Journal of Cellular Biochemistry 102:1308–1317 (2007)

HSPA1A is an Important Regulator of the Stability and Function of ZNF198 and Its Oncogenic Derivative, ZNF198–FGFR1

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Abstract Mass spectroscopy analysis demonstrated that the HSPA1A protein is found in complex with the ZNF198 protein which is involved in a chromosome rearrangement with the FGFR1 gene in an atypical myeloproliferative disease. HSPA1A is a member of the HSP70 family of genes which has been shown to be inducible in a variety of circumstances. Exogenous expression of the ZNF198–FGFR1 fusion kinase gene as well as ZNF198 in a model cell system results in a large (>650-fold) increase in HSP70 mRNA levels. Using KNK437, a specific inhibitor of HSP70 transcription, we have demonstrated that an important function of HSPA1A is to stabilize the ZNF198 and ZNF198–FGFR1 proteins. In the absence of HSPA1A, specific functions of ZNF198–FGFR1 such as STAT3 phosphorylation is also lost. Treatment of cells with KNK437 in the presence of MG132, an inhibitor of proteasomal degradation of proteins, suggested that only the ZNF198–FGFR1 protein is subject to the proteasomal degradation pathway, while ZNF198 is not. These observations suggest an important role for HSPA1A in ZNF198 and ZNF198–FGFR1 mediated cellular function. J. Cell. Biochem. 102: 1308–1317, 2007.

Key words: HSPA1A; ZNF198–FGFR1; myeloproliferative disease; KNK437

Reciprocal chromosomal translocations in leukemias often result in the generation of chimeric proteins with constitutive functions that promote the malignant phenotype [Rabbitts, 1994]. We have previously described a novel fusion gene between the kinase domain of FGFR1 and a novel zinc finger protein, ZNF198, in an atypical myeloproliferative disease [Still and Cowell, 1998]. The ZNF198 gene is 1377 amino acids long with 10 zinc fingers, a proline rich domain, and a consensus C-terminal nuclear localization signal. Subsequent studies have shown that several different proteins can also combine with FGFR1 [Popovici et al., 1999; Guasch et al., 2000; Macdonald et al., 2002] which appear to facilitate multimerization and constitutive activation of its kinase domain. Downstream targets of the fusion kinase have been shown to include members of the STAT family of transcription factors [Smedley et al., 1999; Baumann et al., 2003; Heath and Cross, 2004]. In the BaF/3 hematopoietic cells, expression of ZNF198–FGFR1 induces IL-3 independent proliferation of otherwise IL-3 dependent cells. We have recently demonstrated that the wild-type endogenous ZNF198 protein can complex with the fusion kinase, which potentially affects the endogenous function of ZNF198 [Kunapuli et al., 2006]. As such, understanding the function of ZNF198 could lead to important insights into the way in which expression of the fusion gene affects intracellular homoeostasis.

In yeast 2-hybrid assays, ZNF198 was shown to bind to the UBE2A/B [Kunapuli et al., 2003] and SUMO-1 proteins [Kunapuli et al., 2006]. UBE2 is involved in DNA repair functions and recruits RAD18 to facilitate this process. In cells expressing the fusion kinase, although UBE2 could form a complex with ZNF198–FGFR1, RAD18 could not and these cells showed increased sensitivity to UV-B irradiation suggesting that ZNF198 potentially plays a role in DNA repair. Mass spectroscopy analysis of proteins that interact with ZNF198 initially

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Received 11 July 2006; Accepted 9 March 2007
DOI 10.1002/jcb.21362

identified proteins such as SFPQ (PSF), PTBP1, hnRNPA2/B1, and hnRNPH3 [Kasyapa et al., 2005] further involving it in RNA splicing and maturation. ZNF198 has also been observed to bind to UBTF, a nucleolar protein that functions as a trans-activation factor in rDNA transcription [Ollendorff et al., 1999; Kasyapa et al., 2005].

The ZNF198 protein has been shown to localize to punctate nuclear structures [Baumann et al., 2003; Kasyapa et al., 2005]. In contrast, the ZNF198–FGFR1 protein localizes to the cytoplasm. We recently demonstrated using co-localization studies [Kunapuli et al., 2006] that some of the ZNF198-containing nuclear bodies are PML bodies and that ZNF198 is present in a protein complex with PML. The presence of the fusion kinase results in loss of PML bodies [Kunapuli et al., 2006]. During these studies it became clear that ZNF198 was also localized in other nuclear structures that were not PML bodies [Kasyapa et al., 2005]. Expression microarray studies demonstrate that ZNF198–FGFR1 expression alters the profile of various protease inhibitors, in particular, plasminogen-activator inhibitor type 2 (PAI-2/SERPINB2) [Kasyapa et al., 2006]. PAI-2 binds to the ZNF198–FGFR1 fusion kinase and presumably helps in stabilizing the protein in the cytoplasm. From the same microarray analysis, we now show that a member of the inducible heat shock protein (HSP)-70 family, HSPA1A, is highly induced in HEK-293 cells expressing ZNF198–FGFR1. Cells over expressing ZNF198 also show increased levels of HSPA1A compared to the parental cells. Mass spectroscopy has been used to demonstrate that ZNF198 forms a complex with HSPA1A. Furthermore, using KNK437, an inhibitor of HSP70, the ZNF198-containing structures in the nucleus are disrupted suggesting that this protein–protein interaction is required for the stability of ZNF198 and for its specific localization in the nucleus.

