The Oncogenic Fusion Protein-tyrosine Kinase ZNF198/Fibroblast Growth Factor Receptor-1 Has Signaling Function Comparable with Interleukin-6 Cytokine Receptors*

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The reciprocal t(8;13) chromosome translocation results in a fusion gene (FUS) in which the N-terminal half of the zinc finger protein ZNF198 is combined with the cytoplasmic domain of the fibroblast growth factor receptor-1 (FGFR1). Expression of FUS is suggested to provide growth-promoting activity to myeloid cells similar to the activity of hematopoietic cytokine receptors. This study determined the specificity of FUS to activate signal transduction pathways. Because no tumor cell line expressing FUS was available, the mode of FUS action was identified in cells transiently and stably transfected with an expression vector for FUS. FUS acted as a constitutively active protein-tyrosine kinase and mediated phosphorylation of STAT1, 3, and 5 but not STAT4 and 6. The same specificity but lower activity was determined for normal FGFR1. STAT activation by FUS, similar to that by interleukin-6-type cytokines, promoted STAT-specific induction of genes. The functionality of FUS, as well as the relative recruitment of STAT isoforms, was determined by the dimerizing function of the zinc finger domain. Replacement of the ZNF198 portion by the Bcr portion as present in the t(8;22) translocation shifted the signaling toward a more prominent STAT5 activation. This study documents that both gene partners forming the oncogenic define the activity and the signaling specificity of the protein-tyrosine kinase of FGFR1.

Reciprocal chromosomal translocations in specific types of leukemia have consistently led to the isolation of genes important for the oncogenic process (1). An atypical chronic form of myeloproliferative disease (MPD)1 was described some years ago (2) that is associated with T-cell leukemia/lymphoma and peripheral blood eosinophilia. Cytogenetic analysis of bone marrow aspirated from these patients showed a consistent reciprocal chromosome translocation t(8;13)(p11;q12). In some cases this rearrangement was the only cytogenetic abnormality. In our initial studies we identified the position of the translocation breakpoints using fluorescent in situ hybridization (3) and then used somatic cell hybrids to clearly define the location of the breakpoints on both chromosomes (4, 5). The 8p11 translocation breakpoint was subsequently shown to interrupt the FGFR1 gene, and in all of the patients reported so far, these breakpoints cluster within intron 8. The chromosome breakpoint in 13q12 was reported by several groups to involve a zinc finger-containing gene, ZNF198 (also called RAMP8 and FIM), where the breakpoint is consistently located in intron 17. Despite some discrepancies in early reports (6, 7), the full-length structure of the ZNF198 gene and the nature of the fusion gene were resolved (8, 9), which demonstrated that the chimeric gene resulted from an in-frame fusion of the ZNF198 zinc finger motif and proline-rich domain (PRD) with the intracellular domain containing the tyrosine kinase region of FGFR1.

ZNF198 is a widely expressed gene and is predicted to encode a 1377-amino acid nuclear protein with a molecular mass of 155 kDa (7–10). Prominent features of ZNF198 are the five zinc finger motifs and a PRD within the central portion of the protein and an acidic domain at the C-terminal end of the protein. The zinc finger motif is unusual in that its structure is characteristic of protein-protein interactions rather than a transcription factor. Despite these motifs, the function of this protein is unknown.

FGFR1 is a transmembrane receptor protein-tyrosine kinase belonging to the fibroblast growth factor receptor family (11). Through fusion of the cytoplasmic kinase domain to the ZNF198, the resulting chimeric protein, ZNF198/FGFR1 (hereafter termed FUS) is assumed to exert signaling functions accounting for the oncogenic event in myeloid cells (12). Stable expression of FUS in Ba/F3 cells confirmed the growth-promoting activity by providing the cells with IL-3-independent survival (13) or even proliferation (14). The same cells indicated an elevated signaling that included the phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase pathways (14, 15). The signaling specificity of the fusion kinase has been proposed based on the known function of FGFR1 (11, 16). However, the type of signaling could not be accurately predicted because the ligand-activated FGFR1 functions at the plasma membrane, whereas the fusion kinase acts as a cytoplasmic and, to some extent, nuclear protein. The analysis of another fusion kinase that contains the cytoplasmic domain of FGFR1, FOP/FGFR1, demonstrated the capability of the kinase to act in Ba/F3 cells via pathways that include STAT1 and...
STAT3, ERK, and phosphatidylinositol 3-kinase/Akt (17). The constitutive activity of FGFR1 kinase, whether part of ZNF198/FGFR1, FOP/FGFR1, or Bcr/FGFR1, has been attributed to the oligomerizing activity provided by each of the N-terminal fusion partners (9, 13, 15, 18).

