Interactions between *RPS19*, mutated in Diamond-Blackfan anemia, and the PIM-1 oncoprotein

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Background and Objectives. Diamond Blackfan anemia (DBA) is a congenital disease characterized by defective erythroid progenitor maturation. Patients' bone marrow progenitor cells do not respond to erythropoietic growth factors, such as erythropoietin. Mutations in the gene encoding for ribosomal protein (RP) S19 account for 25% of cases of DBA. The link between defective erythropoiesis and *RPS19* is still unclear. Two not mutually exclusive hypotheses have been proposed: altered protein synthesis and loss of unknown extraribosomal functions.

Design and Methods. We used yeast two-hybrid screening and a human liver cDNA library obtained at 19-24 weeks of gestation, when hepatic erythropoiesis is efficient, to search for proteins interacting with RPS19.

Results. We found that RPS19 binds PIM-1, an ubiquitous serine-threonine kinase whose expression can be induced in erythropoietic cells by several growth factors, such as erythropoietin. The PIM-1/RPS19 interaction was demonstrated both *in vitro* and in living cells and led to phosphorylation of RPS19 in an *in vitro* kinase assay. We also showed that in human 293T cells PIM-1 interacts with ribosomes and may be involved in translational control. Three DBA-associated *RPS19* mutations alter the binding between RPS19 and PIM-1.

Interpretation and Conclusions. A link between erythropoietic growth factor signaling and RPS19 has been identified for the first time.

Key words: Diamond-Blackfan anemia, ribosomal protein S19, PIM-1, erythropoiesis

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Diamond Blackfan anemia (DBA) (MIM 205900) is a congenital disease characterized by defective erythroid progenitor maturation.[1-4] Most cases are sporadic, though a dominant or, more rarely, a recessive inheritance is observed in 10% of patients. The main clinical sign is profound isolated normochromic or macrocytic anemia, with normal numbers and function of the other hematopoietic cells. Defective erythropoiesis is revealed in the bone marrow by a very low number of erythropoietic precursors, and functionally by a reduction of burst-forming unit-erythroid (BFU-E) progenitor cells.[5] DBA patients have high levels of erythropoietin, irrespective of the degree of their anemia. The failure of their hematopoietic progenitors to respond to erythropoietin, both *in vitro* and *in vivo*, suggests erythropoietin insensitivity, and a defect in the erythropoietin-receptor (EPOR) pathway has thus been widely accepted as an explanation of defective erythropoiesis.[6] This has also been suggested by the fact that phenotype reversal can be induced *in vitro* by the addition of stem cell factor (which uses a different transduction pathway from erythropoietin) to interleukin-3 and erythropoietin to CD34$^+$ bone marrow cells from DBA patients.[5,7,8] However, *EPOR* and other genes encoding for erythropoietic growth factors have been ruled out as potential candidates.[9,10,11]

More than 50% of patients respond to steroid therapy, though the mechanisms involved are unknown.[4] Options in steroid-resistant patients are chronic red cell transfusions or allogeneic stem cell transplantation.[12] A number of patients experience spontaneous remission, irrespective of the type of treatment.[4] Patients with DBA also show an increased risk of malignancies.[1] One-third of patients have malformations, usually involving the upper limbs, head, the urogenital or cardiovascular system, and short stature.

One gene on chromosome 19q13.2, encoding ribosomal protein (RP) S19,

accounts for 25% of patients with either the dominant or the sporadic form of DBA.[13,14,4] A second DBA locus has been identified on human chromosome 8p22-p23 by linkage analysis;[15] however, the lack of linkage of DBA to either the 8p or the 19q critical regions in some families suggests that other genes are involved. DBA is the first human disease known to be caused by mutations in a ribosomal structural protein. The malformations and short stature occurring in DBA could be the consequence of defective protein synthesis during embryogenesis. Interestingly, *Drosophila minute* phenotypes, characterized by delayed larval development, diminished viability, reduced body size, diminished fertility and thin bristles, are due to *RP* mutations.[16,17]

The link between defective erythropoiesis and *RPS19* is still unclear. The finding that most *RPS19* mutations completely suppress the expression of the allele has suggested that haploinsufficiency is the main cause of abnormal erythropoiesis in DBA patients.[18,19] However, some patients carry missense mutations in the *RPS19* gene. Deficient nucleolar localization, which may lead to abnormal ribosome incorporation, has been found for three missense mutants,[20,21] and hence this may not be a univocal disease mechanism.

RPS19 expression is greater in the early stages of erythropoiesis, which are characterized by intense proliferation, than in the late stages, characterized by maturation of erythroid precursors.[22] The role of *RPS19* is also shown by the increase of BFU-E formation after overexpression of transfected oncoretroviral vectors containing the wild-type cDNA in $CD34^+$ bone marrow cells from DBA patients[23] and by the decrease of *in vitro* erythropoiesis when *RPS19* expression is impaired by RNA interference.[24] Like other structural ribosomal proteins in humans and other organisms,[25] RPS19 may have extraribosomal functions. Free RPS19 interacts with fibroblast growth factor 2 *in vitro* and the RPS19 dimer released by apoptotic cells induces monocyte chemotaxis.[26,27]

We used yeast two-hybrid screening[28] and a human liver cDNA library obtained at 19-24 weeks of gestation, when hepatic erythropoiesis is efficient, to search for proteins that interact with RPS19.