MATERIALS AND METHODS

Cell Culture and Transfection Studies

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 to confirm the correct reading frame for the constructs. HEK 293 clones stably expressing the various constructs were generated as described previously [Kasyapa et al., 2006].

HEK-293 cells stably expressing the ZNF198 or the ZNF198–FGFR1 fusion kinase were maintained in DMEM with 10% FBS in 5% CO_2. Specific gene expression was confirmed using RT-PCR and protein expression was confirmed by Western blotting using an anti-GFP monoclonal antibody (Covance, NJ).

RT-PCR Analysis

RNA from cell cultures was prepared using the Trizol (Invitrogen, Carlsbad, CA) method as described previously [Kasyapa et al., 2005]. The purity of the RNA was determined calorimetrically. The SuperScript One-step RT-PCR kit (Invitrogen), with platinum Taq, was used to amplify RNA in the samples. For HSPA1A, the primer set used was human HSPA1A:

forward- 5′-AGCTGCTGCAGGACTTCTTC-3′
reverse- 5′-CGTTGGTGATGGTGATCTTG-3′
which gives a 474 bp product specific to this member of the hSP70 family of genes.

The β-actin primers used, which generated a 646 bp product, were:

forward- 5′-AAATCTGGCACCACACCTTC-3′
reverse- 5′-GCACTGTGTTGGCGTACAG-3′

Immunoprecipitation and Western Blotting

Cells grown to 80% confluency were washed twice with PBS and lysed in RIPA buffer (50 mM Tris containing 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate; pH 7.2) with 0.2% protease and phophatase inhibitor cocktails (Sigma, MO) on ice for 10 min. When immunoprecipitations (IP) were performed, the lysates were first passed through rabbit IgG-coupled sepharose to remove proteins that bind IgG non-specifically, and then 500 μl of the supernatant was combined with 3 μl of anti-GFP rabbit polyclonal serum (Clontech, CA) and incubated overnight at 4°C with slow mixing. The immune complexes were applied to Protein A-Sepharose and centrifuged. The Sepharose beads were then washed five times with PBS and the proteins were eluted by

incubation in SDS-sample buffer at 100°C for 3 min. Aliquots were subjected to SDS–PAGE and Western blot analysis.

Antibodies for the various studies were obtained from: HSPA1A, beta actin, PTBP1, and PML (Santa Cruz, CA); monoclonal SUMO-1 antibody (Zymed, CA); anti-GFP polyclonal antibody (Clonetech) and monoclonal antibodies (Covance); STAT3, p-STAT3 antibodies (Cell Signaling Technologies, MA); SERPINB2 (American Diagnostica, CT); SFPQ/PSF (Sigma, St. Louis, MO).

Gene Expression Analysis

RNA extracted from individual HEK-293 cell clones was used to prepare cRNA for hybridization to the Affymetrix U133A oligonucleotide arrays as described previously [Kunapuli et al., 2004; Kasyapa et al., 2006]. The gene expression profile from the ZNF198/FGFR1 expressing cells was compared with that obtained from HEK-293 clones stably expressing the GFP protein from the pEGFP vector. To assess gene expression differences, the base-line corrected data were imported into the Affymetrix Data Mining Tool (DMT 4.0) using the publishing tool, MicroDataBase (MDB 3.0). After the genes were sorted using a count and percent tool, a list of only those that showed altered expression in at least two of the ZNF198/FGFR1 clones (two cross-comparisons) was compiled. A cut-off of an average twofold or greater change was selected. All functional annotation and chromosomal locations were obtained using NetAffx [Liu et al., 2003].

Confocal Microscopy

Stable transfectants carrying GFP-tagged ZNF198 and ZNF198/FGFR1 genes were fixed in 4% paraformaldehyde on coverslips and blocked with PBS containing 0.05% Triton X-100 and 5% fetal calf serum. Cells were then incubated with (1:50) monoclonal PML antibody (Santa Cruz) overnight at 4°C. Cells were washed for 3×15 min with PBS containing 0.05% Triton-X 100 and incubated at room temperature with a goat anti-mouse antibody-Texas red conjugate for 1 h. An anti-mouse IgG-Texas red conjugate (Jackson Laboratories) was used as the secondary antibody. The cells were then examined using a Leica confocal laser-scanning microscope. Images were collected using a 60× oil immersion lens using an excitation wavelength of 488 nm for GFP and FITC and 543 nm for Texas red.

