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SH2B3 (LNK) mutations from Myeloproliferative Neoplasms patients have mild loss of function against wild type JAK2 and JAK2 V617F

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Summary

Somatic point mutations in the PH domain of SH2B3 (LNK), an adaptor protein that is highly expressed in haematopoietic cells, were recently described in patients with myeloproliferative neoplasms. We studied the effect of these mutations on the JAK2 signalling pathway in cells expressing either wild type JAK2 or the JAK2 V617F mutation. Compared to wild type SH2B3, PH domain mutants have mild loss of function, with no evidence for a dominant-negative effect. Mutants retain binding capacity for JAK2, an established SH2B3 target, as well as for the adaptor proteins 14-3-3 and CBL. Our data suggest that the loss of SH2B3 inhibitory function conferred by the PH domain mutations is mild and may collaborate with JAK2 V617F and CBL mutations in order to promote either the development or the progression of myeloproliferative neoplasms.

Keywords

MPN; *JAK2* mutation; *SH2B3 (LNK)* mutation; 14-3-3; CBL

Introduction

Activation of the JAK2-STAT pathway plays a major role in the pathogenesis of the Philadelphia negative (Ph-) myeloproliferative neoplasms (MPN). This is most commonly attained by an activating *JAK2* V617F mutation which is found in more than 90% of patients with polycythaemia vera and approximately 50% of patients with essential thrombocythaemia and primary myelofibrosis (Baxter, *et al* 2005, James, *et al* 2005, Jones, *et al* 2005, Kralovics, *et al* 2005, Levine, *et al* 2005). Alternatively, JAK2-STAT activation occurs with less prevalence due to either other *JAK2* mutations (for example, in exon 12) (Pardanani, *et al* 2007, Scott, *et al* 2007) or activating mutations of the thrombopoietin receptor, *MPL* (Pardanani, *et al* 2006, Pikman, *et al* 2006). Recently, mutations in *SH2B3 (LNK)*, a negative regulator of JAK2-STAT signalling pathway, were described in approximately 6% of patients with chronic phase MPN (Ha and Jeon 2011, Hurtado, *et al*

2011, Lasho, *et al* 2011, Oh, *et al* 2010a, Oh, *et al* 2010b) and up to 10% of blast phase MPN patients (Pardanani, *et al* 2010). SH2B3 is a member of an adaptor protein family with structural homology to SH2B2 (APS) and SH2B1 (SH2B). It is highly expressed in haematopoietic cells (Huang, *et al* 1995, Li, *et al* 2000) and inhibits signalling through the tyrosine kinase receptors KIT (Gueller, *et al* 2008, Simon, *et al* 2008), CSF1R (FMS) (Gueller, *et al* 2010) and PDGFR (Gueller, *et al* 2011), as well as the non-tyrosine kinase receptors MPL (Gery, *et al* 2007, Seita, *et al* 2007, Tong and Lodish 2004) and the erythropoietin receptor (EPOR) (Tong, *et al* 2005). SH2B3 directly binds to wild type (WT) JAK2 and JAK2 V617F, and decreases their autophosphorylation and downstream signalling through STAT5, MAPK/ERK and the PI3K/AKT pathways (Bersenev, *et al* 2008, Gery, *et al* 2009, Tong, *et al* 2005). Moreover, knockout of *Sh2b3* cooperates with JAK2 activating variants in the formation of myeloproliferative disease in a murine bone marrow transplantation model (Bersenev, *et al* 2010), supporting the importance of SH2B3 in the inhibition of the JAK2-STAT pathway. Although loss of SH2B3 function is not sufficient for the transformation of haematopoietic cells, *SH2B3* mutants may affect either disease phenotype or response to therapy. Therefore, understanding SH2B3 involvement in MPN pathogenesis may have therapeutic significance. To delineate the mechanism of somatic *SH2B3* mutations in the pathogenesis of MPN, we studied the effect of different *SH2B3* PH domain mutations that have been identified in MPN patients (E208Q, A215V and G220V) on the JAK2 signalling pathway.