Design and Methods

Yeast two-hybrid screen

The human *RPS19* cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) from peripheral blood leukocytes using the primers 5'-GTGAATTCATGCCTGGAGTTACTGTAAAAG-3' and 5'- GTCTCGAGCCAGCATGGTTTGTTC-TAATG-3' (GenBank database accession nNM_001022). The products were digested with EcoRI and XhoI and inserted into the pLexA vector (CLONTECH Laboratories, Inc.). The expression library was the Human Fetal Liver MATCHMAKER LexA cDNA Library (Clontech) obtained from normal, whole livers pooled from 32 male or female Caucasian fetuses spontaneously aborted at 19-24 weeks of gestation. Plasmids were introduced into the yeast strain EGY48[p8op-lacZ] and interacting proteins were double-selected for growth on His/Leu/Trp/Ura-deficient plates and β-galactosidase production. Interactions were confirmed by transforming yeast cells with DNA from isolated clones. DNA sequencing was carried out on an automated Applied Biosystem apparatus (Applied Biosystems, Foster City, CA, USA).

Analysis of interactions between wild-type and mutant RPS19 and PIM-1 in yeast

Competent cells of EGY48 [p8op-lacZ] were prepared using the lithium acetate method.[29] Transformations were performed using heat shock treatment described in the Matchmaker Library Protocol (Clontech). The EGY48 [p8op-lacZ] yeast strain was transformed with 2 μg of pB42 *PIM-1* to obtain EGY48 PIM-1, and yeast transformants were plated on SD/-Ura/-Trp and incubated for 2 days at 30°C. cDNA corresponding to natural mutants found in DBA (R56Q, R62W, R101H, and the in-frame insertion 53_54insAGA) were inserted into the pLexA vector.

A single colony of EGY48*PIM-1* was inoculated in SD/-Ura/-Trp and grown overnight at 30°C with agitation. For each transformation 1 mL of EGY48*PIM-1*competent cells was mixed with 2 μg of DNA corresponding to wild-type RPS19 and its natural mutants. Yeast transformants were plated on SD/-Ura/-His/-Trp and incubated for 3 days at 30°C. Interactions between wild-type and mutant RPS19 with PIM-1 were qualitatively analyzed by streaking single colonies on SD/-Ura/-Trp/-His/Gal/Raf/X-Gal for 4 days to check for the activation of the lacZ and Leu reporter genes (*data not shown*).

The interactions were quantified by measuring the β-galactosidase activity in solution with yeast β-galactosidase assay kit (75768; Pierce Chemical) at room temperature, with O-nitrophenyl β-D-galactopyranoside as the substrate. Briefly, single colonies of yeast co-transformed with *PIM-1* and natural *RPS19* mutants were used to inoculate 5 mL of –Ura/-Trp/-His/Gal/Raf liquid media and were allowed to grow to mid-log phase overnight at 30°C. The OD_{600} was measured and 350 μL of each cell culture were mixed with 150 μL of yeast protein extraction reagent and with 150 μL of 2X β-galactosidase assay buffer. The reactions were incubated at room temperature until a color change was observed and stopped by adding 300 μL of 2X β-galactosidase assay stop solution. The total reaction time was recorded. The cell debris was pelleted and the

OD_{420} of the supernatants was measured. The β-galactosidase units were calculated using the formula $U=(1000\times OD_{420})/(t \times v \times OD_{600})$, where v = volume of culture used in the assay in milliliters, and t = time of assay in minutes. All assays were performed in parallel with three colonies from each transformation. Each experiment was performed in triplicate. The results were then averaged. Each interaction was also evaluated by pooling six colonies from each transformation. The statistical analysis was performed using Wilcoxon's signed rank test for paired data.

Plasmids and expression vectors

RPS19 expression plasmids were constructed by inserting RT-PCR products into pcDNA3 (Invitrogen, Milan, Italy) downstream from the sequence coding for the FLAG-tag (pFLAG-RPS) or into pGEX4.T1 (Amersham Pharmacia, pGST-RPS). The natural *RPS19* mutants R56Q, R62W, R101H, and an in-frame insertion (53_54insAGA, which is expected to insert an arginine after residue 18) were prepared by RT-PCR from peripheral blood lymphocytes of DBA patients after informed consent or by PCR-dependent mutagenesis. The mutation nomenclature used is that described by den Dunnen and Antonarakis.[30] The full-length *PIM-1* cDNA was obtained by RT-PCR from HeLa cells using the following primers: 5'-CCGGAATTCCCTCTT-GTCCAAAATCAACTCGCT-3' and 5'-CCGCTC-GAGCTATTTGCTGGGCCCCGGCG-3' (GenBank database accession nNM_002648). The PCR fragment was digested with EcoRI and XhoI and inserted into pGEX-4T.1 (pGST-PIM). All constructs were sequenced.

Cell lines and transfections

Human embryonic kidney 293T cells (ATCC #CRL-11268) were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. For DNA transfections, 3×10^6 cells were plated in 90-mm diameter dishes and transfected with the Lipofectamine 2000 kit (Invitrogen, Milan, Italy) according to the manufacturer's intructions. After 48 hr, the cells were harvested in ice-cold AKT buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl,1% Triton X-100,10% glycerol, 0.5 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin) for 20 min on ice and sonicated three times. Cell debris was removed by centrifugation and cleared lysates were analyzed further. Human erythroleukemia K562 cells (ATCC#CCL-243) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C with 5%CO_2.

Antibodies and co-immunoprecipitations

Anti-RPS19 sera were prepared (Sigma Genosys, Cambridge, UK) against the RPS19-derived peptide, LDRIAGQVAAANKKH, by injections in a rabbit. After four immunizations, antibody was purified on peptide affinity columns. Antibody activity was tested by immunoblotting with recombinant RPS19.

Antibodies specific for PIM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the hemagglutinin (HA) or the FLAG tags (Sigma, St. Louis, MO, USA) were used as directed by the manufacturers. Immunoblots were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence according to the supplier's instructions (Amersham, Arlington Heights, IL, USA).