Two-Dimensional Gel Electrophoresis

For two-dimensional electrophoresis, IP 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 together 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 (Bio-Rad). 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, Bio-Rad) 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 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 1.5 ml microcentrifuge tube that had been washed with 50% acetonitrile (ACN) and 0.1% TFA. Gel pieces were destained by incubating at 37°C for 45 min in 0.2 ml in 50% ACN containing 50 mM NH_4HCO_3. This process was repeated twice. The gel pieces were then incubated for 5 min at

room temperature in 100μl of 100% ACN. ACN was removed and the gel pieces were dried using a Speedvac for 10 min at room temperature. The protein in the dried gel pieces was digested using freshly prepared trypsin (trypsin Gold, Mass Spec Grade from Promega, Madison, WI) at 20 ng/μl in 25 mM NH_4HCO_3. The digestion was performed at 37°C overnight. The gel pieces were centrifuged for 1 min and the supernatant was transferred to a fresh tube. The peptides in the gel pieces were extracted by incubating for 60 min in 50 ml of 50% ACN and 0.1% TFA at room temperature with constant shaking. This process was repeated once more and the extracts were pooled and concentrated using a Speedvac until the volume was reduced to 10 μl. The peptide mixture was further purified and concentrated using ZipTip c_{18} reagents (Millipore).

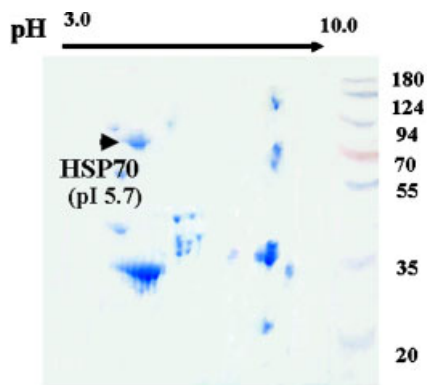
Protein Identification by MALDI-MS Analysis

For MALDI-TOF MS analysis, the ZipTip-concentrated peptides were plated on the MALDI target in 60% ACN in the presence of 1 μl of α-cyano-4-hydroxycinnamic acid (CHCA, Sigma). Mass measurements were carried out using a Biflex IV TOF/TOF mass spectrometer system from Bruker Daltonics. The mass spectra generated were internally calibrated using the trypsin autolysis peaks (m/z 842.51 and 2211.07). Monoisotopic masses were assigned to individual peaks and used for database searches using the MASCOT (http://www.matrixscience.com) search engine. This program uses the list of peptide mass values from the enzymatic digest for protein identification. The data were compared with the NCBI non-redundant protein sequence database using the protein sequences.

RESULTS

Analysis of HSPA1A in 293 Cells Expressing ZNF198 or ZNF198–FGFR1

Since functional aspects of a protein can often be revealed by identifying its interacting protein partners, we used MALDI-TOF mass spectrometry (MS) to analyze proteins that co-IP with ZNF198. An anti-GFP antibody was used in IP procedures with HEK-293 cell lysates expressing the GFP-tagged ZNF198 protein. Individual proteins were then resolved using 2D gel electrophoresis (Fig. 1) and Comassie blue-stained spots were isolated individually from the gel. One of these proteins showed a molecular weight (MW) of ~70 kDa and a PI = 5.7. MS analysis of the protein isolated from this spot identified four peptides which showed homology with the HSPA1A (HSP70-1) and HSPA1B (HSP70-2) proteins, providing 8% coverage (Fig. 1). Since these four peptide sequences are identical in both proteins, MS analysis alone could not distinguish between them, which is not surprising since, despite being derived from different genes, both proteins have the same amino acid sequences [Milner and Campbell, 1990]. To confirm the MS data, we used the IPs from HEK-293 cells expressing GFP-ZNF198 and analyzed these proteins using Western blotting and an HSP70 antibody. This analysis showed the presence of HSP70 in the IP from cells expressing exongenous GFP-ZNF198, but not in cells expressing GFP alone (Fig. 2A). IPs from cells expressing the GFP-ZNF198–FGFR1 fusion protein also showed the presence of the 70 kDa protein after Western blot analysis suggesting that the binding site is located in the proximal 913 amino acids of ZNF198. Using the polyclonal antibody against ZNF198 [Kunapuli et al., 2003], we were able to show that, in parental 293 cells, HSP70 is also in complex with ZNF198 demonstrating that this is not an artifact of over