Because the expression of ZNF198/FGFR1 in Ba/F3 cells displayed some of the properties associated with the action of hematopoietic cytokines, transfected the receptor tyrosine kinase signaling that is executed by the fusion kinase and whether this signaling is identifiable by the specificity of transcriptional regulation of cytokine-responsive genes. By using various experimental cell models we have identified a signaling capacity that is compatible with that of IL-6 cytokines.

**MATERIALS AND METHODS**

**Constructs**—The various expression vectors for constructs containing ZNF198 and FGFR1 sequences are described in Fig. 1. The full-length ZNF198 cDNA was amplified from a fetal bone marrow cDNA library (Clontech), and ZNF198/FGFR1 (FUS) cDNA was amplified from a patient RNA sample (4). The cDNAs were inserted into the pcDNA3 vector. The ATG codon from each of these constructs was mutated to ATCC and then cloned into the pEGFPc2 vector by using EcoRI and NotI sites yielding GFP-ZNF198 and GFP-FUS, respectively (19). The full-length FGFR1 cDNA was amplified from a human genomic DNA using a BsrGI-modified FGFR1 forward primer (AAAGATATCTCATGAGCTGCGTTAGG-GCC) and a NotI-modified FGFR1 reverse primer (ATTGGATACCATGGCACCAGCCAGAGG-GAGACGTTTTT) and then cloned into the BamHI/NotI sites of pcDNA3. To generate GFP-FUS lacking the PRD, GFP-FUS (APRD), the ZNF198/FGFR1 construct was amplified by using the KpnI/ZNF198 forward primer (CGGGTGACCCCATGCCTGCGCAG-GAGACGTTTTT) and the XbaI/ZNF198 reverse primer (TGCTCTAGACGGACTCATTTTGTTGCGATAGTCTG). The FGFR1 portion was ampliﬁed using the XbaI/FGFR1 forward primer (TGCTCTAGACGGACTCATTTTGTTGCGATAGTCTG) and cloned into the pcDNA3.1 GFP-CT TOPO vector. All of the constructs were verified by sequencing.

**Expression of Chimeric Kinase Constructs**—The expression vector containing the cDNAs encoding ZNF198, FUS (ZNF198/FGFR1), and FGFR1, as shown in Fig. 1, was transfected into MCF7 cells to determine their expression levels and to verify that the correct sized proteins were produced. The Western blot assays confirmed the immune detectable presence of the ZNF198 (Fig. 2A) and the FGFR1 epitopes (Fig. 2B) in bands corresponding to the expected full-length proteins. Strong expression was seen for the 155-kDa FUS, the 172-kDa GFP-tagged FUS, and 177-kDa GFP-tagged ZNF198. Expression of FUS (Δ7–17) protein, lacking most of the zinc ﬁnger motif, was also detected, but at a relatively low level. Of note is that the turnover of the untagged, but not the GFP-tagged, FUS protein led to the accumulation of a stable breakdown product of ~40 kDa that contained the FGFR1 epitope (Fig. 2B, ΔFUS). The relative amount and the molecular size of the degradation product detectable in the transfected cells were dependent on...
the cell line. Among the cell lines tested in this study, the degradation product was most abundant in MCF7 cells and lowest in 293 cells (see Fig. 11). When the same cell extracts were probed with an antiphosphotyrosine (anti-PY) antibody, the full-length constructs containing the FGFR1 kinase domain were phosphorylated (Fig. 2C). The comparison of immunoblot signals for FGFR1 and phosphotyrosine indicated that both the untagged and GFP-tagged FUS were highly phosphorylated. In contrast, the tyrosine phosphorylation of the overexpressed FGFR1 and FUS(Δ7–17) was low, and no phosphorylation was detectable for ZNF198. The relative expression of the transfected vectors and relative level of phosphorylation of the different proteins, as seen in MCF7 (Fig. 2), were also observed in other cells lines (see below), ruling out appreciable cell typespecific effects on expression and action of these proteins.

The detailed morphology of adherent MCF7 and HepG2 cells facilitated the visualization of the subcellular localization of the transfected proteins (Fig. 3). GFP-ZNF198 was localized in both cell types primarily to the nucleus with higher concentrations in distinct subnuclear structures, including PML bodies. This pattern did not alter during the extended period of culture. In contrast, GFP-FUS revealed a temporal change in distribution. During the first 12–24 h the FUS appeared to be predominantly cytoplasmic. During the subsequent culture period, GFP-FUS became more broadly distributed within the cells, with local accumulation at numerous sites within the cytoplasm. These accumulations gave the cells a “spotty” appearance (Fig. 3, GFP-FUS, 36 h). At later time points, there was also a detectable staining of the nucleus. The subcellular distribution of GFP-FUS and GFP-ZNF198 is distinct from that of GFP (Fig. 3, GFP). GFP showed the uniform cytoplasmic and nuclear distribution. A virtually identical subcellular distribution as seen for the transfected ZNF198 and FUS was observed with other cell types, including COS-7, NIH3T3 (data not presented), and 293 cells (Fig. 4B and Ref. 13).