Materials and Methods

Mice and cell culture

Sh2b3-deficient 129/Sv mice were a generous gift from Dr. T. Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada) (Velazquez, *et al* 2002). Bone marrow (BM) cells were obtained 5 days post-intraperitoneal (IP) 150 µg/kg fluorouracil (5FU) treatment, and were cultured in Iscove's modified Eagle's medium (IMDM) containing 20% fetal bovine serum (FBS), 50 µM β-mercaptoethanol, 10 ng/ml mouse (m) interleukin 3 (IL3) and mIL6 and 50 ng/ml murine stem cell factor (mSCF). The 293T cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA), and the packaging cell line Plat-E was generously provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan) (Morita, *et al* 2000). These cell lines were grown in Dulbecco's minimal essential medium with 10% FBS. The BaF3 stably expressing EPOR cells (BaF3-E) were previously described (Gery, *et al* 2009) and were maintained in RPMI-1640 medium containing 10% FBS with 10 iu/ml recombinant erythropoietin (Epo). The HEL cell line was obtained from ATCC and grown in RPMI-1640 medium containing 10% FBS.

SH2B3 mutants and expression vectors

The pcDNA3.1/V5 human *SH2B3* vector and *SH2B3* RE were previously described (Gery, *et al* 2007, Gueller, *et al* 2008). The bicistronic retroviral MSCV-IRES-GFP (MIG) murine *Sh2b3* vectors (WT and R364E) were generously provided by Dr. W. Tong (Children's Hospital of Philadelphia, Philadelphia, PA) (Tong and Lodish 2004). The pcDNA3.1 WT *JAK2* and *CBL* vectors were kindly provided by Dr. Z.J. Zhao (University of Oklahoma, Oklahoma City, OK) (Zhao, *et al* 2005) and Dr. H. Serve (University Hospital Münster, Münster, Germany) (Sargin, *et al* 2007), respectively.

The *SH2B3* mutations E208Q (EQ), A215V (AV) and G220V (GV) in the pcDNA3.1/V5 human *SH2B3* vector and E182Q (EQ) and G194V (GV) in the MIG murine *Sh2b3* vectors were generated by polymerase chain reaction (PCR) based, site-directed mutagenesis and confirmed by DNA sequencing.

Transfections

293T and Plat-E cells were transfected with either BioT (Bioland Scientific, Paramount, CA) or ProFection® Mammalian Transfection System (Promega, Madison, WI) (293T for viral production) following the manufacturer's instructions. Transfection of BaF3-E cells was done with the Nucleofector technology (Lonza, Cologne, Germany).

Retroviral infection

Plat-E cells were transfected with the MIG vectors, and viral supernatants were collected after 48 h. 293T cells were transfected with the MIG vectors in conjunction with pGag-Pol and RD114 *Env* as previously described (Cosset, *et al* 1995). Viral titres were determined by serial dilutions of the viral supernatant and infection of either NIH 3T3 or 293T cells. Supernatants with equal viral titres were used in each experiment. HEL cells were spin-infected on 2 consecutive days with retroviral supernatants containing 6 µg/ml polybrene (Sigma, Saint Louis, MO) at 1900 rpm (Beckman GS-CR, Brea, CA) for 60 min at 25°C. BM cells were infected on 2 consecutive days on RetroNectin™-coated dishes, following the manufacturer's instructions (Takara Shuzo Co., Otsu, Japan).

Western blot and immunoprecipitation

Cells were washed twice with phosphate-buffered saline (PBS) and lysed on ice with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40) containing complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 mM NaF and 10mM Na₃VO₄). Proteins were subjected to 4–15% gradient sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Sigma). Immunoblots were incubated with primary antibodies followed by incubation with appropriate secondary immunoglobulin G antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Sunnyvale, CA). SuperSignal West Pico and West Dura Chemiluminescent substrates (Pierce Biotechnology, Rockford, IL) were used for detection. The following antibodies were used for immunoprecipitation and Western blot analysis: LNK/SH2B3, JAK2, phospho-ERK (Tyr 204), ERK, STAT5 and CBL (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT5A/B (Y694/699) (Millipore, Billerica, MA); V5 (Invitrogen, Camarillo, CA) and β actin/ACTB (Sigma). For immunoprecipitation, lysates were incubated in lysis buffer containing protease and phosphatase inhibitors, with specific antibodies and protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 16 h. Precipitated proteins were washed 3 times with lysis buffer, eluted with SDS sample buffer and subjected to Western blot analysis. Analysis of bands was done with the ImageJ software.