MATAKGIAIG IDLGTTYSCV GVFQHGK **VEI IANDQGNR TTPSYVAFTDTE R**
LIGDAAKNQ VAMNPQNTVF DAKRLIGRKF NDPVV QADMK LWPFQVIN EG GKPK VLVSY K
GENKAFY PEE ISSMVLTKLK ETAEAFLGH P VTNAVI TVPA YFNDS QRQAT K**DAGVIAGLN**
VLRIINEP TA AAIAYGLDKG GQG ERHVLIF DLGGG TFDVS ILTIDDGIF E VK**ATAGDTHL**
GGEDFDNRLV SH FVE EFKRK HKKDIS QNKR AVR RLRTACE RA KRTLSSST QANLEI DSLY
EGIDFYTS ITRARFEELC CAD LFRG TLEP VE KALRDAKMDK AKIHDIVLV G GSTRIPKVQR
LLQDYFN GRDLNKSINP DEA VAYGAAVQA AILMGDKSEKV QDLLLLD VAP LSLGLETAGG
VMTALIKRN STIPTKQTQIF TTYSDNQPG V LIQV YEGERA MTKDN NLLGR FDLTGIPP AP
RGVPQ IEVTFDID ANGILN V TATDKSTGKV NKITITNDKG RLSKEEI ERM VLD AEKYKA E
DEV QREKIAAKN ALESYAFN MKSVVS DEGL KGKIS ESDKN KILDKCNELL SWLEVNQL AE
KDEFDH KRKELEQVCNPIIT KLYQG GCTGP AGSTG YVPGR PATGPTIEEV D

Fig. 1. Two-dimensional gel from the IP of GFP tagged ZNF198 with anti-GFP antibodies. The location of the spot representing HSPA1A is indicated by the arrow. Four peptides were identified from the spectra produced from this spot and their location within the amino acid sequence of HSPA1A is show below. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

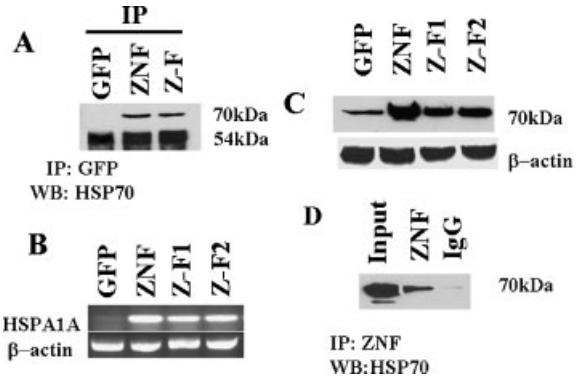


Fig. 2. In (**A**) IP of the exogenous ZNF198 and ZNF198–FGFR1 (Z-F) proteins with GFP antibodies demonstrates the presence of HSPA1A (70 kDa) in complex with these proteins, but not in cells expressing the GFP protein alone. The 54 kDa band seen in this gel results from the IgG cross reaction between the primary and secondary antibodies. In (**B**) semi-quantitative RT-PCR analysis (22 cycles) shows high level increases in HSPA1A mRNA levels in cells expressing exogenous ZNF198 as well as in two independently derived clones (Z-F1 and Z-F2) expressing ZNF198–FGFR1. At the protein level (**C**) the increased HSPA1A was significantly higher in cells expressing ZNF198 compared with ZNF198–FGFR1. IP of the endogenous ZNF198 protein from wild-type 293 using a polyclonal antibody (**D**) reveals that HSP70 is also present in a complex with this protein.

expressing an exogenous GFP-tagged protein (Fig. 2D).

Analysis of the HSPA1A levels in cells over-expressing either ZNF198 or ZNF198–FGFR1 showed large increases in its mRNA (Fig. 2B) and protein levels (Fig. 2C). We had previously performed an Affymetrix U133A expression array analysis [Kasyapa et al., 2006] comparing cells over expressing the various GFP-fused proteins. Analysis of this data, specifically for HSP70 family members, demonstrated that HSPA1A expression was increased ~670-fold in cells expressing exogenous ZNF198 and ~620-fold in cells expressing exogenous ZNF198–FGFR1 when compared to the cells expressing exogenous GFP alone. HSPA1A is the most inducible member of the HSP70 family. Other members of this family (such as HSPA1B and HSPA6) showed no significant expression changes between the various cell lines in our microarray analysis. HSPA1B was apparently increased ~sevenfold in both ZNF198 and ZNF198–FGFR1 expressing cells (Table I) although semi-quantitative RT-PCR analysis using primers specific for HSPA1B showed relatively little increase in expression levels (data not shown). Thus, these results demonstrate that there is an over-expression of HSPA1A in cells expressing either ZNF198 or ZNF198–FGFR1, thus raising the question whether HSPA1A binds to these proteins and stabilizes them.

Effect of KNK437 on HEK-293 Cells Expressing ZNF198 or ZNF198–FGFR1

To investigate the significance of HSPA1A over-expression, HEK-293 cells stably expressing ZNF198 or ZNF198–FGFR1 were treated with increasing concentrations of KNK437 (Calbiochem, CA) for 48 h. KNK437 is a specific inhibitor of transcriptional activation of HSP70 genes [Yokota et al., 2000]. Cells treated with KNK437 showed reduction of HSPA1A protein levels in a dose dependent manner after 48 h (Fig. 3). Protein levels of both ZNF198 (Fig. 3A) and ZNF198–FGFR1 (Fig. 3B) were also reduced in these cells in a dose dependent manner becoming first evident at 25 μM, but becoming more pronounced at 100 μM. In all subsequent experiments, therefore, we used the 100 μM concentration of KNK437. Interestingly, the GFP protein levels in 293 cells expressing exogenous GFP alone was not inhibited (Fig. 3C), indicating that KNK437 exerts its effect only on ZNF198 and ZNF198–FGFR1 rather than the GFP associated with these proteins. These results suggest that expression of HSPA1A is important for the stability of both the ZNF198 and ZNF198–FGFR1 proteins.