Cleared lysates from co-transfected cells (5 mg total proteins) were pre-absorbed to protein-G beads (Amersham, Arlington, Heights, IL, USA) for 1 hour at 4°C. Supernatants were first incubated with anti-FLAG antibody (5 μg) for 16 hours at 4°C on a rocker, and then with new protein-G beads for 1 hour at 4°C. Immunocomplexes were recovered by centrifugation at 3000 rpm for 1 min, washed four times with 1 mL of AKT buffer, loaded onto a 12% polyacrylamide SDS-gel and analyzed by immunoblotting.

GST-fusion proteins and in vitro binding assay (GST pull-down assay)

Recombinant GST-PIM-1 fusion protein was produced in *E. coli* cells (strain JM109) and bound to glutathione-Sepharose 4B resin (Sigma St. Louis, MO, USA) as previously described.[31] For pull-down assays, lysates (5 mg of total protein) from 293T cells transfected with the indicated *RPS19* contructs, were incubated with 50 μL of GST-PIM-1 affinity resin (~100 μg of recombinant protein) in AKT buffer for 16 hr at 4°C on a rocker. The GST-RPS19 wild-type or mutant fusion proteins were similarly produced in *E. coli* cells (strain BL-21) and used to prepare affinity resins. For pull-down assays, K562 cell lysates (5mg of total protein) were incubated with GST-RPS19 affinity resins as described above. Bound proteins were washed with TM 0.1 buffer (50 mM Tris-HCl, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), eluted with TM 0.5 buffer (50 mM Tris-HCl, pH 7.9, 500 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), and precipitated with 10% trichloracetic acid. The pellet was washed twice with acetone, resuspended in SDS-PAGE loading buffer (63 mM Tris-HCl pH 6.8, 5% glycerol, 1% SDS, 2.5% bromophenol-blue), resolved by SDS-PAGE and subjected to immunoblotting with a monoclonal anti-PIM-1 antibody (Santa Cruz Biotechnology).

Kinase assay

To determine whether PIM-1 was able to phosphorylate RPS19, GST fusion proteins were individually affinity purified and eluted from the resins; next, 2.5 μg

of GST-PIM-1 (20 μL of bead slurry) were washed twice in kinase buffer (20 mM PIPES, pH 7.0, 5 mM $MnCl_2$, 7 mM β-mercaptoethanol) and then resuspended in kinase buffer and mixed with 10 μg of GST-RPS19. The reaction was started by adding 10 mM ATP and 10 μCi of γ-^{32}P-ATP. Histone H1, an effective substrate for a number of serine/threonine kinases (Upstate #14155), was used as a positive control, whereas GST alone was used as a negative control. Reactions were incubated at 30°C for 30 min, boiled in SDS-PAGE loading buffer, resolved on an SDS gel, and subsequently analyzed by autoradiography.

Sucrose gradient fractionation

Human embryonic kidney 293T cells were lysed in lysis buffer [10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1% sodium deoxycholate, aprotinin 1 μg/mL, leupeptin 1 μg/mL, pepstatin A 1 μg/mL, PMSF 100 μg/mL]. After incubation on ice for 1 min, the extract was centrifuged for 1 min in a cold centrifuge and the supernatant was frozen in liquid nitrogen or loaded directly onto a 5%-65% linear sucrose gradient containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$ and centrifuged in a Beckman SW 41 rotor for 3 hr at 37,000 rpm. Twelve fractions were collected while monitoring the absorbance at 260 nm. Proteins from each fraction were precipitated with 10% trichloracetic acid. The pellet was washed with acetone, dried, and resuspended in SDS-PAGE loading buffer (63 mM Tris-HCl pH 6.8, 5% glycerol, 1% SDS, 2.5% bromophenol-blue) . The first five fractions (polysomes) were pooled and loaded entirely on a single lane, whereas only 1/10 of fraction 11 and 12 was loaded on the gel.

The proteins were separated on 12% SDS polyacrylamide gel, transferred onto a polyvinilydene fluoride membrane and incubated with either rabbit anti-RPS19, or a monoclonal anti-PIM-1 antibody (Santa Cruz Biotechnology) or rabbit anti-S6K1 (Santa Cruz Biotechnology, Sc-230). Immunoblots were detected with SuperSignal reagent (Pierce).

Screening for mutations of PIM-1 in DBA patients

PIM-1 gene mutations were sought in 116 DBA patients: 17 patients carried mutations in *RPS19* and 99 did not. Patients with these mutations were studied to ascertain whether *PIM-1* is a modifier gene. Gene analysis was performed on genomic DNA isolated from peripheral blood leukocytes with standard techniques. Coding sequences and intron-exon boundaries were amplified by PCR and sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Restriction digestion of the specific PCR product was used to trace segregation of the identified DNA change in a mutated family and test 50 unrelated normal individuals. Genetic analyses were performed on patients and controls after informed consent had been given.

Results

RPS19 interacts with PIM-1

RPS19 gene mutations account for 25% of DBA cases. Even so, the correlation between *RPS19* functions and normal erythropoiesis is still unknown. We employed yeast two-hybrid screening to identify cellular proteins that interact with RPS19 and were thus likely to link it to pathways involved in the regulation of erythropoiesis. We used a full-length cDNA for *RPS19* to screen a cDNA library of human embryonic liver cells obtained at 19-24 weeks of gestation, when hepatic erythropoiesis is active. Approximately 10^7 yeast transformants, co-expressing the pLexA-*RPS19* bait and the cDNA clones, were tested in the two-hybrid assay and 38 clones that activated two separate reporter genes (β-galactosidase and leucine) in an *RPS19*-dependent fashion were recovered. Four independent clones (a-d) harbored in-frame fragments coding for PIM-1 proteins lacking different N-terminal portions. The most extended clone, designated *PIM-a*, encoded a PIM-1 protein lacking the first 47 codons. To rule out the possibility that the PIM-1/RPS19 interaction was an artifact due to the absence of this N-terminal sequence, we constructed a *PIM-1* full-length cDNA and confirmed the interaction in yeast cells (*data not shown*).