Phospho-flow analysis

At 2 days after the transfection of BaF3-E cells with *Sh2b3* constructs, cells were serum-starved for 6 h, stimulated with 10 iu/ml of Epo for 15 and 60 min, immediately fixed with freshly-prepared paraformaldehyde solution in a final concentration of 1.6% and permeabilized with ice-cold methanol, as previously described (Krutzik, *et al* 2004, Krutzik and Nolan 2003). BM progenitor cells were serum-starved for 2 h and then stimulated with 10 iu/ml IL3. HEL cells were serum-starved for 8 h. Cells were stained with phycoerythrin (PE)-conjugated STAT5 (pY694) and Alexa Fluor 647 conjugated ERK 1/2 (pY202/204) (BD Biosciences, San Jose, CA) antibodies. Analysis was done on GFP positive cells with the LSRII flow cytometer and the FlowJo software.

Colony assays

BM cells from *Sh2b3*^{+/+} and *Sh2b3*^{-/-} mice (3 of each) were harvested 5 days after treatment with IP 150 µg/kg 5FU and cultured for one day in IMDM with 20% FBS, 50 µM β-mercaptoethanol, 10 ng/ml mIL3 and mIL6 and 50 ng/ml mSCF. Cells were infected twice on RetroNectin™-coated dishes with either MIG or MIG-*Sh2b3* retroviral supernatants and after two days sorted for GFP expression. Positive cells (10³) were plated in triplicates in semisolid methylcellulose (MethoCult GF M3434, Stem Cell Technologies, Vancouver, Canada). Colony formation was assessed after 8–9 days, by scoring the number of total colonies. BaF3-E cells were transfected with either MIG or MIG-*Sh2b3* using the Nucleofector system and sorted for GFP expression after 2 days. Positive cells (10³) were plated in triplicates in soft agar with 10 iu/ml Epo. Colony formation was assessed after 10 days.

Statistical analysis

Colonies and cell numbers number were compared between the different SH2B3 constructs using unpaired student T test and one-way analysis of variance (ANOVA).

Results

Sh2b3 PH domain mutants inhibit growth to a lesser extent than WT Sh2b3

We have previously shown that WT SH2B3 inhibits Epo-dependent growth of BaF3-E cells (Gery, *et al* 2009). To test whether the SH2B3 PH domain mutations result in loss of SH2B3 function, BaF3-E were transfected with either WT *Sh2b3*, the SH2 domain RE mutant (which was shown to abolish SH2B3 function) or the *Sh2b3* PH domain mutants EQ and GV. Colony forming efficiency with either Epo or IL3 was assessed (Fig 1A and B). As expected, colony formation of BaF3-E cells was inhibited by WT SH2B3 compared with control vector transfected cells, while the RE mutation abolished the ability of SH2B3 to inhibit colony formation. SH2B3 EQ and GV were less inhibitory compared to WT SH2B3. We also compared the effect of the different SH2B3 mutants on colony formation of BM progenitor cells. WT SH2B3 significantly inhibited colony formation compared to a vector control when reintroduced in *Sh2b3* knock-out (KO) BM. Reintroduction of SH2B3 GV resulted in less clonal inhibition compared to WT SH2B3, while SH2B3 EQ caused a non-significant decrease in clonal inhibition (Fig 1C). Overexpression of either SH2B3 EQ or GV in WT *Sh2b3* BM resulted in less clonal inhibition compared to WT SH2B3 (Fig 1D).

Effect of Sh2b3 PH domain mutants on Jak2 downstream signalling

We next examined the effect of SH2B3 mutants on downstream signalling of JAK2. BaF3-E cells overexpressing either WT or mutant SH2B3 were serum-starved for 6 h, stimulated with Epo and studied for changes of STAT5 and ERK phosphorylation using phospho-flow (Fig 2). As also previously shown (Tong, *et al* 2005), we found that WT SH2B3 resulted in a slower and lower maximal STAT5 phosphorylation and completely abolished ERK phosphorylation after Epo stimulation. Epo stimulation of BaF3-E cells overexpressing either SH2B3 RE or a vector control resulted in similar levels of STAT5 and ERK phosphorylation. SH2B3 EQ inhibited maximal STAT5 and ERK phosphorylation slightly less than WT SH2B3, while the inhibition of Stat5 and Erk phosphorylation by SH2B3 GV was noticeably less than WT SH2B3. Given that MPN is a disease of myeloid progenitors and WT JAK2 is also a mediator of IL3 signalling, we studied IL3 signalling in progenitor-enriched BM cells with forced expression of either WT SH2B3 or one of the SH2B3 mutants (Fig 3). All SH2B3 mutants inhibited Stat5 phosphorylation less than WT SH2B3 suggesting that the SH2B3 PH domain mutants exhibit less functional inhibition of this stimulatory pathway.