TABLE I. Summary of Gene Expression (Fold) Changes, Derived from Affymetrix HU133Plus2 Arrays, for HSP70 Family Members Seen in HEK 293 Cells Expressing the Exogenous ZNF198 or ZNF198–FGFR1 Fusion Kinase Compared With Cells Expressing the Exogenous GFP Gene Alone

	Name	Symbol	ZNF198	ZNF198–FGFR1
1	Heat shock 70 kDa protein 1A	HSPA1A	668.14	623.09
2	Heat shock 70 kDa protein 2	HSPA1B	−2.69	−2.34
3	Heat shock 70 kDa protein 6 (HSP70B′)	HSPA6	−4.65	−4.58
4	Heat shock transcription factor 1	HSF1	−4.72	−4.75

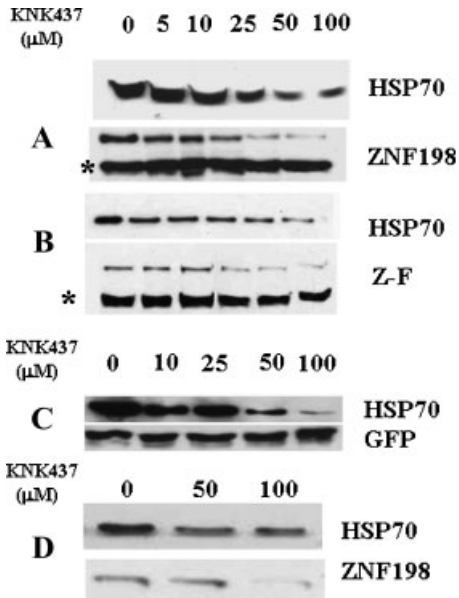


Fig. 3. Treatment of 293 cells expressing exogenous GFP-tagged proteins with varying concentrations (0–100 μM) of KNK437 leads to a reduction in HSP70 levels (**A**; **upper**) which parallels loss of ZNF198 protein (A; **lower**) in cells over-expressing ZNF198. In cells expressing the ZNF198–FGFR1 fusion gene (Z-F) , the same reduction in the chimeric protein levels (**B**; **lower**) parallels loss of the HSP70 protein (B; **upper**). A non-specific band consistently seen with the GFP antibody in cell lysates is indicated by (*) in (A) and (B) and is used to demonstrate equal loading of the lanes. In 293 cells expressing exogenous GFP (**C**), KNK437 treatment has no effect on the GFP protein levels (**lower**), while HSP70 levels are reduced (**upper**) as in A and B. Using a ZNF198-specific antibody [Kunapuli et al., 2004], treatment of wild-type 293 cells with KNK437 (**D**) results in a reduction of the endogenous levels of ZNF198 which also parallels the reduction in HSP70 levels.

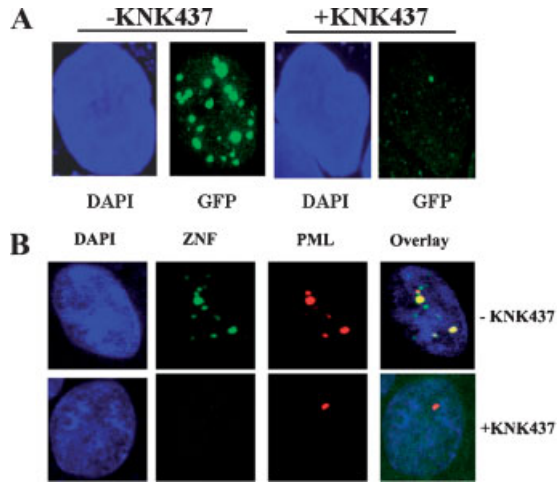


Fig. 4. **A**: Confocal microscopy of 293 cells expressing GFP-tagged ZNF198 shows typical punctate structures in the nucleus. When these cells are treated with KNK437 for 48 h, the punctate structures are mostly lost. In (**B**) co-localization of ZNF (green) and PML (red) proteins is shown in PML bodies in normal cells (-KNK). Treatment of these cells with KNK 437 results in loss of PML bodies.

To further explore the effect of KNK437 on endogenous ZNF198 levels, we treated HEK-293 cells with KNK437 for 48 h and analyzed the protein levels of HSPA1A and endogenous ZNF198. Both ZNF198 as well as HSP70 were inhibited when 50 and 100 μM concentration of KNK437 was used (Fig. 3D). This result indicates that HSP70 is essential for the stable presence of ZNF198.