Phenotypic Changes in Cell Expressing FUS—To assess the effects of FUS on proliferation and cellular phenotype, we established 293 cell lines stably expressing FUS. During these experiments, several important aspects about the ability of cells to support expression of FUS became evident. Although cells, which were initially selected for resistance to G-418, showed prominent FUS and GFP-FUS expression as detectable by immunoblotting and, in the latter case, by fluorescent microscopy, with serial passage of the pool cultures, the percentage of FUS-expressing and GFP-positive cells in the population declined. In contrast, 293 cells transfected with the expression vector for GFP, GFP-ZNF198, or FGFR1 and selected for G-418
resistance maintained long term protein expression. This observation suggested that the expression of the active FUS kinase interfered with growth selection. The difficulty in isolating stable clonal lines was best illustrated by following the visual expression of GFP-FUS. Generally, the intensely green fluorescent cells present after transfection lost adhesion to the culture substratum and either apoptosed or, in a few instances (−1 × 10^−3 of the GFP-positive cells), formed slowly growing spheres. From this we concluded that high level expression of FUS was cytotoxic and thus prevented the recovery of stable lines with high expression levels. Among the cells that maintained adherence, more than 90% of the clonal lines generated from these cells demonstrated unstable phenotypes. The expression of FUS was heterogeneous, with frequent occurrence of GFP-FUS proteins being truncated to forms ranging in size from 28 to 60 kDa. In all of those cells, the subcellular distribution of GFP changed to an evenly distributed pattern, the same distribution observed in cells expressing normal GFP, as shown in Fig. 4B (right panel). On a few occasions, however, we were able to recover truly stable subclonal lines that expressed the full-length fusion kinase in all cells (Fig. 4, A and B, left panels). From the initially transfected culture of 1 × 10^6 cells, we succeeded in establishing 12 individual subclonal lines expressing full-length GFP-FUS (numbered 1–12). Our attempt to isolate stable lines expressing untagged FUS by sequential screening of clones for kinase expression by immunoblotting proved to be nonproductive. Although we could determine expression of FUS in pool and few initial clones, the FUS expression in these cells proved to be instable and, without the benefit of a vital marker, we were unable to recover the stable subclonal line.

The GFP-FUS cell lines differ in their level of kinase expression with the highest level recorded for Clone 1 and lowest for Clone 8 (Fig. 4A). The expression of GFP-FUS as detected using the anti-GFP immune reaction (Fig. 4A, right panel) or the anti-FGFR1 immune reaction (not shown) correlated with the immune reaction with anti-phosphotyrosine (Fig. 4A, left panel). These cells also demonstrated a close correlation of FUS expression with altered culture morphology. With increasing expression of GFP-FUS, the cells formed more tightly interacting cell clusters with reduced adherence to the tissue culture substratum (Fig. 4C, left three panels). A similar change of culture morphology with prominent clusters and release of cell aggregates was noted in the pool cultures expressing FUS but...
not in the pool cultures expressing FGFR1 (Fig. 4C, right two panels).

Although the GFP-tagged ZNF198 in stably transfected cells localized to subnuclear structures (13, 19), GFP-FUS in stably expressing 293 cells, as in transiently transfected cells, was distributed throughout the cells. Higher concentrations in certain cytoplasmic structures, however, led to a punctuate appearance of the cells (Fig. 4B, center panel). This pattern differed from the uniform distribution of GFP that is seen in stably GFP-transfected 293 cells (Fig. 4B, right panel). Interestingly, a similar punctate pattern of subcellular distribution was seen in cells transfected with the truncated version of ZNF198, ZF1-GFP, consisting of the N-terminal part of FUS but lacking the FGFR1 portion (see Fig. 10). From these results we concluded that the region of the normal ZNF198 responsible for nuclear localization of the protein resides in the C-terminal portion where the nuclear localization sequence has been described (13) and that the N-terminal portion determines the cytoplasmic localization and the accumulation into aggregates.