PIM-1 interacts with RPS19 in vitro and in human cells and phosphorylates it

To rule out the possibility that the PIM-1/RPS19 interaction could be mediated by yeast proteins, we performed an *in vitro* pull-down assay. GST-PIM-1 affinity resin was incubated with lysates from human embryonic kidney 293T cells transfected with pFLAG-RPS19 plasmid DNA. Bound proteins were separated by SDS-PAGE and analyzed by western blot using an anti-FLAG antibody. As shown in Figure 1A, tagged RPS19 protein was specifically retained by the GST-PIM resin. A co-immunoprecipitation assay was used to confirm the PIM-1/RPS19 interaction in living human cells. 293T cells were co-transfected with pFLAG-RPS19 and pHA-PIM DNA, and cell lysates were immunoprecipitated with an anti-FLAG antibody. Immunocomplexes were resolved by SDS-PAGE and analyzed by western blot using an anti-HA antibody. As shown in Figure 1B, a band corresponding to the expected HA-PIM-1 protein was exclusively revealed in lysates from cells co-transfected with pFLAG-RPS19 (Figure 1B, right panel).These data demonstrate that HA-tagged PIM-1 interacts with FLAG-RPS19.

Many data show that K562 cells are erythroid-

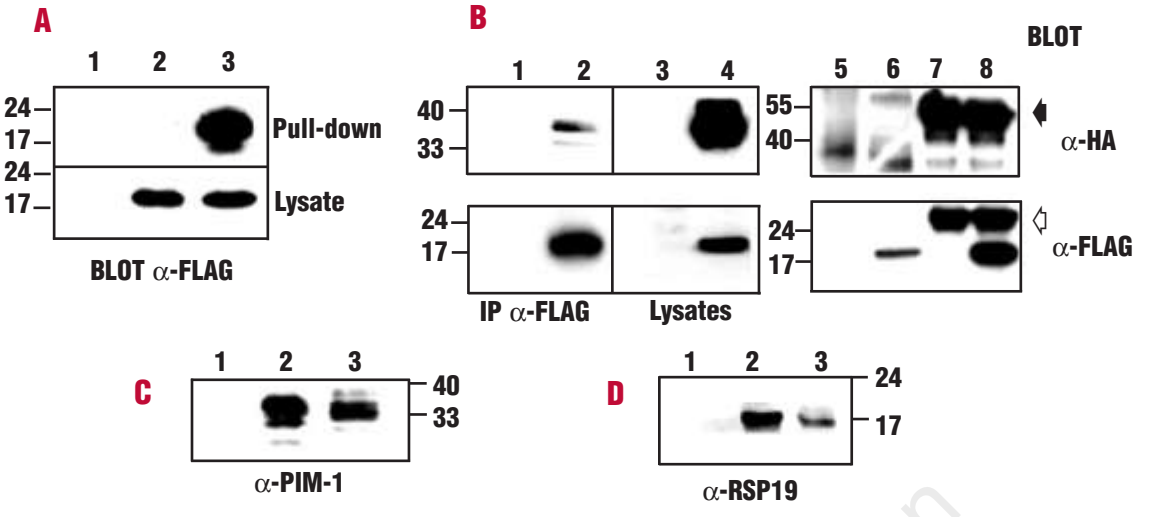

Figure 1. PIM-1 binds RPS19. A. 293T cells were transiently transfected with pcDNA3.1 empty vector (lane 1) or pFLAG-RPS19 construct (lanes 2 and 3) and cell lysates were incubated with either GST (lane 2) or GST-PIM-1 affinity resin (lanes 1 and 3). The bound proteins (upper panel) and total cell lysates (lower panel) were immunoblotted using an anti-FLAG antibody. B. 293T cells were transiently transfected with pcDNA3.1 empty vector (lanes 1, 3) or pFLAG-RPS19 plus pHA-PIM-1 (lanes 2, 4). Cell lysates were immunoprecipitated (left panel) with the anti-FLAG antibody and the immunocomplexes were immunoblotted with the anti-HA or anti-FLAG antibodies. Total lysates are shown in the middle panel. In the right panel 293T cells were transfected with empty vector (lanes 5, 7) or with pFLAG-RPS19 (lanes 6, 8); lysates were immunoprecipitated with anti-FLAG antibody (lanes 7, 8) and all samples were immunoblotted with anti-HA antibody (upper panel) or anti-FLAG antibody (lower panel). C and D. Lysates from human erythroleukemia K562 cells were incubated with either GST alone (C and D: lane 1) or GST-RPS19 (C: lane 2) or GST-PIM-1 affinity resins (D: lane 2). Total cell lysates (C and D: lane 3) were immunoblotted using the anti-PIM-1 antibody (C) or the anti-RPS19 antibody (D). Bold and empty head arrows indicate immunoglobulin heavy and light chains, respectively. Molecular weights are shown.

derived cells capable of hemoglobinization when exposed to erythropoietin.[32] Therefore, we also verified the RPS19/PIM-1 interaction in these cells, which represented a more pertinent model than embryonic kidney 293T cells. A pull-down assay was performed on K562 whole cell extracts with a GST-RPS19 affinity resin, and the bound proteins were analyzed by immunoblotting with an anti-PIM-1 antibody (Figure 1C). To ascertain whether the endogenous RPS19 was able to interact with PIM-1, we also performed the reverse pull-down assay using the GST-PIM-1 affinity resin; in this case the bound proteins were analyzed by immunoblotting with a RPS19 polyclonal antibody (Figure 1D). Taken together, these results confirm that RPS19 and PIM-1 interact each other, and suggest that they form a stable complex in living cells. Since PIM-1 is a serine/threonine kinase, we evaluated whether RPS19 is phosphorylated by PIM-1 in an *in vitro* kinase assay. The results shown in Figure 2 demonstrate that RPS19 is a substrate for PIM-1 activity *in vitro* and suggest that their interaction may be of functional significance.

