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Research Article

Mass spectroscopy identifies the splicing-associated proteins, PSF, hnRNP H3, hnRNP A2/B1, and TLS/FUS as interacting partners of the ZNF198 protein associated with rearrangement in myeloproliferative disease

Chitta S. Kasyapa, Padmaja Kunapuli, John K. Cowell*

Department of Cancer Genetics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

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Abstract

ZNF198 is fused with FGFR1 in an atypical myeloproliferative disease that results in constitutive activation of the kinase domain and mislocalization to the cytoplasm. We have used immunoprecipitation of a GFP-tagged ZNF198 combined with MALDI-TOF mass spectroscopy to identify interacting proteins. P splicing factor (PSF) was identified as one of the proteins and this interaction was confirmed by Western blotting. Other proteins identified were the spliceosomal components hnRNP A2/B1, hnRNP H3, and TLS/FUS. PSF is also known to interact with PTB, another member of the hnRNP family of proteins, and we further demonstrated that PTB interacts with ZNF198. The interaction between TLS/FUS and ZNF198 was confirmed using Western blot analysis. In 293 cells expressing the ZNF198/FGFR1 fusion protein, neither PSF nor PTB binds to the fusion protein, possibly because of their differential localization in the cell.

Keywords: Mass spectroscopy; RNA maturation; ZNF198/FGFR1; RNA upstream binding factor; Leukemia; TLS

Introduction

The ZNF198 gene was originally identified because of its fusion to the FGFR1 kinase domain in a chimeric fusion gene in tumors from patients with a variant form of myeloproliferative disease [1–3]. This zinc-finger gene is located in 13q12 and consists of 1377 amino acids which are organized into three major domains: an N-terminal domain consisting of five zinc-finger motifs, a proline-rich region, and an acidic C-terminal domain [2]. ZNF198 has been shown to localize exclusively in the nucleus and carries a nuclear localization signal which is lost in the chimeric fusion kinase resulting in a cytoplasmic localization of the fusion protein [4]. The zinc-finger motif has a structure characteristic of a protein–protein interacting domain which is responsible for dimerization of the fusion kinase and presumably, therefore, the wild-type ZNF198 protein also exists as a multimer. In a recent report [5], we provided evidence that the endogenous ZNF198 dimerizes with the fusion protein, raising the possibility that the role of the fusion kinase in oncogenic transformation may include the disruption of the normal function of ZNF198 in these cells.

The function of the wild-type ZNF198 protein is only just beginning to emerge. We have shown previously that ZNF198 interacts with the HHR6A and 6B proteins as well as RAD18 [6]. These proteins are known to be involved in repair of DNA replication errors and cells expressing the fusion kinase protein are more sensitive to UV-induced cell death, presumably because of the disruption of the involvement of ZNF198 in DNA repair. Immunohistochemical studies demonstrated that ZNF198 is localized to a number

* Corresponding author. Fax: +1 716 845 1698.
E-mail address: John.Cowell@RoswellPark.org (J.K. Cowell).

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of differently sized punctate structures in the nucleus. It was reported earlier that ZNF198 localizes to the nucleolus within the nucleus [7] and interacts with upstream binding factor (UBF) which plays an important role in rRNA transcription.

The ZNF198 protein, therefore, appears to interact with a number of different proteins and, in an attempt to identify other interacting partners of ZNF198, we created HEK 293 cells which stably express either a GFP-tagged full-length ZNF198 protein or a GFP-tagged ZNF198/FGFR1 fusion kinase protein [4,6]. Using anti-GFP antibodies, we have now been able to immunoprecipitate (IP) ZNF198 from these cells and resolve the associated proteins from this IP on two-dimensional gels. Proteins isolated in this way have been recovered and analyzed using MALDI-TOF mass spectroscopy. We now report that PSF, PTB, and other proteins that are the components of the spliceosome interact with ZNF198 but not with the fusion kinase, indicating a possible involvement of ZNF198 in RNA processing and its possible contribution to oncogenesis in atypical myeloproliferative disease.