**FUS Activates Specific STAT Proteins**—The transfected FUS is a constitutively active protein-tyrosine kinase that shows a high level of autophosphorylation (Figs. 2C and 4A). One of the consequences of FUS expression is the phosphorylation and activation of the STAT proteins. The STAT specificity of this action was determined in the 293 cell lines stably expressing FUS (Fig. 5A), example of Clone 8; others not presented). A 2–3-fold increase in the expression of STAT1, STAT3, and STAT5 (Fig. 5B). The expression level of STAT4 and STAT6 was low in 293 cells, and thus their activation by FUS could not be determined. To compare the action of FUS on STATs with that of an internal reference, we selected the effect of a short term (15 min) treatment of the cells with LIF as a marker. The repertoire of STAT-activating cytokine receptors in 293 cells is very limited, with LIF receptor being the only effective member of the receptors for IL-6 cytokines. Although total cell lysates of LIF-treated parental 293 cells did not reveal an enhanced tyrosine phosphorylation of cellular proteins that approached the level seen in FUS-expressing cells (Fig. 5A), the activation of STAT3 was still prominently detectable (Fig. 5B). There was no further increase of active STAT3 in FUS-transfected cells following LIF treatment, suggesting that the activation of this pathway was already maximally induced by FUS. LIF treatment was not appreciably effective on STAT1 and STAT5 in 293 cells, indicating that FUS exerted a broader spectrum of signaling than the LIF receptor.

In FUS-expressing cells, as well as after LIF treatment, the level of phosphorylated ERK was minimally enhanced over background. As a reference for high level activation of the ERK pathway, we used the effect of a 15-min treatment with phorbol ester. In both parental and FUS-expressing 293 cells, phorbol 12-myristate 13-acetate activated ERK to a level that exceeded severalfold that of control or LIF-treated cells (Fig. 5B). This finding suggests that FUS, like LIF treatment, was activating ERK to a submaximal level. Although the activation of the phosphatidylinositol 3-kinase/Akt pathway has been associated with the action of FGF receptors (29), in FUS-expressing cells, the level of phosphorylated Akt was essentially the same as in untreated parental cells (Fig. 5B).

FUS was effective in activating STAT signaling, although an increased expression of STAT proteins was not evident (Fig. 5B for STAT3; others not shown). A 2–3-fold increase in the expression of LIF receptor α and a 5-fold increase of EGFR were consistently seen in the different FUS-expressing clones. Despite these increases, a higher signaling through those receptors was not detectable in the presence of the prominent FUS signaling (Fig. 5B for LIF; not shown for EGF).

To compare the signaling activity of GFP-FUS with that of the untagged FUS and FGFR1 in 293 cells, we also analyzed...
the kinase expression and STAT3 phosphorylation in pool cultures of growth-selected 293 cells transfected with the vectors for those proteins (Fig. 5C). In this analysis, the pool culture expressing GFP served as a control. Although the cultures represented homogenous population of cells, the average expression of the FGFR1 epitope was approximately the same (Fig. 5C, top panel). The comparison of the anti-FGFR1 signal to that of anti-phosphotyrosine (Fig. 5C, middle panel) confirmed the similar strong autophosphorylation of the two FUS forms and the rather low level phosphorylation of FGFR1. Proportional to the level of autophosphorylation was the phosphorylation of STAT3 (Fig. 5C, bottom panel).

Because STAT4 and STAT6 are relevant for hematopoietic cells, but these forms are expressed at low level in 293 cells, we investigated the STAT specificity of FUS in the setting of overexpression of each STAT isoform in COS-7 cells. Co-transfection of FUS and the individual STAT isoforms indicated that FUS mediated phosphorylation of STAT1, STAT3, and STAT5 (Fig. 6A). STAT4 and STAT6 were not detectably modified even though the cells showed high level expression of each of the transfected STAT proteins (Fig. 6B). This restricted pattern of STAT activation distinguishes FUS from other oncogenic protein-tyrosine kinases, such as v-Src or Bcr/Abl that are capable of mediating phosphorylation of every STAT isoform (20, 30). The qualitative pattern of STAT1, 3, and 5 recruitment by FUS predicted an effective regulation because these cells not only accommodate signaling through those STAT proteins. To test this prediction, we employed the assay system of transiently transfected HepG2 cells. The assay relied on the induction of reporter gene constructs that are under the control of STAT-specific response elements. HepG2 cells are optimal for assessing transcriptional gene regulation because these cells not only accommodate signaling by many different hematopoietin receptors but also support high level transcriptional induction (26). Expression of FUS alone was sufficient, through the endogenous STAT proteins, to induce the expression of STAT-specific reporter gene constructs (Fig. 7). A comparison of untagged FUS and GFP-tagged FUS did not reveal any detectable qualitative or quantitative difference in regulatory activity between the two kinase versions (data not presented). The induction of reporter gene expression by FUS was accomplished using one reporter gene construct (p3xHPX-IL-6RE-CAT) together with an empty expression vector, or expression vector for FGFR1, v-FMS, or FUS (each 5 μg/ml) as indicated at the tops of the panels. The subcultures of the transfected cells were treated for 24 h with the medium alone (Control) or medium containing the factors listed at the bottom. A, the CAT activity detected by thin layer chromatography of the reaction mixture or medium containing the factors listed at the bottom. A, the CAT activity detected by thin layer chromatography of the reaction mixture or medium containing the factors listed at the bottom., B, the normalized values (means ± S.D.) of three independently performed experiments are reproduced.