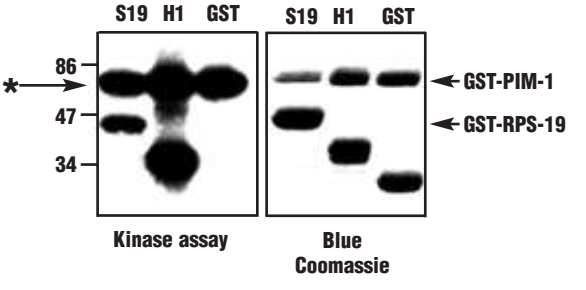

Figure 2. PIM-1 phosphorylates RPS19. GST-RPS19 and GST-PIM-1 were expressed in *E. coli* and affinity purified. Eluted GST-PIM-1 protein was used in the kinase assay, using as substrate either GST-RPS19 or histone-1 (positive control) or GST proteins (negative control). Phosphorylated proteins were separated by 10% SDS-PAGE and visualized by autoradiography (left panel). Coomassie staining (right panel) shows the proportion of recombinant protein used in the kinase assay. Data are representative of three independent experiments. *Autophosphorylation.

DBA natural mutants alter the binding with PIM-1

Various kinds of RPS19 mutations are found in DBA patients and many completely suppress the expression of the allele.[14,18,19] To determine whether DBA-associated mutations affect the binding of RPS19 to PIM-1, we analyzed the DBA missense mutants R56Q, R62W, R101H and an in-frame insertion, 53_54insAGA, by pull-down experiments. Lysates from 293T cells transfected with wild-type *RPS19* or mutant cDNAs were incubated with the GST-PIM-1 affinity resin and bound proteins analyzed by immunoblotting. The results, representative of several independent experiments, are shown in Figure 3A. All the mutant proteins bind to PIM-1, but with different strengths. In particular, whereas R101H showed reduced binding, R62W and R56Q showed increased binding, as compared to the

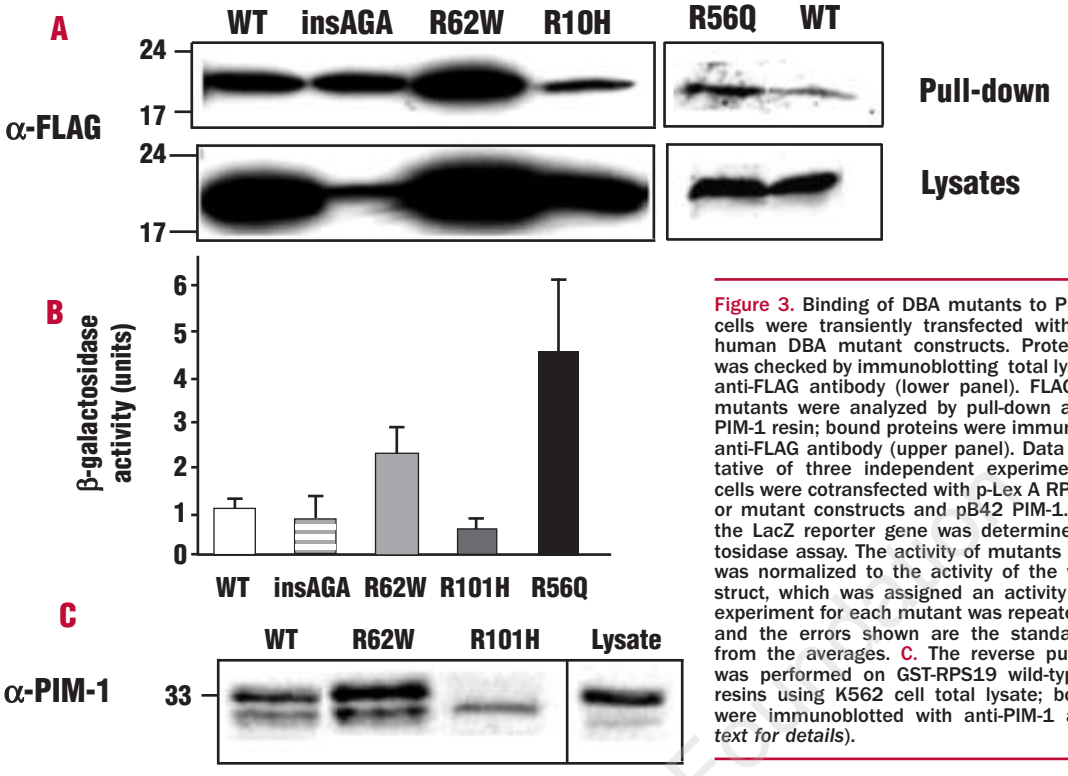

Figure 3. Binding of DBA mutants to PIM-1. **A.** 293T cells were transiently transfected with wild-type or human DBA mutant constructs. Protein expression was checked by immunoblotting total lysates with the anti-FLAG antibody (lower panel). FLAG-RPS19 DBA mutants were analyzed by pull-down assay on GST-PIM-1 resin; bound proteins were immunoblotted with anti-FLAG antibody (upper panel). Data are representative of three independent experiments. **B.** Yeast cells were cotransfected with p-Lex A RPS19 wild-type or mutant constructs and pB42 PIM-1. Activation of the LacZ reporter gene was determined by β-galactosidase assay. The activity of mutants in each assay was normalized to the activity of the wild-type construct, which was assigned an activity of 1.0. Each experiment for each mutant was repeated nine times, and the errors shown are the standard deviations from the averages. **C.** The reverse pull-down assay was performed on GST-RPS19 wild-type or mutant resins using K562 cell total lysate; bound proteins were immunoblotted with anti-PIM-1 antibody (*see text for details*).

