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Regulation of SRF protein stability by an autophagy-dependent pathway

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Abstract

Serum response factor (SRF), a key transcription factor, plays an important role in regulating cell functions such as proliferation and differentiation. Most proteins are unstable, and protein stability is regulated through the ubiquitin-proteasome system (UPS) or the autophagy lysosome pathway (ALP). Whether SRF is degraded and what mechanisms control SRF protein stability remain unexplored. Western blot analyses of cells treated with cycloheximide (CHX), a protein synthesis inhibitor, showed that SRF was degraded in a time-dependent manner. Moreover, we observed that SRF undergoes autophagy-dependent destruction, which is accelerated by serum deprivation. Through bioinformatics screening, we found that SRF contains the GSK3 β phosphorylation motif (T/SPPXS): SPDSPPRSDPT, which is conserved from zebrafish to humans. Serum deprivation stimulated GSK3 β activation that then potentiates SRF degradation through the autophagy lysosome pathway. Since SRF is important for numerous cellular activities, our results suggest that the autophagy-dependent SRF degradation pathway may provide a new avenue to modulate SRF-mediated cell functions.

Keywords

Serum response factor (SRF); Degradation; Autophagy lysosome pathway

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Author contributions

JL, FQJ and ZGJ wrote the manuscript. JL, FQJ, and MY designed and performed experiments. ZGJ conceived the study. All authors approved the final version of the manuscript.

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1. Introduction

Serum response factor (SRF), a ubiquitously expressed MADS-box transcription factor with multiple roles in various physiological processes, was first identified in 1986 [1]. It was originally discovered as a gene regulator effected by binding a palindromic DNA sequence named serum response element (SRE) when cells were stimulated with serum [1,2]. SRF has multiple evolutionarily-conserved roles, including regulating cardiac homeostasis [3,4], maintaining skeletal muscle function [5], and participating in the regulation of cancer, digestive system disease, and nervous system disease [6,7]. Mounting evidence suggests that SRF plays a necessary role in a vast number of cellular processes, such as cell growth, cell differentiation, and cell adhesion and migration, which are processes directly related to cytoskeleton dynamics [8,9].

SRF expression and activation are tightly controlled by multiple mechanisms. SRF activation is mainly correlated to one of two classes of cofactors, ternary complex factor (TCF) family and myocardin-related transcription factors (MRTFs) [10–13]. Other cofactors such as the GATA, Nkx2–5 and the CRP family can also regulate SRF activation. Like other transcription factors, the level of SRF in cells is tightly controlled by the rate of synthesis and degradation, and the appropriate level of SRF may be maintained by the targeted degradation pathway. Further understanding how SRF is regulated may provide new potential strategies targeting SRF-dependent diseases.

Most transcription factors are unstable and usually targeted to degradation through the ubiquitin-proteasome system (UPS) or autophagy lysosome pathway (ALP). The UPS is generally responsible for the degradation of most short-lived regulatory and mis-folded proteins [14]. Autophagy is involved in the cell degradation of organelles and large protein aggregates with the formation of autophagosomes and eventually fuse with lysosomes [15]. SRF plays an indispensable role in multiple cells, so it is important to investigate the molecular mechanisms regulating SRF stability/degradation. Whether autophagy or the ubiquitin-proteasome is responsible for the degradation of SRF is still unknown.

In the present study, we observed that autophagy, but not the ubiquitin-proteasome, facilitates SRF's degradation and that this degradation is induced by serum deprivation. Serum-deprivation-stimulated GSK3 β activation affects the degradation of SRF by regulating autophagy.

2. Materials and methods

2.1. Antibodies and reagents

Anti-HA-tag (Covance, D14EF01045), anti-GFP-tag (Proteintech, 66002-1-1g), anti-SRF, anti- β -Actin (Santa Cruz, sc-47778), anti-p-GSK3 β (Cell Signaling technology, 9336), cycloheximide (CHX), MG132, lactacystin, 3-methyladenine (3-MA), and NH₄Cl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Constitutively active GSK3 β (GSK3 β -CA) was obtained from Addgene, HA-SRF plasmid was obtained from Addgene, GFP-SRF plasmid was obtained from Addgene.