Sh2b3 PH domain mutants inhibit JAK2 V617F-dependent cells to a lesser extent than WT Sh2b3

JAK2 V617F is the most common mutation found in MPN. MPN cells with mutant *JAK2* can also have *SH2B3* PH domain mutations. We therefore studied the effect of the SH2B3 PH domain mutants in HEL cells which constitutively express the JAK2 V617F mutant. Overexpression of WT SH2B3 inhibited the growth of HEL cells (Fig 4A). The inhibitory effect on growth was partially lost with SH2B3 EQ and GV PH domain mutants. Analysis of JAK2 downstream signalling pathway by phospho-flow (Fig 4B–C) demonstrated that the SH2B3 PH domain mutants inhibited unstimulated STAT5 phosphorylation slightly less than WT SH2B3 while WT and mutant SH2B3 inhibited ERK phosphorylation to similar extents. Similar results were also observed in Western blot analysis (Fig 4D).

SH2B3 mutants retain JAK2 binding capacity as well as binding to CBL and 14-3-3

The exact mechanism for SH2B3 inhibitory activity is not known; however, this effect appears to require direct contact between SH2B3 and its target. Therefore, loss of SH2B3 function could occur via decreased binding affinity to its targets. On the other hand, stronger binding to possible SH2B3 modifiers may also decrease its inhibitory effect.

Both 14-3-3 and CBL have recently been suggested to modify the activity of SH2B3 family members (Jiang, *et al* 2012, Yokouchi, *et al* 1999). Therefore, we examined SH2B3 WT and PH domain mutants binding to JAK2, 14-3-3 and CBL (Fig 5). JAK2 and either SH2B3 WT or mutants were co-expressed in 293T cells and protein lysates were immunoprecipitated with a SH2B3 antibody and western blotted with a JAK2 antibody. WT SH2B3 bound JAK2 while the SH2B3 RE mutation nearly lost this binding ability, as expected. SH2B3 PH domain mutants (EQ, GV, and AV) were able to bind JAK2 similar to WT SH2B3 (Fig 5A). In order to study the binding of SH2B3 mutants to 14-3-3, SH2B3 WT and mutants were expressed in 293T cells, endogenous 14-3-3 was immunoprecipitated and Western blotted with a SH2B3 antibody (Fig 5B). WT SH2B3 and all the PH domain mutants interacted with 14-3-3 to a similar extent. In addition, both CBL and either SH2B3 WT or the PH domain mutants EQ and GV were expressed in 293T cells, protein lysates were immunoprecipitated with a SH2B3 antibody and Western blotted and probed with either a CBL or a 14-3-3 antibody (Fig 5C). SH2B3 WT, EQ and GV bound CBL, as well as 14-3-3. We have previously demonstrated a role for the SH2B3 PH domain in plasma membrane localization (Gery, *et al* 2007). We therefore assessed the localization of the SH2B3 PH domain mutants with confocal microscopy. WT SH2B3 and the SH2B3 PH domain mutants EQ and GV all localized to both the cytoplasm and the cell membrane (Supplementary Fig 1).

Discussion

Since its characterization approximately 15 years ago (Huang, *et al* 1995, Li, *et al* 2000, Takaki, *et al* 1997), the adaptor protein SH2B3 has been identified as an important negative regulator of cytokine signalling in haematopoietic cells, and mutations in *SH2B3* were recently found in MPN patients (Ha and Jeon 2011, Hurtado, *et al* 2011, Lasho, *et al* 2011, Oh, *et al* 2010a, Oh, *et al* 2010b, Pardanani, *et al* 2010). In most cases, *SH2B3* PH domain mutations identified in MPN patients are heterozygous (Oh, *et al* 2010a, Oh, *et al* 2010b, Pardanani, *et al* 2010) suggesting that SH2B3 haploinsufficiency is sufficient to contribute to MPN pathogenesis. Alternatively, as these mutations retain the SH2B3 N-terminal dimerization domain, they could act by binding and sequestering WT SH2B3, resulting in a dominant negative effect. We found that introduction of SH2B3 mutants into *Sh2b3* WT BM cells did not result in enhanced colony formation, suggesting that the mutants do not relieve the functions of endogenous SH2B3, and do not act in a dominant negative manner. Furthermore, the mutants inhibited growth of *Sh2b3* KO BM cells, albeit to a lesser extent