wild-type protein. We also employed a semi-quantitative yeast β-galactosidase assay to evaluate these differences, as reported by other authors with other interactors.[33] The results (Figure 3B) show that the R101H mutant significantly reduced the interaction ($p<0.01$), whereas the R56Q and R62W mutants increased it ($p<0.01$). This behavior was shown in many independent experiments and was not due to differences in protein expression in yeast cells, as checked by western blot analysis (*data not shown*). Nevertheless, to exclude the possibility that stoichiometric differences among the RPS19 proteins could have affected their binding to PIM-1, we performed reverse pull-down assays using either the GST-RPS19 wild-type or R62W or R101H affinity resins. Equal amounts of K562 whole cell extract were incubated with equivalent amounts of GST-RPS19 affinity resins and bound endogenous PIM-1 was detected by immunoblotting with anti-PIM-1 antiserum (Figure 3C). In these conditions, too, compared to the wild-type protein, R101H showed reduced binding, whereas R62W showed increased binding. The results confirmed those observed in previous experiments and illustrate differences in the ability of the RPS19 proteins to interact with PIM-1.

At this stage, we do not know whether the mutations affect the on/off rate of the interaction because of structural differences or altered intracellular distribution of RPS19 proteins. Further analyses are needed to ascertain the functional role of these differences.

PIM-1 interacts with polysomes

To check whether RPS19 and PIM-1 interact on the ribosome, we analyzed the sedimentation profile of cytoplasmic extracts from 293T cells. Extracts were fractionated on a linear sucrose gradient to separate polysomes (fractions 1-5), 80S ribosomes, 60S and 40S ribosomal subunits and ribosome-free cytosol (as indicated in Figure 4A). Collected fractions were subjected to SDS-PAGE and immunoblot analysis with specific antibodies. Preliminary experiments on HEK 293 cells transiently transfected with pHA-PIM-1 indicated that at least part of the tagged PIM-1 was associated with polysomes and 40S ribosomal subunit (*not shown*).

We then repeated the analysis on untransfected cells with antibodies against PIM-1, RPS19, and a known cytosolic serine-threonine kinase, S6K1,[34] which phosphorylates another ribosomal protein, RPS6. The results (Figure 4B) indicate that S6K1 sediments in cytosolic ribosome-free fractions whereas RPS19 is distributed in fractions 1 to 9 (polysomes to 40S subunit), as expected. Interestingly, most PIM-1 distributes together with polysomes, as well as in 80S and 40S fractions, demonstrating that the interaction with RPS19 occurs on ribosomal particles. A lower molecular weight band recognized by anti-PIM-1 antibodies was visible in the cytosolic ribosome-free fractions. This could be a PIM-1 isoform reported by other authors in the nucleus and the cytoplasm.[35,36] However it constitutes a small fraction of the total cytoplasmic PIM-1. Therefore our results are

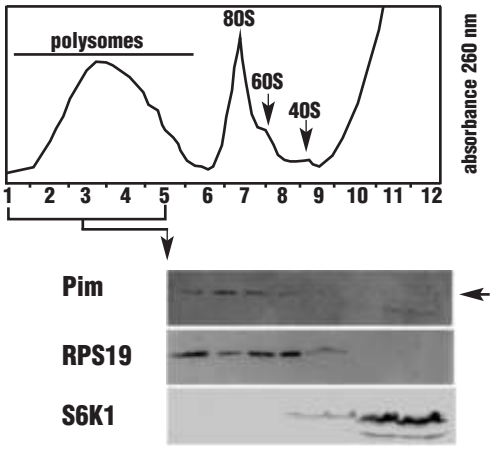

Figure 4. PIM-1 associates with ribosomes. A. Cytoplasmic extracts from 293T cells were fractionated onto a 5-65% sucrose gradient. The absorbance profile and the polysomal and ribosomal fractions are indicated. B. Fraction aliquots were precipitated by trichloroacetic acid and analyzed by western blot using antibodies against PIM-1, RPS19, and S6 kinase-1. Only 1/10 of fractions 11 and 12 was analyzed. Arrow indicates a slower migrating PIM-1 isoform (*see text*).

consistent with the possibility that the RPS19-PIM-1 reaction in the cytoplasm takes place on translationally active ribosomes. However, since both RPS19 and PIM-1 are also located in the nucleus, these data cannot rule out the possibility that the interaction takes place in the nucleus, as well.

Mutation screening of PIM-1 in DBA patients

To check whether *PIM-1* is a candidate gene for DBA forms not due to *RPS19* mutations, we performed mutational screening in 99 DBA patients and identified two missense mutations: P311T (C→A) and C17Y (G→A). These mutations were not found in 50 normal controls.

The patient heterozygous for P311T has an intra-atrial defect (ostium secundum type), was diagnosed in infancy, is transfusion-dependent and a steroid non-responder. Her reportedly healthy parents are not available for mutation analyses. We, therefore, analyzed the mutant's ability to bind RPS19 and to phosphorylate either RPS19 or histone-1, in pull-down or *in vitro* phosphorylation assays, respectively. These abilities were retained by the mutant PIM-1 (*data not shown*).

Mutation C17Y was found in a family which included two DBA patients: the mother and one of her two daughters. The affected mother is very short (<2SD) but without malformations. She was transfusion-dependent as a child and is now steroid-dependent. The affected daughter was anemic in infancy and responded to prednisone. After a few years, her hemoglobin levels normalized and she is now in complete remission. Her sister and father have always had normal hemoglobin levels. The C17Y mutation was carried by the affected daughter and her healthy father (both were heterozygotes); thus it does not segregate with the disease. Since there is a definite phenotypic variation between the mother and daughter, it is possible that C17Y is a phenotype modifier and reduces the severity of the disease. However, we did not observe a difference in binding strength to RPS19 between the C17Y mutant and wild-type protein in a pull-down assay (*data not shown*). We also searched for mutations in 17 patients who carried a mutation in RPS19 to test the hypothesis of *PIM-1* being a *modifier gene*, i.e. one capable of modifying the DBA phenotype and thus explaining the variable expressivity and/or incomplete penetrance of this disease. No mutations were identified in these patients.