2.2. Cell culture and transfection

The COS-7 cells (ATCC) and the rat aortic smooth muscle cells (RASMCs, Sigma-Aldrich, St. Louis, MO, USA) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 µg/mL streptomycin at 37 °C and 5% CO₂. For transient transfection, the COS-7 cells were grown to ~70%–80% confluence, and the cells were transfected by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, a mixture of 2 µg plasmid DNA and 10 µL of lipofectamine 2000 in 2 mL of OPTI-MEM I medium (Invitrogen, Carlsbad, CA, USA) was introduced in cells, and after 4 h incubation at 37 °C the transfection mixture was replaced by complete culture medium and incubated for 24 h after transfection.

2.3. Pharmacological treatment

Cells were treated with proteasome inhibitors MG-132 (5 µM) and lactacystin (10 µM) or autophagy inhibitor 3-Methyladenine (3-MA, 10 mM) and NH₄Cl (20 mM). Cycloheximide (CHX), a protein-synthesis inhibitor, was used at 30 µg/mL at 0, 2, 4, and 8 h for the time-course assay of protein degradation.

2.4. Western blotting

Cells were harvested into RIPA buffer supplemented with pro-tease inhibitors. The whole cell lysate was separated on sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) and then blotted to PVDF membranes. The membranes were incubated with primary antibodies at 4 °C overnight. Secondary antibodies were as follows: anti-mouse IgG (IRDye 680RD goat anti-mouse IgG, LI-COR), anti-rabbit IgG (IRDye 800CW goat anti-rabbit IgG, LI-COR). Signals were detected by Odyssey scanner (LICOR, Biosciences, USA). Analysis of optical density was assessed using ImageJ software.

2.5. Confocal imaging

Cells were cultured on glass bottom dishes, transfected with fluorescent constructs and visualized directly on a confocal microscope. All experiments were repeated at least three times.

2.6. Statistical analysis

The data are presented as the means ± SEM. When only two value sets were compared, statistical analysis was made by Student's t-test; when the data involved three or more groups, statistical analysis was made by one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* values less than 0.05 (*P* < 0.05) were considered as a significant difference.

3. Results

3.1. SRF undergoes degradation in COS-7 cells and rat aortic smooth muscle cells (RASMCs)

Many proteins in cells are short-lived proteins and can be degraded quickly. The protein synthesis inhibitor CHX is usually used to test the degradation of proteins. To evaluate the

degradation of SRF, we first transfected HA-SRF plasmid in COS-7 cells for 24 h, then the cells were treated with 30 $\mu\text{g/mL}$ CHX for various times, separately. We then harvested total cell lysates and used the anti-HA antibody to determine the levels of SRF protein. We found that SRF protein was degraded in COS-7 cells in a time-dependent manner (Fig. 1A).

The degradation of SRF was also validated in cultured smooth muscle cells (SMCs). SRF is a key signal transduction factor in SMCs and can regulate many functions of SMCs, such as proliferation, migration, and apoptosis. So, we next examined SRF degradation in cultured RASMCs. The RASMCs were also treated with CHX (30 $\mu\text{g/mL}$) for 0, 2, 4, 8 h, and we observed time-dependent SRF degradation (Fig. 1B).

3.2. Inhibition of autophagy promotes the accumulation of SRF protein in cells

Proteins are degraded in cells mostly through two pathways: the ubiquitin proteasome system and the autophagy-dependent pathway. To investigate the pathway(s) involved in SRF degradation, proteasome inhibitors or autophagy inhibitors were used. COS-7 cells were transiently transfected with HA-SRF and incubated with proteasome inhibitors MG-132 and lactacystin. The results show that both inhibitors did not abolish the degradation of SRF (Fig. 2A), indicating that proteasomal degradation is not responsible for SRF degradation. We then assessed the potential involvement of the autophagy pathway in SRF degradation. COS-7 cells were transfected with HA-SRF and incubated with or without autophagy inhibitor 3-Methyladenine (3-MA) and NH_4Cl . The Western blot results showed a substantial accumulation of SRF protein levels upon the treatment with 3-MA and NH_4Cl (Fig. 2B). These data suggest that the autophagy- but not proteasome-dependent pathway contributes to the degradation of SRF.

3.3. Serum deprivation sensitizes SRF degradation

Since the importance of autophagy in SRF degradation was demonstrated by the treatment of the autophagy inhibitors, we next examined the influence of autophagy itself on SRF stability. Serum deprivation is a frequently performed procedure to stimulate autophagy. In this procedure, COS-7 cells were transfected with HA-SRF plasmid and cultured in serum deprived medium for 24 h. Upon the serum deprivation treatment, the SRF level in the COS-7 cells was significantly decreased (Fig. 3A). The dynamics of SRF protein levels in response to serum-free treatment over a 16 h period were also monitored. The result showed that serum starvation induced SRF protein degradation in a time-dependent manner (Fig. 3B). These data indicated that stimulating autophagy by serum starvation induces SRF degradation.