compared with WT SH2B3, which is also consistent with a non-dominant negative effect. *Sh2b3* KO mice exhibit a MPN-like phenotype, while *Sh2b3* heterozygous mice have an intermediate phenotype (Velazquez, *et al* 2002), further supporting the haploinsufficiency model. Finally, some of the *SH2B3* mutations occurring in haematopoietic disorders are frame shifts or stop codons that presumably destroy the function of SH2B3, again associated with loss of function (Hurtado, *et al* 2011, Oh, *et al* 2010b, Pardanani, *et al* 2010, Roberts, *et al* 2012). Oh *et al* (2010a) recently showed that thrombopoietin-dependent growth, as well as STAT5 phosphorylation of BaF3 cells that stably expressed MPL, was less inhibited by the SH2B3 EQ mutant while a deletion mutant lacking both SH2B3 SH2 and PH domains completely lost the SH2B3 inhibitory effect. Together these results support a slight loss of function for the SH2B3 PH domain point mutants.

Interestingly, a genome-wide association study (Gudbjartsson, *et al* 2009) found that a known non-synonymous germline polymorphism in the *SH2B3* PH domain (rs3184504, SH2B3 W262R) was associated with increased haemoglobin, white blood cell and platelet counts. Furthermore, some of the *SH2B3* nucleotide changes discovered in MPN patients are germline changes (Jiang, *et al* 2012, Oh, *et al* 2010b). The effect of SH2B3 W262R polymorphism on cell growth was recently studied (McMullin, *et al* 2011). When overexpressed in 32D/EPOR cells, the SH2B3 W262R variant inhibited Epo-dependent cell growth to the same extent as WT SH2B3. In contrast, SH2B3 W217A, a point mutation in the SH2B3 PH domain generated by Tong *et al* (2005) caused mild loss of SH2B3 function on 32D/EPOR cell growth (McMullin, *et al* 2011, Tong, *et al* 2005). This observation complements our results and suggests that the effect of the *SH2B3* mutations may be specific to the cell type and the cytokine receptor. Germline *SH2B3* changes, therefore, could affect the MPN phenotype and possibly the therapeutic response to novel agents.

In general, activating mutations in the JAK2/STAT pathway (e.g. *JAK2*, *MPL* mutations), are considered mutually exclusive (Cross 2011). However, *SH2B3* mutations can occur concurrently with *JAK2* V617F (Lasho, *et al* 2011, Pardanani, *et al* 2010), suggesting that these mutations may be cooperative in nature. Our finding, that SH2B3 PH domain mutations show a mild loss of function, support this hypothesis and furthermore, raise the possibility that *SH2B3* mutations in haematopoietic malignancies might often be associated with additional mutations in the JAK/STAT pathway. Interestingly, *SH2B3* deletional mutations associated with activating mutations in *IL7R* (which signals via JAK1 and JAK3) were recently reported in high-risk acute lymphoblastic leukemia (Roberts, *et al* 2012). These findings demonstrate the importance of the clonal context in which *SH2B3* mutations occur and underscore the complexity of genomic alterations in haematopoietic malignancies.

Mutant JAK2 (JAK2 V617F) is inhibited by WT SH2B3 (Gery, *et al* 2009, Tong, *et al* 2005). We found that the SH2B3 PH domain mutants were weaker than WT SH2B3 at inhibiting growth and STAT5 phosphorylation of cells that harbour JAK2 V617F (HEL). The coexistence of *SH2B3* mutations with *JAK2* V617F in MPN samples, and the suggested higher prevalence of these mutations in the blast phase of MPN compared to the chronic phase (Pardanani, *et al* 2010), suggest that *SH2B3* mutations may collaborate in the development and / or progression of MPN.

