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Increased stability of heterogeneous ribonucleoproteins by a deacetylase inhibitor

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ABSTRACT

Splicing factors are often influenced by various signaling pathways, contributing to the dynamic changes of protein isoforms in cells. Heterogeneous ribonucleoproteins (hnRNPs) regulate many steps of RNA metabolism including pre-mRNA splicing but their control by cell signaling particularly through acetylation and ubiquitination pathways remains largely unknown. Here we show that TSA, a deacetylase inhibitor, reduced the ratio of Bcl-x splice variants Bcl-xL/xS in MDA-MB-231 breast cancer cells. This TSA effect was independent of TGFβ1; however, only in the presence of TGFβ1 was TSA able to change the splicing regulators hnRNP F/H by slightly reducing their mRNA transcripts but strongly preventing protein degradation. The latter was also efficiently prevented by lactacystin, a proteasome inhibitor, suggesting their protein stability control by both acetylation and ubiquitination pathways. Three lysines K87, K98 and K224 of hnRNP F are potential targets of the mutually exclusive acetylation or ubiquitination ($K^{Ac/Ub}$) in the protein modification database PhosphoSitePlus. Mutating each of them but not a control non-$K^{Ac/Ub}$ (K68) specifically abolished the TSA enhancement of protein stability. Moreover, mutating K98 (K98R) and K224 (K224R) also abolished the TSA regulation of alternative splicing of a Bcl-x mini-gene. Furthermore, about 86% (30 of 35) of the multi-functional hnRNP proteins in the database contain lysines that are potential sites for acetylation/ubiquitination. We demonstrate that the degradation of three of them (A1, I and L) are also prevented by TSA. Thus, the deacetylase inhibitor TSA enhances hnRNP F stability through the $K^{Ac/Ub}$ lysines, with some of them essential for its regulation of alternative splicing. Such a regulation of protein stability is perhaps common for a group of hnRNPs and RNA metabolism.

1. Introduction

Alternative pre-mRNA splicing is tightly controlled by *cis*- and *trans*-acting elements/factors for proper protein and cell functions [1–3], and aberrant splicing causes genetic diseases [4,5]. Many of the *trans*-acting splicing factors are regulated by various intracellular signaling pathways [6,7], but in few cases the effect of interplay between different pathways in the regulation of splicing has been studied [8].

Of the protein modifications in cell signaling pathways, acetylation and ubiquitination each has recently been found to regulate splicing [8–16]. In these studies, acetylation/deacetylation effect on alternative splicing has been examined mostly from the point of histone modifications coupled with chromatin remodeling and transcription. However, more and more evidence indicate that this modification is also common to many other proteins of diverse functions in cells [14], including splicing factors [8]. Control of alternative splicing and/or splicing factors by protein degradation or ubiquitination has been observed as well [9–11,16,17]. Moreover, crosstalk between the acetylation and ubiquitination pathways controls important cellular proteins such as p53 through competition for the posttranslational modification of particular lysines ($K^{Ac/Ub}$) [18,19]. However, the effect of crosstalk between the two pathways on splicing factors has not been reported.

Heterogeneous ribonucleoproteins (hnRNPs) are a family of RNA binding proteins that regulate many steps during RNA metabolism including processing, transport, localization, translation and degradation [20]. Human hnRNP F and H (H1) proteins share about 78% identity and many common features, including shuttling between the nucleus and cytoplasm [21–23], binding to G tracts, playing roles in 3′ end processing and translation [24–26], and regulation of alternative splicing of common targets [27–29], particularly of the cell survival/death-promoting splice variants Bcl-xL/xS [27]. They also have different preferences to certain G

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tracts depending on the sequence composition and location or cellular context [30,31].

In this report, we found that the degradation of hnRNP F/H, likely through the proteasome/ubiquitination pathway, is prevented by trichostatin A (TSA), a deacetylase inhibitor [32], and identified critical amino acids that are essential for the regulation and their effect on splicing. Similar regulation by TSA is also found for other hnRNP proteins.

2. Materials and methods

2.1. Plasmid construction

To make the Myc-tagged hnRNP F plasmid pMychnRNPF, a cDNA fragment containing hnRNP F codons for amino acid residues 3–415 (GenBank accession #NM_001037287) was amplified using RT-PCR from PC12 cells and inserted into the KpnI site of pCMV–Myc vector (Clontech Co.) in frame with the NH_2 terminal Myc peptide. The hnRNP F mutations K68R, K87R, K98R and K224R were created using PCR with Pfu DNA polymerase. DNA sequencing and Western blots were carried out to verify the plasmids and their protein expression. Splicing reporter Bcl-x20 and its mutations of the G tracts have been described previously [33].

2.2. Pathway inhibitors, chemicals

TSA (T1952) and cycloheximide (C7698) were purchased from Sigma-Aldrich Co., lactacystin (#426100) from Calbiochem and ubiquitin isopeptidase inhibitor G5 (sc-356181) from Santa Cruz Biotech. Co.

2.3. Cell culture, treatment and transfection

MDA-MB-231 cells were cultured in a Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin–glutamine solution at 37 °C with 5% CO_2, as described previously [33], except that TGFβ1 (2.5 ng/ml) were added in the media unless otherwise indicated. The TGFβ1-grown cells were passaged in the presence of TGFβ1 or kept frozen before being used for experiments. Cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instruction. They were grown in 6- or 12-well plates and transfected with different plasmids (Myc–hnRNP F or its mutants, HA-Ubiquitin or Bcl-x20) for 24 h before any treatment was initiated.