To further investigate the effect of serum deprivation on the SRF degradation. COS-7 cells were transfected with HA-SRF plasmid and treated with CHX (30 $\mu\text{g/mL}$) plus serum deprivation. As expected, the SRF protein was degraded much faster under the condition of the serum deprivation than that of normal serum culture (Fig. 3C). The same result was obtained by confocal microscopy assay to examine the level of SRF protein immunostaining in cultured cells (Fig. 3D). These results together indicate a crucial role of autophagy in promoting the degradation of SRF.

3.4. GSK3 β affects the degradation of SRF via autophagy

Glycogen synthase kinase-3 β (GSK3 β) has been recognized as a regulator of autophagy, especially serum starvation-induced autophagy [16]. Through bioinformatics screening, we first found that SRF contains the GSK3 β phosphorylation motif (T/SPPXS):SPDSPPRSDPT, which is conserved from zebrafish to humans (Fig. 4A). GSK3 β is activated under serum starvation conditions, evidenced by the decrease of GSK3 β phosphorylation at Serine 9 (p-GSK3 β) in COS-7 cells (Fig. 4B), which confirmed the inhibition of GSK3 β by growth factors in previous studies [17,18]. These results prompted us to assess the effect of GSK3 β in the degradation of SRF. When the constitutively active GSK3 β (GSK3 β -CA) mutant was introduced into the HA-SRF transfected COS-7 cells, the abundance of SRF protein decreased significantly (Fig. 4C). Immunofluorescent staining using anti-GFP antibody on GFP-SRF transfected COS-7 cells also revealed that GSK3 β induced SRF degradation in cells (Fig. 4D). Moreover, the pharmacological activation of GSK3 β by wortmannin-induced inhibition of the PI3K-Akt pathway also triggered SRF degradation (Fig. 4E). To determine whether GSK3 β -induced degradation of SRF is mediated by the autophagy-dependent mechanism, we treated cells with the autophagy inhibitor 3-methyladenine (3-MA) and NH₄Cl. As expected, inhibition of autophagy partially reversed the effect of the GSK3 β on the SRF protein degradation (Fig. 4F). Taken together, these results support the hypothesis that the effect of GSK3 β on the degradation of SRF is mediated by the autophagy-dependent pathway.

4. Discussion

SRF is an essential transcription factor that has important functions in multiple biological processes. It is integral to a vast number of cellular processes, from cell-proliferation, to muscle differentiation and development. Cardiomyocyte or smooth muscle cell-specific knockouts of the SRF gene in mice is embryonic lethal due to impaired cardiac function and heart failure. SRF has also been implicated in gastrointestinal system disease and central nervous system disease. The level of SRF is tightly controlled by the rates of synthesis and degradation and directly related to its function in many cell types. In this study, we provide a new mechanism, autophagy-mediated degradation, for the regulation of SRF.

We found that SRF undergoes degradation in both COS-7 cells and rat aortic smooth muscle cells, which means that SRF is an unstable protein just like other important transcriptional regulators. Although extensive studies have shown that many transcription factors were the substrates for modification by ubiquitin and degraded by ubiquitin-proteasome system, in our present study, the proteasome-specific inhibitor, MG132 and lactacystin had no effect on the SRF protein levels. The autophagy lysosome system has an equally importance role in the cellular degradation of protein. Therefore, we used the autophagy inhibitors 3-MA and NH₄Cl to test the effect on SRF's degradation. The results indicated that inhibitors of autophagy markedly decrease the degradation of SRF. These data suggest that SRF is regulated by degradation through the autophagy lysosome pathway but not the ubiquitin-proteasome system.

The autophagy-lysosome pathway is a cell survival mechanism, which is usually considered as an unselective cytoplasmic bulk degradation system and responsible for the degradation

of whole organelles and long-lived proteins [19]. Metazoans depend on extracellular growth factors to regulate cellular homeostasis. Cellular autophagy usually initiates upon growth factor withdrawal or in serum-free conditions [20,21]. In this study, we show that upon serum deprivation, SRF is rapidly degraded by endosomal micro-autophagy. Although extensive studies have focused on autophagic turnover of cytoplasmic materials, little is known about the role of autophagy in degrading transcriptional factors [22]. Our finding in this study suggests that SRF is one of such protein that undergoes degradation through autophagy.