To assay for the effect of this treatment, we used confocal microscopy to visualize the ZNF198 and HSP70 proteins. In normal cells, ZNF198 forms punctuate bodies in the nucleus [Baumann et al., 2003; Kunapuli et al., 2003]. In HEK293 cells, there are usually between 4-20 ZNF198-containing nuclear bodies many of which we have previously shown to be PML nuclear bodies [Kunapuli et al., 2006]. After KNK437 treatment, a significant reduction in the number of these punctate structures occurred after 24 h of treatment, which was further reduced to less than 10% of normal after 48 h (Fig. 4A). Analysis of these cells with anti-PML antibodies show almost complete absence of PML bodies as expected (Fig. 4B). If the KNK437 agent was removed from the culture within the first 48 h, the punctuate ZNF198 bodies were able to reform (data not shown). Cell viability studies using Trypan blue exclusion assays demonstrated that 99% viability was retained in cultures of HEK293 cells carrying either exogenous ZNF198 or ZNF198–FGFR1 even in the presence of KNK437 for 48 h. After 72 h viability was reduced to 83 and 79%, respectively, in the two populations. Treatments for longer than 72 h resulted in progressive loss of cell adherence in vitro and ultimately cell death. Even though Trypan blue exclusion ability was retained after 48 h, if KNK437 exposure was maintained beyond this time the cells eventually died, suggesting that irreversible changes had been induced in the cells after this period which led to cell death.

Effect of KNK437 on ZNF198 Interactions

We reported earlier that ZNF198 is sumoylated [Kunapuli et al., 2006] but that ZNF198–FGFR1 is not, because the position of the translocation breakpoint excludes the sumoylation site from the chimeric protein. To investigate the effect of KNK437 on ZNF198

sumoylation, HEK-293 cells expressing GFP-ZNF198 were treated with 100 μM KNK437 for 48 h and cell lysates were immunoprecipitated with an anti-GFP antibody and SUMO1 levels analyzed using Western blotting (Fig. 5). IP of the GFP-ZNF198 protein using anti-GFP antibodies showed that, although ZNF198 levels are reduced in the presence of KNK437, the anti-GFP antibody could still IP the remaining ZNF198 protein in these cells (Fig. 5A). ZNF198 was sumoylated in cells expressing GFP-ZNF198 and this modification was not inhibited by KNK437.

We have also shown previously that ZNF198 interacts with a number of other proteins such as UBE2 [Kunapuli et al., 2003], SFPQ, PTBP1, and UBTF [Kasyapa et al., 2005]. Since one function of HSPA1A is to bind to, and stabilize proteins, we investigated whether, during the first 48 h of KNK437 treatment, ZNF198 could still form a complex with these proteins. As shown in Figure 5, none of the protein–protein interactions analyzed were affected by the drug treatment. These results suggest that although the ZNF198 protein pool is being depleted in the presence of KNK437 over 48 h, the remaining full-length protein can still interact with its defined partners.

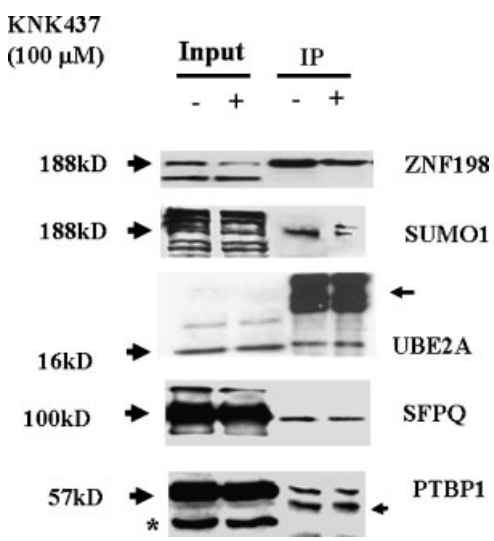


Fig. 5. Analysis of the interaction of various proteins with ZNF198 in the presence of 100 μM of KNK437. In 293 cells expressing GFP-ZNF198, IP using anti-GFP antibodies shows that even though ZNF198 levels are reduced in the parental cells ZNF198 can still be IPed from these cells. In the same cells, SUMO1, UBE2A, SFPQ, and PTBP1 are found in a complex with ZNF198 even in the presence of KNK437. Arrowheads on the right indicate the IgG interaction (54–65 kDa) between antibodies from the IP and probe.

Effect of KNK437 on the Functions of ZNF198–FGFR1

ZNF198–FGFR1 has been shown to have constitutive tyrosine kinase activity in the cytoplasm of cells that express it, and that specific targets of this kinase include STAT1, STAT3, and STAT5B [Baumann et al., 2003]. Since KNK437 treatment results in reduced levels of ZNF198–FGFR1 (Fig. 3), we investigated whether it could also inhibit phospho-activation of STAT3, as well as expression of SERPINB2 protein levels, which we have shown is also important for stability of the fusion kinase [Kasyapa et al., 2006] in HEK-293 cells expressing ZNF198–FGFR1. Both STAT3 phosphorylation levels (Fig. 6) as well as SERPINB2 protein levels (Fig. 6) were reduced in the presence of 100 μM KNK437, although STAT3 protein levels did not change (Fig. 6). These results further suggest that HSPA1A is important for the stability of ZNF198–FGFR1 and that reduced levels of this protein compromises the downstream consequences of its action.

