeling assay that directly measures Spry2 protein synthesis rate are needed to validate our speculation.

The process of mRNA translation or protein synthesis is conventionally divided into three main stages: initiation, elongation and termination, and the initiation step is often the target of translational regulation [20]. Early reports have already described that TNF- α can inhibit the initiation stage of translation through different mechanisms. For example, TNF- α is capable of directly inhibiting the activities of two eukaryotic initiation factors, eIF4E and eIF2B, and thereby suppressing the initiation of protein translation. In addition, TNF- α is also able to affect the upstream signal transduction pathways that are known to mediate translational control, such as mammalian target of rapamycin (mTOR) protein [21,22]. Therefore, it will be of future interest to further determine if the translational suppression of Spry2 in response to TNF- α occurs at the initiation stage of translation and what physiological mediators are involved in.

TNF- α is a multifunctional cytokine that induces a broad spectrum of responses, including cell growth, differentiation and apoptosis. In this report, we showed that there was a correlation between the decrease in Spry2 protein abundance and the TNF- α -induced apoptosis in 3T3 cells, and we therefore proposed that decreased Spry2 levels might contribute to the initiation of apoptosis or increase susceptibility to apoptosis. As evidence supporting the role of Spry2 in the regulation of apoptosis, it was recently reported that silencing of endogenous Spry2 by siRNA abolished the anti-apoptotic action of serum in adrenal cortex adenocarcinoma cells. Mechanistically, the depletion of endogenous Spry2 led to a decrease in the amount of EGFR, which in turn attenuated the activation of ERK and AKT pathways in response to serum or EGF [23]. However, this mechanism may not explain why Spry2 can also regulate the TNF- α -induced cell apoptosis, which doesn't require signal transduction from EGFR. In the present work, we showed that overexpression of Spry2 significantly protected cells from the apoptotic effect of TNF- α without affecting the mitogenic action of TNF- α . This was surprising because we previously showed that overexpression of Spry2 inhibited the proliferation of Swiss 3T3 cells in response to TGF- β stimulation [13]. How did Spry2, which is well known as a growth inhibitor, paradoxically enhance survival of the same cell type from apoptosis? And why was the TNF- α -induced cell proliferation not affected by overexpression of Spry2? One explanation is that Spry2 may serve plural functions and control multiple cell behaviors. In particular, regulation of intracellular Spry2 abundance alone is essential but not sufficient to determine the fate of the cell, thus it only gives the cell potential to undergo either proliferation or apoptosis or maintain viability in quiescent state, but the outcome seems to be directly dependent on the influence of other factors, such as the stimuli received by the cell (TGF- β vs TNF- α), or activation of other genes, or the phase of the cell cycle. Additional studies are needed to test this hypothesis and determine the precise mechanism by which Spry2 protects cells from apoptosis.

In summary, our data indicated that down-regulation of Spry2 could be induced by TNF- α . This down-regulation was mediated through p38 MAPK, but neither JNK nor p42/p44 MAPK nor NF- κ B pathway, and it played an important role in regulating apoptosis. All these information may enhance our understanding of the complex functions of Spry2 in both physiological conditions and pathological diseases.

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Fig. 1. TNF- α down-regulates the protein abundance of Spry2 in Swiss 3T3 fibroblasts. A, dose-dependent down-regulation of Spry2 by TNF- α . Swiss 3T3 cells were treated with various concentrations of TNF- α for 24 h. B, kinetics of the inhibitory effect of TNF- α on Spry2 expression. Swiss 3T3 cells were treated with 10 ng/ml TNF- α for indicated times. Controls were maintained in serum-free DMEM for up to 24 h without TNF- α treatment. Equal amount of total protein extracts was subject to immunoblotting with Spry2- and β -actin-specific antibodies. β -actin was used as a loading control.

Fig. 2.

The TNF- α -induced down-regulation of Spry2 expression depends on p38 MAPK signaling cascade. A, SB-431542 only abrogated the TGF- β -induced, but not the TNF- α -induced Spry2 down-regulation. B, PD98059, SP600125 and BAY 11-7028 had no specific inhibitory effects on the TNF- α -induced Spry2 down-regulation. C, SB203580 abolished the TNF- α -induced down-regulation of Spry2 expression in a dose-dependent manner. Swiss 3T3 cells were pretreated with indicated chemical inhibitors at concentrations shown for 1 h, followed by incubation with or without 10 ng/ml TNF- α or TGF- β 1 for 24 h. Control was pre-treated with DMSO.

Fig. 3. Down-regulation of Spry2 by TNF- α is due to decrease in Spry2 translation but not Spry2 protein stability. A, TNF- α did not change the turnover rate of Spry2 protein. Swiss 3T3 cells were treated with (+) or without (–) 10 ng/ml TNF- α for 5 h, followed by the addition of 30 μ g/ml cycloheximide (CHX) over a 4-h time course. B, inhibition of new protein synthesis abolished the effect of TNF- α on Spry2 protein level. Cells were pretreated with or without 30 μ g/ml CHX for 1 h prior to the addition of 10 ng/ml TNF- α for 7 h.

Fig. 4.

Over-expression of Spry2 inhibited the apoptotic effect of TNF- α on Swiss 3T3 cells. Cells were transiently transfected with either control LacZ expression vector or Spry2 expression vector, followed by incubation in serum-free medium for 24 h. Thereafter, cells were treated with (+) or without (-) 10 ng/ml TNF- α for additional 16 h. Cell apoptosis assay were performed as described under "Materials and methods." We assigned the value of 1 to the average absorbance of control (no TNF- α treatment) samples. The average absorbance of the TNF- α treated samples are represented as fold over control samples. Data shown are representative of three independent experiments with each experiment performed in triplicate. The *bar graphs* represent the mean $\pm$ SD (n = 3). * Indicates the statistical significance of Spry2 transfection compared with control vector transfection, $p < 0.05$.