The effects of GSK3 β on autophagy induction have been highly controversial [23]. GSK3 is activated by the removal of its inhibitory Ser9 phosphorylation upon deprived of serum [16]. We found that GSK3 β , when disinhibited by default in cells deprived of growth factors, can facilitate the degradation of SRF. GSK3 β has been found to regulate proteolysis of a vast number of important signaling proteins and transcription factors. The results from our recent study add a new member to the GSK3 β -targeted proteins. These findings uncover a new pathway that the activation of GSK3 β leads to SRF protein degradation through the regulation of autophagy.

In summary, we found that SRF protein undergoes degradation. We further explored the intrinsic mechanism of SRF degradation. Our data implies that the GSK3 β regulated autophagy pathway mediates the turnover of SRF protein. Our finding provides an important new avenue to influence SRF protein level for uncovering the molecular basis of SRF in cellular processes.

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Fig. 1.

SRF undergoes degradation in COS-7 cells and RASMCs. **(A)** SRF protein stability was assessed by cycloheximide (CHX) chase in COS-7 cells. COS-7 cells were transfected with HA-SRF plasmid for 24 h, then the cells were harvested at the indicated time points (0, 2, 4, 8 h) after CHX addition and then analyzed by immunoblotting with anti-HA antibody and β-actin antibody. **(B)** SRF protein stability was assessed by CHX chase in RASMCs. Immunoblotting showing SRF degradation in RASMCs treated with CHX for 0, 2, 4, 8 h. β-Actin served as the loading control (* $p < 0.05$, ** $p < 0.01$, $n = 3$).

Fig. 2.

Autophagy inhibition, but not proteasome inhibition, diminished the degradation of SRF. **(A)** COS-7 cells were transfected with HA-SRF plasmid, 20 h after transfection, CHX, or CHX combination with MG132 (5 μ M) or lactacystin (10 μ M) was added to one set of cultures and incubated for an additional 2–8 h. Cell lysates were prepared at indicated time points and the protein level of SRF was examined by Western blot analysis. **(B)** Immunoblotting showing SRF degradation in COS-7 cells treated with CHX, CHX in combination with the autophagy inhibitors 3-MA (10 mM) and NH₄Cl (20 mM). β -Actin was used as loading control. (* p < 0.05, ** p < 0.01, n = 3).

Fig. 3.

Serum deprivation sensitized SRF degradation. (A) COS-7 cells transfected with HA-SRF plasmid were cultured in 10% serum-containing medium or in medium containing 5%, 1% or 0% serum for 16 h. Equal amounts of cell extracts were subjected to immunoblotting analysis as shown. (B) Total lysates of HA-SRF transfected COS-7 cells after serum starvation for various time points (0, 0.5, 1, 2, 4, 8, 16 h) were prepared and immunoblotted as shown. (C) HA-SRF-expressing COS-7 cells were incubated in 10% serum-containing medium or in medium containing 5%, 1% or 0% serum, combined with CHX treatment for the indicated times, temporal regulation of SRF was analyzed by Western blot. (D) GFP-SRF-expressing COS-7 cells incubated in 10% serum-containing medium or in medium containing 0% serum for 16 h and visualized by confocal microscopy.

Fig. 4.

GSK3 β affects the degradation of SRF via the autophagy-dependent pathway. **(A)** SRF has a putative GSK3 phosphorylation site. **(B)** COS-7 cells were cultured in 10% serum-containing medium or in medium containing 5%, 1% or 0% serum for 16 h, GSK3 β -inhibitory phosphorylation at serine 9 (*p*-GSK3 β) was analyzed by Western blot. **(C)** COS-7 cells were transfected with HA-SRF and/or retrovirus containing sequence for a constitutively active form of GSK3 β (GSK3 β -CA). Whole cell extracts were subjected to Western blot analysis. **(D)** GFP-SRF-expressing COS-7 cells were transfected with GSK3 β -CA and visualized by confocal microscopy. **(E)** HA-SRF-expressing COS-7 cells were treated with Wortmannin (10 nM) for 24 h. Whole cell extracts were subjected to Western blot analysis. **(F)** GFP-SRF-expressing COS-7 cells were treated with GSK3 β -CA, or GSK3 β -CA plus 3-MA (10 mM) and NH₄Cl (20 mM). Whole cell extracts were subjected to Western blot analysis. β -Actin was used as loading control. (* p < 0.05, n = 3).