**FUS Induces Transcription of STAT-responsive Genes**—The strong STAT activation by FUS predicted an effective regulation of genes that are responsive to cytokines that signal through those STAT proteins. To test this prediction, we employed the assay system of transiently transfected HepG2 cells. The assay relied on the induction of reporter gene constructs that are under the control of STAT-specific response elements. HepG2 cells are optimal for assessing transcriptional gene regulation because these cells not only accommodate signaling by many different hematopoietin receptors but also support high level transcriptional induction (26). Expression of FUS alone was sufficient, through the endogenous STAT proteins, to induce the expression of STAT-specific reporter gene constructs (Fig. 7). A comparison of untagged FUS and GFP-tagged FUS did not reveal any detectable qualitative or quantitative difference in regulatory activity between the two kinase versions (data not presented). The induction of reporter gene expression by FUS was accomplished using one reporter gene construct (p3xHPX-IL-6RE-CAT) together with an empty expression vector, or expression vector for FGFR1, v-FMS, or FUS (each 5 μg/ml) as indicated at the tops of the panels. The subcultures of the transfected cells were treated for 24 h with the medium alone (Control) or medium containing the factors listed at the bottom. A, the CAT activity detected by thin layer chromatography of the reaction mixture or medium containing the factors listed at the bottom. A, the CAT activity detected by thin layer chromatography of the reaction mixture or medium containing the factors listed at the bottom., B, the normalized values (means ± S.D.) of three independently performed experiments are reproduced.
In 293 cells stably expressing GFP-FUS, we noted that FUS, probably through STAT activation, was effective in elevating the immunodetectable expression level of SOCS3 (data not shown). However, based on the finding in Fig. 8B, it is expected that induced SOCS3 is unable to interfere with FUS kinase action.

The Zinc Finger Domain of ZNF198 Mediates the Oligomerization of FUS—The activation of the FGFR1 kinase in its native membrane receptor form is accomplished through ligand-induced dimerization of the receptor. Because FUS is a constitutively active FGFR1 kinase, it is assumed that the ZNF198 domain must act as an activator, such as promoting oligomerization of the fusion protein (9, 13, 14). To test in what form FUS proteins are present in transfected cells, we performed FPLC analysis of lysates of FUS-expressing MCF7 cells (Fig. 9). The proteins extracted under non-denaturating conditions were size-fractionated on a Superose 12 column. Western blot analysis of the individual fractions for proteins reacting with the anti-FGFR1 antibody (Fig. 9, upper panel) and with antiphosphotyrosine (not shown) demonstrated that, in each case, the FUS protein was primarily detected in fractions that eluted slightly ahead of the 300-kDa thyroglobulin marker. Only a very minor fraction of FUS protein eluted at the 150-kDa position expected for the monomeric FUS protein. In separate experiments (not shown) we confirmed that GFP-FUS also eluted as a dimeric complex of ~350 kDa.

As already mentioned above (Fig. 2B), untagged FUS was subject to proteolytic degradation in the transfected MCF7 cells, resulting in the accumulation of a 40-kDa product that lacked most of the ZNF198 portion but still retained the FGFR1 domain (Fig. 9, ΔFUS). This fragment was eluted on the Superose 12 column as a monomeric protein and detectable in fractions 33–38 in Fig. 9 (upper panel). These data indicated that full-length FUS is present in a complex that consists of at least a FUS dimer. The distinct elution profile of both FUS and the ΔFUS degradation product further support the interpretation that the zinc finger domain was required for dimerization. An equivalent FPLC analysis of the extract from cells expressing the full-length ZNF198 demonstrated that this protein was also eluted with a mass of ~400 kDa (data not shown).