Discussion

DBA is rare, but is important in hematology as a paradigm of an intrinsic genetic disorder of the committed erythroid progenitor. Many of its clinical and pathogenic aspects are still unclear. Mutations in the *RPS19* gene account for about 25% of cases.[4] DBA is the first and so far the only human disease known to be due to a structural ribosomal protein defect. However, the link between *RPS19* and erythropoiesis remains to be clarified. A generic deficiency of protein synthesis or a defect of a distinct, so far unknown, physiologic function of *RPS19* have been proposed.[13] These hypotheses are not mutually exclusive.

We employed a yeast two-hybrid screen to look for cellular partners that could link *RPS19* to biological pathways involved in erythropoiesis. Screening of a human fetal liver cDNA library identified the PIM-1 oncoprotein as a prominent RPS19 interactor. Our data also show that RPS19 interacts with PIM-1 both *in vitro* and in living cells, including the erythroleukemia-derived K562 cell line. *In vitro* this interaction leads to RPS19 phosphorylation. The functional role of the interaction in living cells is also suggested by the observation that in human 293T cells PIM-1 interacts with polysomes, the 80S and the small ribosomal subunit.

The proto-oncogene *pim-1* (MIM164960) was first isolated in mouse T-cell lymphomas as a preferred integration site of the Moloney leukemia virus.[37] It encodes for a serine/threonine kinase with autophosphorylating activity and a very short half-life.[31,38] PIM-1 is ubiquitously expressed in mammalian tissues, but is strongly induced as an early response gene by cytokines having hemopoietin-type receptors,[39-43] such as erythropoietin.[44,45] Ligand-mediated activation of these receptors rapidly activates one or more members of the Jak family of receptor-associated tyrosine kinases. Once acti-

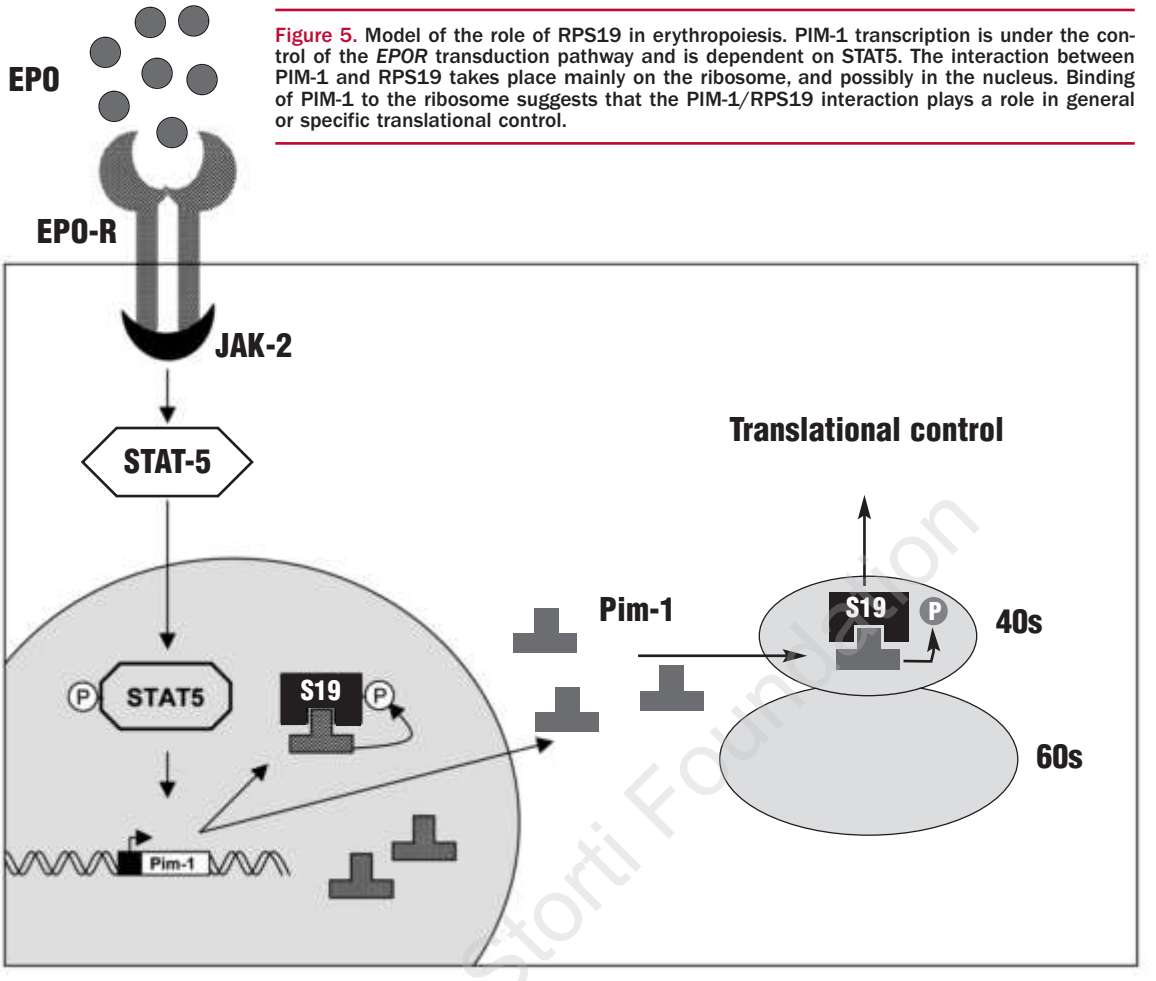

Figure 5. Model of the role of RPS19 in erythropoiesis. PIM-1 transcription is under the control of the *EPOR* transduction pathway and is dependent on STAT5. The interaction between PIM-1 and RPS19 takes place mainly on the ribosome, and possibly in the nucleus. Binding of PIM-1 to the ribosome suggests that the PIM-1/RPS19 interaction plays a role in general or specific translational control.