Effect of MG132 on KNK437 Mediated Inhibition of ZNF198 and ZNF198–FGFR1

KNK437 inhibits HSPA1A transcriptional activation [Yokota et al., 2000]. In turn, HSPA1A is important for the proper folding of the proteins it interacts with, and is known to facilitate the ubiquitin-mediated proteasomal degradation of misfolded proteins. The data presented above show that levels of both the ZNF198 and ZNF198–FGFR1 proteins were

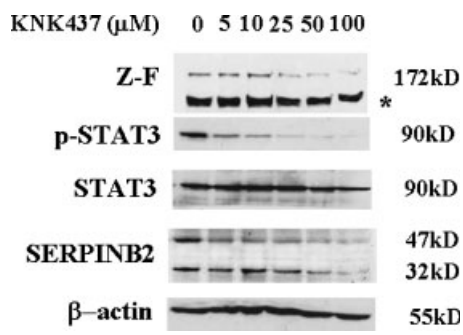


Fig. 6. When 293 cells expressing the ZNF198–FGFR1 fusion gene (Z-F) are treated with KNK437, there is a reduction in protein levels of ZNF198–FGFR1 with increasing concentration. This loss of ZNF198–FGFR1 is paralleled with a reduction in phospho-STAT3 levels but not of the STAT protein as well as the two isoforms of SERPINB2. Equal loading of the Western blot is shown by actin levels.

greatly reduced when cells were treated with KNK437. To determine whether, in the absence of HSPA1A, degradation of these proteins occurs through the proteasomal pathway, we treated cells over expressing ZNF198–FGFR1 and ZNF198 with the MG132 proteasomal inhibitor, which blocks the degradation of ubiquitinated proteins [Lee and Goldberg, 1998]. Treatment with MG132, however, also results in upregulation of HSP70 proteins in its own right [Kim et al., 1999]. When cells expressing the exogenous ZNF198–FGFR1 gene were treated with KNK437, HSPA1A expression levels were reduced as expected (Fig. 7). In parallel, ZNF198–FGFR1 protein levels were also reduced in the presence of KNK437 but were not reduced when the cells were treated with a combination of KNK437 and MG132. These observations suggest that KNK437-mediated degradation of ZNF198–FGFR1 is facilitated through the proteasomal degradation pathway in the absence of HSP70. In cells expressing the exogenous ZNF198 protein, KNK437 also results in reduced ZNF198 protein levels but when ZNF198 expressing cells were treated simultaneously with KNK437 and MG132, the same reduction in ZNF198 protein levels was also seen. These observations could not be accounted for by progressive cell death in the treated cells since, at 48 h, cell viability as determined by trypan blue exclusion was 99% and reduced only to 74–77% after 72 h continuous treatment with both drugs. Thus, while it seems that the fusion kinase protein is degraded through the proteasomal pathway, the same is not true for the ZNF198 protein.

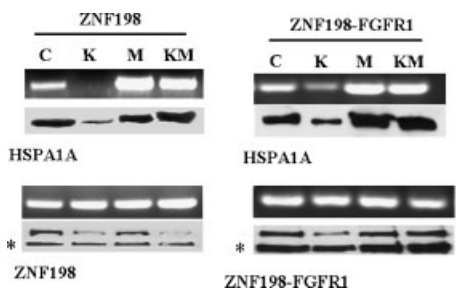


Fig. 7. Semi-quantitative RT-PCR analysis (22 cycles) of cells expressing exogenous ZNF198 (**left**) shows loss of HSPA1A expression in the presence of KNK437 (+K). Treatment of these cells with MG132 alone (M) or in combination with KNK437 (KM) does not reduce HSPA1A expression levels. During the various treatments of these cells, expression levels of ZNF198 are unaffected (**lower left**). Protein levels of GFP-ZNF198 (as revealed by Western blotting using anti-GFP antibodies) in cells treated with KNK437 are reduced compared to the untreated cells (lower left) and the same effect is seen in cells treated with KM. The typical non-specific band for GFP is seen in all lysates and reveals equal protein loading in the lanes. When cells expressing the ZNF198–FGFR1 protein (**right**) are treated with KNK437, there is a similar reduction in HSPA1A expression and protein levels. Reduced protein levels, however, are only seen when they are treated with KNK437 but not when they are treated with KNK437 and MG132 together (**lower right**).