To demonstrate that the zinc finger domain but not the N-terminal domain of ZNF198 was necessary for dimerization, we analyzed FUS with an internal deletion of exons 7–17 of ZNF198, FUS(Δ7–17) (Fig. 1). As already shown in Fig. 2C, under condition of high expression, FUS(Δ7–17) showed low level auto-phosphorylation, suggesting an activation by oligomerization. Size separation of extracts from FUS(Δ7–17)-transfected cells revealed, however, that all immune-detectable FUS protein migrated as a monomeric protein (Fig. 9, lower panel). We concluded from this observation that the zinc finger domain of ZNF198 was necessary for forming stable FUS complexes, and by this interaction the fully activated kinase and signaling function of FUS was obtained. The low kinase activity detected with FUS(Δ7–17) was attributed to either a low affinity or nonspecific interaction that became effective under conditions of overexpression. In separate experiments (not shown), we determined that induction of co-transfected CAT reporter gene constructs could only be detected in transfected cells expressing FUS(Δ7–17) at high levels.

Because the ZNF198 portion of FUS also contains a PRD that has been suggested to be responsible for FUS oligomerization (14), we assessed the contribution of PRD to the signaling function of FUS by deletion. GFP-FUS lacking the PRD, GFP-FUS(ΔPRD), was generated (Fig. 1) and transfected into 293 and HepG2 cells to determine expression and signaling. The results from those experiments (data not presented) indicated...
that GFP-FUS(ΔPRD) behaved indistinguishably from GFP-FUS in respect to subcellular localization, with accumulation in cytoplasmic structures, expression level and tyrosine phosphorylation, activation of STAT proteins, and induction of STAT-responsive gene constructs. We concluded from these findings that it is the zinc fingers and not the PRD that are responsible for the activation of the FUS kinase.

ZNF198 Has Competitive Inhibitory Activity on FUS—The ability of ZNF198 to direct complex formation suggested that, in cells co-expressing ZNF198 and FUS, heterodimers could be formed between the two proteins and that these heterodimers might be less active than the FUS homodimer. The predominant nuclear localization of ZNF198 would suggest a restricted capability of that protein to interact with cytoplasmic FUS. An alternative scenario would suggest that the heterodimers were directed to the nucleus by the nuclear localization signal of ZNF198. By using co-transfection of FUS and ZNF198, either as a normal or GFP-tagged version, we could not detect any appreciable ZNF198-dependent relocation of FUS to the nucleus or any FUS-dependent retention of ZNF198 in the cytoplasm. Nevertheless, using phosphotyrosine analysis of the two proteins, we could identify that FUS and ZNF198 were able to interact, resulting in the tyrosine phosphorylation of ZNF198 (Fig. 10A). By including ZNF198 overexpression in the reporter gene regulation assay, we also could discern a modest attenuating effect on FUS action (Fig. 10C). These data document the oligomerizing function of the N-terminal half of ZNF198 but also highlight the fact that the use of this function is curtailed by the prominent control of subcellular localization and turnover of the ZNF198 protein.

Oligomerization of FGFR1 by ZNF198 and the Bcr Domain Generates Similar but Not Identical Signaling Function—Our characterization of FUS demonstrated a highly efficient FGFR1 kinase activity controlled by the ZNF198 zinc finger domain. This activity was also proposed to promote the malignant phenotype in hematopoietic cells (12). Because a number of other oligomerization mechanisms have been found to result in FGFR1 activation, we assessed whether the type of oligomerization would affect the specificity of signaling. Recently, the chimeric kinase Bcr/FGFR1 has been identified as another oncogenic version of FGFR1 (15, 18). Considering that the N-terminal portion of Bcr is directing the formation of a tetrameric complex (33), we investigated whether signaling activity of FUS and Bcr/FGFR1 reflected that difference in oligomerization function. An expression vector for untagged Bcr/FGFR1 was generated (Fig. 1). The expression of this kinase in transfected 293 cells was comparable with that of the untagged FUS (Fig. 11A, left panel). One notable difference was that Bcr/FGFR1 did not give rise to a degradation product as found for FUS. The relative level of autophosphorylation appeared to be similar between the two kinases, although at high expression levels, FUS was more effective in mediating the tyrosine phosphorylation of other cellular proteins (Fig. 11A, right panel).
Portions of the full-length FUS and Bcr/FGFR1 are indicated. The position of the ~65-kDa fragment of FUS (ΔFUS) is given on the left. B, the extracts from the cells transfected with 4 μg of the expression vector in A were analyzed for the level of the signaling proteins listed at the right. Note, the antibodies against the tyrosine-phosphorylated STAT proteins also cross-react with the kinases. C, HepG2 cells were transfected with the CAT reporter constructs (15 μg/ml), p(8xHRRE)-CAT (left panel), or p(5xHPX-IL-6RE)-CAT (right panel) and decreasing amount of expression vectors carrying FUS, Bcr/FGFR1, or FGFR1 (from 5 to 0.02 μg/ml). The expression of the reporter genes relative to the untreated vector control is presented (mean ± S.D. of three separate experiments). W.B., Western blotting.