vated, these kinases phosphorylate members of the signal transducers and activators of transcription (STAT) family of transcription factors. Activated STAT dimerize, translocate to the nucleus and bind specifically to promoter regions of responsive genes. In the erythroid lineage, PIM-1 expression is correlated with the JAK2/STAT5-mediated mitogenic response to erythropoietin.[45] Since it is rapidly induced after cytokine stimulation, PIM-1 has been thought to play a significant role in the transduction of mitogenic signals from cytokines. In agreement with this hypothesis, the proliferative response to cytokines, in particular to interleukin-3, is impaired in *pim-1* null mice.[46] Confocal microscopy has revealed dynamic redistribution of *pim-1* during the cell cycle: it moves from the nucleus to the cytoplasm in interphase.[47] PIM-1 has also been associated with protection of hematopoietic cells from apoptosis induced by genotoxic stress or growth factor withdrawal.[48] Its role in erythropoiesis is also suggested by the observation that mice defective for *pim-1* have smaller red cells, whereas those hyperexpressing it display macrocytosis.[49,50] Its effect on erythropoiesis is supposed to be redundant in mice, since *pim-1*$^{-/-}$ mice show a mild phenotype.[49] On the other hand, hyperexpression of *pim-1* is involved in the development of myeloid leukemias and lymphomas.[50-53] Its effect on proliferation and on apoptosis protection are probably involved in tumorigenesis.[54] These effects are mediated by interactions with multiple substrates, as for other serine-threonine kinases.[35,36,55-64]

We have shown that RPS19 is another interactor for PIM-1. *In vitro* this interaction leads to RPS19 phosphorylation. Several other ribosomal proteins, such as RPS6 and RPL13, undergo phosphorylation by serine-threonine kinases.[65,66] Phosphorylation of RPS6 is considered to have a role in cell cycle control, whereas RPL13 phosphorylation is involved in a specific translational control. The binding of PIM-1 to the ribosome suggests that the PIM-1/RPS19 interaction plays a role in general or specific translational control (Figure 5).

We do not know whether the PIM-1/RPS19 interaction also takes place in the nucleus. However, the fact that in our assay a cytosolic protein which phosphorylates RPS6 (S6K1) was not detected in the ribosomal fractions indicates that the PIM-1/RPS19 association is more stable than a simple kinase-target interaction. The question of the place of *PIM-1* in the pathogenesis of DBA was investigated by determining whether the

PIM-1/RPS19 interaction is altered by RPS19 DBA mutants, and by looking for PIM-1 mutations in DBA patients. To ascertain whether four DBA mutants altered the PIM-1/RPS19 interaction, we used both a pull-down and a yeast β-galactosidase assay. Three mutants (R101H, R62W, and R56Q) showed a altered binding (Figure 3).

Reduced or increased binding may be supposed to disrupt the finely-tuned regulation of the many activities of PIM-1. R101H and *null RPS19* mutations would both impair its possible role in translational control and leave PIM-1 available for other interactions. Since DBA patients are at risk of malignancies,[1] this could be due to the increased availability of the oncoprotein PIM-1. By contrast, mutations that increase the strength of the interaction (R62W, R56Q) might sequester both PIM-1 and RPS19 and make them less available for their other functions, including ribosome biogenesis. Both hypotheses need to be assessed with other experiments. Lastly, mutational screening of *PIM-1* in 116 DBA patients led to the identification of two missense mutations. These do not seem to alter the interaction with RPS19 dramatically. These mutations do not affect important PIM-1 domains.[67,68] However, since they were not found in 50 normal controls, they are not common polymorphisms. Even if our data show that *PIM-1* is not a major DBA gene, its missense mutations may subtly modulate the phenotype in those patients who carry them.

As to the pathogenesis of DBA, our data cannot be used to discriminate between the two hypotheses which consider DBA as a ribosome biogenesis defect or due to the loss of a second function for *RPS19*. It is likely that both mechanisms co-operate to induce the erythroid defect. However, our data show that the function implicated by PIM-1 binding is still connected with the ribosome and that RPS19 may have more than a merely structural role. In conclusion, our study demonstrates that RPS19 is a new interactor of PIM-1. A link between hematopoietic growth factor signaling and *RPS19* has been identified for the first time and may be involved in the pathogenesis of DBA.

ACh: performed most of the experiments reported in the paper; ID, CS: developed the project, designed the experimental procedures and coordinated them; all other authors participated, with the following specific contributions: LG helped ACh to prepare the necessary constructs, she also performed the mutant pull-down assays in 293T cells; ACa performed the yeast two-hybrid system, which identified PIM-1 as a RPS19 interactor; AA performed all the experiments in K562 cells; PS performed the β-galactosidase assay in yeast; EG, AB performed mutation detection analyses of PIM-1 in the 116 DBA patients; EG also performed mutation detection analysis of RPS19 on half of the DBA patients; FL and MA performed the sucrose gradient fractionation assay and the relative western blotting analyses; ND diagnosed the disease and performed mutation detection analysis of RPS19 on half of the DBA patients; UD participated in the design of the experimental work focused on the definition of protein-protein interactions; UR diagnosed DBA in half of the DBA patients, provided their clinical data, coordinated the mutation detection analyses. Figures 1,2,3, and 5 were prepared by ACh, figure 4 was prepared by FL. ID takes primary responsibility for the paper. The authors declare that they have no potential conflict of interest.

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