Experimental procedures

Vectors and plasmid construction

The ZNF198/FGFR1 fusion gene was amplified by PCR from leukemic cells expressing the fusion gene. The PCR product was then cloned into the pEGFP-C2 vector as an N-terminal GFP fusion protein. The ZNF198 gene was amplified from a fetal bone marrow cDNA library and similarly cloned into pEGFP-C2 vector. All the constructs were sequenced before transfection.

Cell culture and transfection studies

HEK-293 cells were maintained in DMEM with 10% FBS in 5% CO_2 and were used to establish cells stably expressing ZNF198 and the ZNF198–FGFR1 fusion kinase independently. Green cells were identified using fluorescence microscopy and colonies, selected in culture medium containing 500 μg/ml of G418, were isolated using ring cloning. Specific gene expression was confirmed using RT-PCR and protein expression was confirmed by Western blotting using an anti-GFP monoclonal antibody (Covance, NJ).

Immunoprecipitations and Western blotting

Cells were grown to 80% confluency and then washed twice with PBS. Lysis was achieved in RIPA buffer (50 mM Tris containing 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.2) with 0.2% protease inhibitor cocktail and 0.1% phosphatase inhibitor cocktail (Sigma, MO) on ice for 10 min. Following centrifugation at 14,000 rpm for 20 min, the supernatant was collected and used for immunoprecipitation (IP). Lysates were precleared using sepharose-coupled rabbit or mouse IgG and then the supernatant was treated with anti-GFP rabbit polyclonal serum (Clontech) at 1:150 dilution or 1:50 dilution of other antibodies. The immune complexes were pulled down with protein-A or protein-G sepharose. The sepharose beads were washed five times with PBS and then proteins were eluted by incubation in SDS-sample buffer at 100°C for 3 min and aliquots were subjected to SDS-PAGE and Western blot analysis. Monoclonal antibodies against PSF (Sigma, St. Louis, MO) and GFP (Covance, New Jersey), and polyclonal antibodies against PTB (Santa Cruz) were used for Western blot analysis. For all IPs presented in this manuscript, the input consists of 40 μg of total protein lysate and the IPs were derived from 200 μg of total lysate, whereafter the entire IP was loaded on the gel.

2-Dimensional electrophoresis

For 2-dimensional electrophoresis, immunoprecipitations were performed using an anti-GFP antibody coupled to protein-A sepharose and immobilized using disuccinimidyl suberate (DSS) (Pierce), thus making the antibody stably attached to protein-A sepharose under the elution conditions of low pH. GFP-fused ZNF198 was immunoprecipitated along with its binding partners and eluted using 100 mM glycine pH 2.5. Immunoprecipitated proteins were concentrated in a lyophilizer and desalted using desalting columns (Biorad). The buffer containing the immunoprecipitated proteins was exchanged with the 2D rehydration buffer (8 M Urea, 2% CHAPS, 50 mM DTT and 0.2% pH 3–10 ampholytes). ReadyStrip IPG strips (pH 3–10, 7 cm, Biorad) were rehydrated overnight in 2D rehydration buffer containing the protein sample. IEF was carried out on a Protean IEF Cell (Bio-Rad) at 20°C with a maximum current setting of 50 mA/strip. Focusing was performed for a total of 10,000 Vh. After the IE focusing was complete, the strips were subjected to a 2 × 10-min equilibration with continuous and gentle shaking. The first step was performed in equilibration buffer I (6 M Urea, 20% glycerol, 2% SDS, 0.375 M Tris–HCl pH 8.8, and 2% DTT) and the second step was performed in equilibration buffer II (6 M Urea, 20% glycerol, 2% SDS, 0.375 M Tris–HCl pH 8.8, and 2.5% Iodoacetamide). The strips were briefly dipped in the SDS-PAGE running buffer and placed on a 1-mm-thick 10% SDS-PAGE gel and sealed in place with 0.5% low melting point agarose. SDS-PAGE was performed at 200 V for 40 min in SDS-PAGE running buffer. The gel was stained with coomassie brilliant blue R250. Stained protein spots were manually excised and used for MALDI-MS analysis.

In-gel digestion

Individual coomassie brilliant blue stained protein spots were excised from the gel, cut into small pieces, placed in a