The recruitment of signaling pathways, however, was reproducibly different (Fig. 11B). Bcr/FGFR1 produced a lower phosphorylation of STAT1, STAT3, and ERK compared with FUS but exhibited a phosphorylation of the STAT5 equivalent to FUS. The functional difference was confirmed at the level of transcripational activation of STAT-specific reporter gene constructs (Fig. 11C). Bcr/FGFR1 was as effective as FUS in activating the STAT3-responsive HRRE construct but could only induce the STAT5-specific reporter to about one-third of that of FUS. In contrast, FGFR1 transfection was severalfold less effective as either fusion kinase in inducing expression of the two reporter gene constructs (Fig. 11C). This finding suggests that the oligomerization reaction not only enabled activation of the FGFR kinase but also determined to some extent the quantitative manifestation of the kinase function toward signal transduction pathways.

DISCUSSION

Structural chromosome rearrangements are one of the hallmarks of human leukemias (1). In many cases these rearrangements are specific for a particular type of leukemia and as such have been used as diagnostic markers. The consequence of these rearrangements is often to generate constitutive activation of genes that are thought to promote the malignant phenotype (35). Many of these chimeric genes are fusion kinases, which are presumably responsible for the activation of specific pathways that lead to loss of the normal controls for growth and differentiation in the particular tumor precursor cell. This is the case with FUS (2), which has been identified in a rare variant form of MPD. To date, no other cancer has been reported that carries this rearrangement, although several variant forms have been described, all of which result in the constitutive activation of the FGFR1 kinase domain (34). Unfortunately, no tumor cell lines carrying this rearrangement are available, which makes it impossible to study the function of FUS in the same cell lineage in which it was identified.

Although Ba/F3 cells have been traditionally used in functional studies for fusion kinases, they are highly evolved mouse cells and do not permit a comprehensive analysis of the signaling function of the kinase (13–15). In the absence of a representative model cell for this MPD, therefore, we have used a series of well characterized cell lines that have allowed us to characterize the signaling capabilities of FUS under controlled conditions to understand more about its function. From these studies it became clear that FUS signaling through STAT pathways was more prominent than reported by others (15, 17, 35). The data also compared the STAT activation profile to that of the normal FGFR1 as well as cytokine receptors, which also operate through the STAT pathway. We were able to show that the STAT signaling by FUS is remarkably similar in specificity to those of IL-6 cytokines, in particular to OSM. Although FUS would be expected to function in a manner similar to FGFR1, because of the embedded FGFR1 kinase in FUS, in fact FUS showed increased phosphorylation of many other cellular proteins as well as having a greater capability to activate the same target genes. Despite the broad range of phosphorylation of cellular proteins noted in FUS-expressing cells, FUS differs from some other oncogenic kinases, such as Src or Bcr/Abl, in its inability to activate STAT4 and 6, demonstrating a substrate-specific action for this kinase (20, 30).

The difference between FUS and both IL-6 cytokine receptors and endogenous FGFR1 in regard to the phosphorylation of cellular proteins is probably related to the fact that FUS is no longer localized to the plasma membrane and so presumably has a broader access to substrates. This enhanced range of targets in turn may be related to the oncogenic activity of FUS.
The greater effectiveness of FUS, compared with cytokine receptors, in modifying cellular proteins may also be assisted by the fact that activity of FUS, like that of Src, Fms, or Abl, but unlike that of hematopoietin receptors is not attenuated by the negative feedback mechanism through the STAT-inducible SOCS members.

It is important to consider that FUS is being ectopically expressed in the experimental systems we have used. How the level of FUS expression relates to that seen in the MPD is not clear, and because of the paucity of FUS-expressing cells from MPD patients, it cannot be determined experimentally as yet. The ability of FUS to elicit a strong signaling action, however, is not limited to cells expressing high levels as achieved in transiently transfected COS7 or 293 cells. Subclones of stably transfected 293 cells, which express very different levels of FUS, demonstrated prominent phosphorylation of cellular proteins (Figs. 4A and 5A). Similarly, HepG2 cells transfected with low amounts of FUS expression vectors showed appreciable transcriptional activation of genes (Fig. 11C). Clearly, a high level overexpression is cytotastic or even cytotoxic. In cases of massive overexpression of FUS, the transfected cells undergo apoptosis within a few days following transfection. Within the cells that have been selected for stable expression and probably also in myeloid cells carrying the translocation, there must be a threshold FUS level that can be tolerated. In many of the transfected cells, exclusion or inactivation of FUS was a frequent event, again attesting to the toxic consequences to the cell. Morphological changes are also manifested at subthalial level of FUS expression, as was seen in pools of transfected cells and in subclones of 293 GFP-FUS cells (Fig. 4C).