Epo stimulation in erythroid progenitors leads to activation of JAK2 and its three major downstream pathways, STAT5, PI3K-AKT and RAS-MAPK. Activation of the RAS-MAPK pathway in erythroid progenitors by oncogenic RAS expressed from its endogenous promoter increases cell sensitivity to Epo stimulation, resulting in a mild block in terminal erythroid differentiation, while overexpression of oncogenic RAS in erythroid progenitors leads to constitutive activation of the major signalling pathways, leading to severe block in terminal erythroid differentiation and cytokine-independent proliferation (Zhang and Lodish

2007). Tokunaga et al (2010) compared the effect of the oncogenic tyrosine kinases BCR-ABL1 and JAK2 V617F on the development of cells of the erythroid lineage. BCR-ABL1 caused an intense activation of RAS and suppressed erythroid cell proliferation while JAK2 V617F resulted in less activation of RAS and did not inhibit erythropoiesis. Interestingly, in human MPN the same oncogenic event, i.e., *JAK2* V617F, is associated with either proliferation of the erythroid lineage and a high haematocrit in polycythaemia vera or with decreased bone marrow erythroid progenitors in primary myelofibrosis and peripheral blood anaemia. We may speculate that additional mutations enhancing RAS-MAPK-ERK signalling may collaborate in this scenario and contribute to a defect in erythroid cell proliferation and maturation. SH2B3 directly attenuates EPOR phosphorylation and JAK2 activation, thereby inhibiting major signalling pathways initiated by Epo/EPOR. In our BM experiments, we tested the effect of WT SH2B3 and SH2B3 mutants on growth of total colonies and could not define the exact effect of SH2B3 on erythroid cells. The slight loss of SH2B3 function conveyed by the PH domain mutations in our studies dampened signalling through STAT5 more than signalling through MAPK-ERK. Whether a more significant loss of SH2B3 function, such as either homozygous or compound heterozygous mutation of the *SH2B3* gene in conjunction with an activating *JAK2* V617F mutation, would be deleterious on erythroid cells production, will require additional studies in the future.

The function of the SH2B3 PH domain is unknown. We have previously shown a role for this domain in binding of un-phosphorylated WT, as well as V617F mutant JAK2 (Gery, et al 2009). We, however, neither detected a decreased interaction between SH2B3 with PH domain mutations and WT JAK2, nor with other known or putative SH2B3 binding partners including 14-3-3 (Jiang, et al 2012) and CBL. The proto-oncogene CBL binds the SH2B3 family member-SH2B2 (Yokouchi, et al 1999) and a similar CBL recognition motif is found in the other family members, SH2B1 and SH2B3 (Hu and Hubbard 2005). Here, we show for the first time that SH2B3 binds CBL. Originally described as an E3 ubiquitin ligase, CBL was also recently recognized as a multi-adaptor protein and a positive regulator of signal transduction (Schmidt and Dikic 2005). *CBL* mutations are found in several myeloid neoplasms, particularly in the MDS/MPN subtype chronic myelomonocytic leukaemia (CMML) (Sanada, et al 2009). Interestingly, *SH2B3* mutations were described in 7% of CMML samples and may co-occur with *CBL* mutations (Oh, et al 2010b), suggesting they may collaborate. CBL is a multifunctional adaptor protein and was previously shown to interact with 14-3-3, JAK2 and SH2B2 (Schmidt and Dikic 2005); we therefore speculate that SH2B3, CBL, JAK2 and 14-3-3 may participate in a multiprotein complex.

We have previously shown that SH2B3 strongly binds through its SH2 domain to phosphorylated JAK2 WT and JAK2 V617F, whereas other SH2B3 regions facilitate weaker interactions with non-phosphorylated JAK2 (Gery, et al 2009). The results of the current study support a different role for the various SH2B3 domains. By binding to phosphorylated tyrosines on the cytokine receptors and JAK2, the SH2B3 SH2 domain is associated with attenuation of downstream signalling in response to cytokine stimulation. The SH2B3 PH domain may attenuate baseline signalling from unstimulated receptors, and may, therefore, be more important in MPN cases that do not harbour the *JAK2* V617F activating mutation.

In conclusion, our study provides important mechanistic insights into the function of the newly identified *SH2B3* PH domain mutants. We show that they are mild loss of function mutations, they do not have a dominant-negative effect, and they can all still bind JAK2. Mutant SH2B3 may collaborate with JAK2 V617F, and CBL and SH2B3 are likely to cooperate in MPN, particularly in CMML. Octa-Arginine mediated delivery of SH2B3 into megakaryocytic leukaemia cell lines was recently shown to be a non-toxic method to inhibit leukaemic cell proliferation (Looi, et al 2011) and may serve as a model to develop SH2B3-

based therapies in MPN. Knowledge of *SH2B3* status in these patients may have clinical and therapeutic implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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M.K.M. designed the research, performed experiments, analysed results and wrote the paper; S.G. designed the research and analysed results; T.T., D.L. and A.N. analysed results; R.A. performed experiments; and H.P.K. designed the research, analysed results and edited the paper.