DISCUSSION

The presence of the ZNF198–FGFR1 fusion kinase protein has a profound effect on gene expression profiles in cells expressing it. This disregulation of gene expression is thought to contribute to the transforming effect of this oncoprotein. Stabilization of these proteins is likely to be an important factor contributing to their oncogenic capacity. The data presented in this report suggest that HSPA1A maintains the stability and function of the ZNF198–FGFR1 protein. Stability of a protein can be achieved in several different ways, in particular through association with various chaperon proteins commonly called HSP. Although initially thought to be only heat inducible, it has now been well established that HSPs can be induced by various other stress stimuli including those caused by oncogenesis. For example, HSP90 is a chaperone that stabilizes various leukemia-associated cellular proteins such as BCR-ABL, RAF1, AKT, KIT, and FLT3 in a conformation that facilitates their proper function [Isaacs et al., 2003; Bagatell and Whitesell, 2004].

HSP70 represents another family of HSP that contains at least 11 highly homologous proteins with closely related functions and which differ by intracellular localization and expression pattern [Tavaria et al., 1996; Daugaard et al., 2005]. Under normal conditions, HSP70 proteins assist in (1) the folding of newly synthesized proteins, (2) assembly of multiprotein complexes, (3) translocation of proteins across cellular membranes, and (4) the channeling of misfolded proteins into the proteasomal degradation pathway [Hartl and Hayer-Hartl, 2002; Mayer and Bukau, 2005]. Of the various members of the HSP70 family, HSPA1A is typically expressed at very low levels in normal cells and appears to be rapidly induced in response to various stimuli. HSPA1A is highly expressed in various cancers and plays a role in cancer cell proliferation [Jaattela, 1999]. Inhibition of HSP70 and HSP90 apparently has a

potential therapeutic value in many cancers but especially in leukemias [Guo et al., 2005]. It is perhaps important to note that only HSPA1A appears to be specifically induced in cells over-expressing both the ZNF198 and ZNF198–FGFR1 proteins. The other inducible members of the HSP70 family, such as HSPA1B and HSPA6 are not affected, suggesting a specific role for HSPA1A in the function of ZNF198 and ZNF198–FGFR1.

ZNF198 is an exclusively nuclear protein that forms complexes with various nuclear proteins. ZNF198 also interacts with UBE2 [Kunapuli et al., 2003], UBTF [Ollendorf et al., 1999; Kasyapa et al., 2005], PML [Kunapuli et al., 2006], and various RNA binding proteins such as SFPQ, PTBP1, and hnRNPA2/B1 [Kasyapa et al., 2005]. The observation that HSPA1A is induced in cells over-expressing ZNF198, and that it interacts with ZNF198, indicates that HSP70 may contribute significantly to the formation of protein complexes involving ZNF198. Interestingly, the observation that the interaction between ZNF198 and its partners is maintained even in the presence of KNK437, suggests that HSPA1A is important for the stability of ZNF198 in these cells. HSP70 is known to enhance the survival of cells and a depletion of HSP70 leads to apoptosis [Beere and Green, 2001]. In our experiments, treatment of cells with KNK437 resulted in the inhibition of ZNF198 protein after 48 h although cell viability was maintained. Prolonged incubation with KNK437 beyond 72 h, however, resulted in irreversible events that led to cell death.

The ZNF198–FGFR1 fusion protein differs from ZNF198 in several ways. ZNF198–FGFR1 does not interact with PML, SFPQ, or PTBP1. At the same time, ZNF198–FGFR1 is localized in the cytoplasm and shows constitutive tyrosine kinase activity that activates various STAT proteins which are suggested to be responsible for the oncogenic activity induced by ZNF198–FGFR1 [Smedley et al., 1999; Baumann et al., 2003; Heath and Cross, 2004]. We have recently reported that SERPINB2, a serine protease inhibitor, is induced several fold in the cells expressing ZNF198–FGFR1 and appears to be essential for the stable presence of ZNF198–FGFR1 [Kasyapa et al., 2006]. In spite of the differences between ZNF198 and ZNF198–FGFR1 in their localization, binding partners and function within the cell, both proteins are associated with the induced expression of HSPA1A and interact with it. This observation indicates that both the ability to induce and to bind to HSPA1A resides in the part of the ZNF198 protein that is retained in ZNF198–FGFR1. Inhibition of HSPA1A using KNK437 prevented ZNF198–FGFR1 from activating STAT3, emphasizing the importance of HSPA1A in maintaining the function of ZNF198–FGFR1. It is interesting, however, that degradation of ZNF198 and ZNF198–FGFR1 is facilitated through different pathways with the proteasomal degradation specifically targeting the fusion kinase. The reason for this difference in the mechanism of degradation of these proteins is not known at this point, although it is possible that a difference in the cellular localization and differential ability to be sumoylated may be important in determining the exact mechanism. Thus, a differential expression pattern of ZNF198–FGFR1 as compared to ZNF198 and its different mode of degradation could potentially be exploited in developing therapy against ZNF198–FGFR1 induced AMPD.

ACKNOWLEDGMENTS

This work was supported by grant CA 76167 from the National Institute of Health and in part by the RPCI Cancer Center support grant CA 16056.

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