Expression of FUS also affects motility and adhesion, which results in the development of poorly attached cells that form dense clusters in the cultures. This phenotype is comparable with that seen for v-Src-expressing cells, where the focal adhesion kinase complex, which control cytoskeleton organization, is dysregulated (36). Analysis of the phosphorylated proteins using anti-PY antibodies in FUS-expressing cells clearly identifies proteins in the 130-kDa size range (Fig. 5A), which include proteins involved in the focal adhesion kinase complexes (data not presented). These data suggest that FUS expression may affect cell architecture by altering cytoskeletal organization. The other unusual observation is that, within some of the cells used, there is an active degradation of the FUS protein that is determined by structural information contained in ZNF198 portion. This observation implies that cells have an endogenous mechanism that recognizes ZNF198 motifs, as part of cytoplasmic proteins, and tags the proteins that harbor those motifs for proteolytic fragmentation. In the case of FUS, the degradation also relieves some of the inhibitory activity of FUS expression. The biochemistry of the degradation process remains to be defined.

Early studies have suggested, using immunoprecipitation of epitope-tagged constructs, that the zinc finger domain serves as a protein-protein interaction motif to activate the kinase (6, 9, 13). Using FPLC analysis we have demonstrated dimerization of the fusion gene and further that this dimerization is lost when the zinc finger, but not the PRD, is removed. These observations are at variance with a previous report (14) suggesting that the PRD alone could be responsible for the dimerization of FUS. In the report by Xiao et al. (14), however, the fusion kinase construct used was significantly smaller (87 kDa) than the native fusion kinase in MPD (155 kDa). It is therefore likely that properties of this smaller fusion protein significantly affect its function because we have clearly demonstrated that the PRD is unnecessary for dimerization in the context of the full-length fusion kinase.

The presence of normal and rearranged proteins in the same hematopoietic cell (ZNF198 is ubiquitously expressed) raises the question whether heterodimers can be formed that affect the normal function of either gene product. Here we have shown that FUS and ZNF198 can form heterodimers and that this interaction results in the phosphorylation of ZNF198. Further, the presence of ZNF198 in the complex suppresses the functional properties of FUS. Exactly what the consequences of this association are for oncogenicity is not clear because the localization of FUS, as a result of this heterotypic dimerization, does not result in significantly increased nuclear localization of FUS. Furthermore, we have shown that reduction of FUS activity to relatively low levels still elicits a signaling response from the homomeric FUS.

Several groups have now reported that, in some cases of this MPD, the FGFR1 kinase domain is fused with the Bcr gene that is usually associated with the Bcr/Abl fusion kinase in AML, as well as the FOP/FGFR1 fusion kinase gene associated with a t(6;8)(q27;p11) rearrangement (9, 12, 33). Both latter fusion genes are also thought to operate through constitutive activation of the kinase function following dimerization. When Bcr is fused with FGFR1, we have demonstrated that, indeed, the N-terminal half of Bcr protein can substitute for the ZNF198 zinc finger motif to oligomerize and activate the kinase. This Bcr/FGFR1 protein, however, although expressed at comparable levels to FUS, is not as efficient in our assays in mediating phosphorylation of other proteins in the cell, including STAT1 and 3 and ERK (Fig. 11). Whether this altered efficiency leads to a milder form of the disease cannot be discerned from the case reports available from the relatively few patients with the variant forms of the fusion kinase gene described so far.

The general suggestion for the mode of action of FUS has been that unlike the FGFR1 kinase, which is restricted to the membrane fraction of the cells, FUS localizes to the cytoplasm, which is responsible for the oncogenic events in the expressing cells. Much of the support for this concept has come from transient transfection assays (13, 14, 35), often in nonhuman cells such as Ba/F3 and COS. Here we have demonstrated that although the fusion protein is predominantly in the cytoplasm during the first 24 h after transfection, this same protein can be found in the nucleus several days later. In stably transfected cells, the protein appears to be generally distributed throughout the cell and often concentrated in subcellular compartments within the cytoplasm when highly overexpressed. Thus, although the majority of the FUS protein is located in the cytoplasm, it is not clear to what extent the cytoplasmic FUS alone accounts for the transforming process (13). Given that the FUS protein is also found in the nucleus in cells stably expressing it, the oncogenic mechanism may be assisted by the action of FUS located in the nucleus. The characterization of other FGFR1-containing fusion gene products with altered subcellular localization and the analysis of FUS-transgenic mice are underway to provide more definitive answers.

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The Oncogenic Fusion Protein-tyrosine Kinase ZNF198/Fibroblast Growth Factor Receptor-1 Has Signaling Function Comparable with Interleukin-6 Cytokine Receptors

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