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Figure 1. Sh2b3 PH domain mutants compared to WT Sh2b3 have a decreased inhibitory effect on BaF3-E and BM colonies

(A,B) BaF3-E cells were transfected with either *Sh2b3* wild type (WT) or mutant *Sh2b3*. GFP-positive cells were sorted 2 days after transfection and plated in equal cell densities with either 10 iu Epo/ ml (A) or 10 iu IL3/ ml (B) in soft agar. Colonies were counted after 10 days. Results represent the mean $\pm$ SD of triplicate samples. (C,D) BM enriched progenitor cells from *Sh2b3* knock-out (KO) (C) or WT (D) mice were infected on 2 consecutive days with either *Sh2b3* WT or mutant *Sh2b3*. GFP positive cells were sorted 2 days after the second infection and equal cell number plated in cytokine rich methylcellulose media (M3434, STEMCELL Technologies Inc., Vancouver, BC). Total colonies number was scored after 8–9 days. Results represent the mean $\pm$ SD of triplicate samples. (E) Western blot demonstrating SH2B3 expression in sorted BaF3-E cells transfected with *Sh2b3* variants.

* Significantly different from WT SH2B3, $p < 0.05$.

Figure 2. Epo-dependent phosphorylation of Stat5 and Erk is inhibited by the Sh2b3 PH domain mutants less than WT Sh2b3

BaF3-E cells transfected with either WT or mutant *Sh2b3* were serum-starved for 6 h and stimulated with 10 iu/ml Epo for 15 and 60 min. Phosphorylation of Stat5 and Erk was assessed in GFP-positive cells by flow analysis, using PE-conjugated STAT5 (pY694) and Alexa Fluor 647 conjugated ERK 1/2 (pY202/204) antibodies. A representative of 2 independent experiments is shown. (A) Gating of BaF3-E GFP-positive cells. (B) STAT5 and ERK phosphorylation. Mean fluorescence intensities (MFIs) for each time point were normalized to un-stimulated cells and are represented as fold-change. (C) Western blot of proteins from BaF3-E transduced with *Sh2b3*.

BM progenitor cells

Figure 3. IL3-dependent STAT5 phosphorylation is inhibited less by the *Sh2b3* PH domain mutants compared to WT *Sh2b3*

Murine 5FU-enriched BM progenitor cells transduced with either WT or mutant *Sh2b3* were serum-starved for 2 h and stimulated with 10 iu/ml IL3 for 15 and 60 min. Phosphorylation of STAT5 and ERK was assessed in GFP-positive cells by flow analysis, using PE-conjugated STAT5 (pY694) and Alexa Fluor 647 conjugated ERK 1/2 (pY202/204) antibodies. A representative of 2 independent experiments is shown. **(A)** Gating of GFP-positive cells. **(B)** STAT5 phosphorylation. MFIs for each time point were normalized to unstimulated cells and are represented as fold-change.

Figure 4. Growth and signal transduction of JAK2 V617F HEL cells is inhibited less by the *Sh2b3* PH domain mutants compared to WT *Sh2b3*

HEL cells (JAK2 V617F) were transduced with either WT or mutant *Sh2b3*. (A) GFP-positive cells were sorted, plated at equal cell densities in liquid culture and counted every 2 days. Results represent the mean $\pm$ SD of triplicate samples. Transduced cells were serum-starved for 8 h and studied for STAT5 and ERK phosphorylation. (B) Gating of GFP-positive cells. (C) STAT5 and ERK phosphorylation. MFIs were normalized to the vector control transduced cells and are represented as fold-change. (D) Western blot analysis of proteins from sorted GFP-positive HEL cells transduced with either a vector control, WT *Sh2b3*, *Sh2b3* RE or EQ. Cells were serum-starved for 8 h and analysed for STAT5 and ERK phosphorylation. Band intensity was normalized to ACTB and represent fold change compared to the vector control.

Figure 5. SH2B3 PH domain mutants retain their binding to JAK2, CBL and 14-3-3
 293T cells were co-transfected with either WT *JAK2* (A) or *CBL* (C) and either WT or mutant *SH2B3*. Protein lysates were immunoprecipitated with V5 antibodies and analysed by Western blot as indicated. (B) 293T cells were transfected with either WT or mutant *SH2B3*. Protein lysates were immunoprecipitated with V5 antibodies and analysed by Western blot as indicated.