2.4. Immunoblot

Whole cell protein lysates were obtained using a RIPA buffer for Western blots as described [34]. Briefly, 10 to 15 μg of protein was loaded onto a 10% SDS-PAGE gel and separated at 150 V for at least 1 h. Proteins were then transferred to PVDF membrane (Millipore Immobilon®-P Transfer Membrane) overnight, then probed for 1 h with anti-hnRNP F/H (1G11, Santa Cruz Biotech.), anti-hnRNP H (sc-10042, N-16, Santa Cruz Biotech.), anti-hnRNP F (a kind gift from Dr. Douglas Black) [35], anti-Myc (Santa Cruz Biotech.), anti-acetyl K (Millipore or Cell Signaling Tech.), anti-PTBP1 (PTB or hnRNP I, BB7) [36], anti-hnRNP L (4D11, Santa Cruz Biotech.) [34,37], or anti-nucleolin (Santa Cruz Biotech.) and appropriate secondary antibodies. The exposed X-ray films of the blots were scanned and quantified using ImageJ (NIH).

2.5. RNA extraction and reverse transcription (RT) PCR

These were carried out as described previously [33]. PCR products were run into 3% agarose gels as indicated in the figures and quantified using ImageJ software (NIH).

2.6. Affinity purification of hnRNP F/H

Two micrograms of hnRNP F/H monoclonal antibody were covalently coupled to Sulfo-Link agarose beads (Pierce Co.) according to the manufacturer's protocol. To the antibody-bead compound, 150 μl of sonicated protein lysates of MDA-MB-231 cells (1/4 of a 100 mm dish of ~90% confluence) in Igepal (0.3%) buffer were added, incubated with rocking at room temperature for 1.5 h and washed with phosphate-buffered saline. The bound protein was boiled and loaded to a SDS-PAGE gel for Western blots using antibodies against acetyllysine (Cell Signaling Tech.) or hnRNP F/H.

3. Results

3.1. The protein stability/degradation of hnRNP F/H is potently regulated by TSA and lactacystin in MDA-MB-231 cells grown in the presence of TGFβ1

In studying the effect of various pathway inhibitors that control the alternative splicing of the *Bcl-x* gene in breast cancer cells, as reported previously in semi-quantitative RT-PCR assays [33], we found that TSA reduced the Bcl-xL with a slight increase of the Bcl-xS in breast cancer MDA-MB-231 cells with or without TGFβ1 (2.5 ng/ml) treatment (Fig. 1, top panel). This caused a decrease of the Bcl-xL/xS level by about 2.5–3 fold ($p < 0.01$, compared to DMSO-treated, $n = 3$). TSA also inhibited the expression of *LYN* (2nd panel from the top, $p < 0.001$, $n = 3$), a proto-oncogene of the tyrosine kinase family, as reported in colon cancer cells [38]. In cells treated with both TSA and TGFβ1, mRNA transcripts of hnRNP F/H, regulators of Bcl-x splicing [27], were slightly reduced (3rd and 4th panels from the top. $p < 0.001$, compared to DMSO-treated, $n = 3$), while they were not changed in cells treated with either compound alone. Taken together,

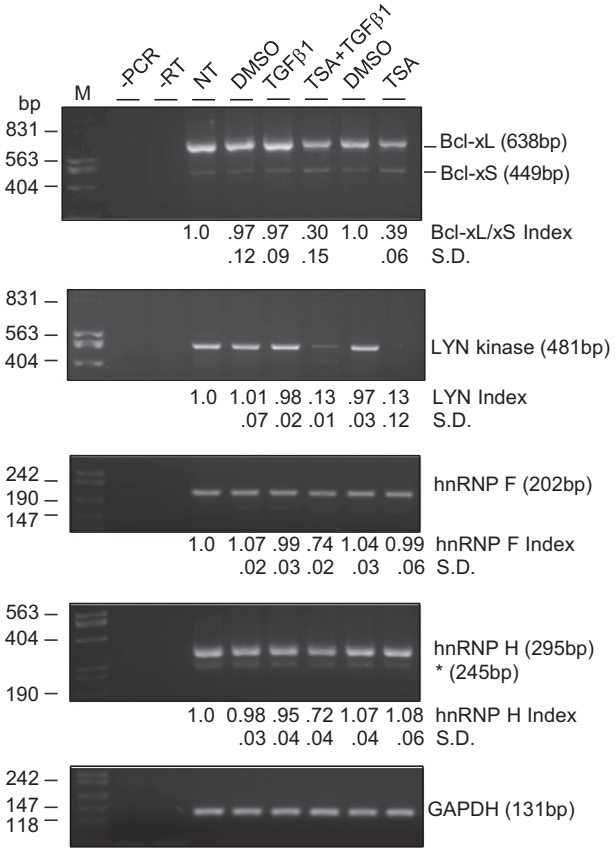

Fig. 1. TSA effect on the alternative splicing of Bcl-x and hnRNP F/H transcripts in MDA-MB-231 cells in the presence or absence of TGFβ1. The top panel is an agarose gel of RT-PCR products of Bcl-x splice variants Bcl-xL and Bcl-xS in cells treated with TSA (5 μM) only or in the presence of TGFβ1 (2.5 ng/ml). Below the gel are the mean index levels of the Bcl-xL/xS ratios, with the NT (non-treated) or DMSO samples as base level 1.0. The following panels are gels for the transcripts of LYN kinase, hnRNP F and H. GAPDH is a RNA loading control. -PCR/-RT: PCR or RT negative control. NT: non-treated. M: DNA size marker in base pairs (bp).