Enhanced calreticulin expression in red cells of polycythemia vera patients harboring the *JAK2*^{V617F} mutation

Polycythemia vera (PV) is a myeloproliferative neoplasm characterized by increased red cell mass and a high risk of thrombosis, considered as a major cause of mortality and morbidity in this disease. The vast majority of PV patients exhibit the V617F activating mutation in the tyrosine kinase JAK2.[1] Microarray and proteomic studies identified sets of deregulated genes and proteins in patients with myeloproliferative neoplasms but no such analyses have been performed with PV red blood cells. Considering the potential role of abnormal protein expression at the red cell surface in promoting abnormal cellular interactions in the circulation, we investigated protein expression at the membrane of erythrocytes from PV patients using a comparative proteomic approach. In this study, we show that PV red blood cells have an unexpectedly high expression of endoplasmic reticulum proteins at the membrane, including calnexin (CANX) and calreticulin (CALR), and present evidence that *JAK2*^{V617F} induces CALR overexpression. Our findings suggest altered protein expression during erythroid differentiation of *JAK2*^{V617F}-positive progenitors leading to circulating red blood cells with potential abnormalities of calcium homeostasis and surface properties.

Gene and protein expression in myeloproliferative neoplasms has been studied to reveal markers of clinical relevance in diagnosis, treatment and patient stratification. These studies were performed using granulocytes and CD34^{+} progenitors from patients with PV, essential thrombocythemia (ET) or primary myelofibrosis.[2-4] They identified sets of upregulated genes and proteins including the transcription factor NF-E2,[4] heat shock protein 70[3] and regulators of the MAPK and PI3K/AKT pathways.[2] Transcriptomic and phospho-proteomic analyses of erythroblasts expanded *in vitro* showed differences in gene expression and protein phosphorylation between those from normal donors and those from PV patients.[5] A number of these genes and proteins are involved in signaling pathways that regulate transcription factors such as GATA1. Based on these findings we hypothesized that differentially regulated genes during erythroid differentiation in PV are not restricted to those involved in cell proliferation and that PV erythrocytes may express a specific repertoire of proteins. This is supported by our findings showing overexpression and activation of the adhesion protein Lutheran/basal cell adhesion molecule (Lu/BCAM) on PV red blood cells.[6,7]

In this study we used a proteomic approach to compare differences in expression of proteins at the erythrocyte membrane between PV and normal red blood cells. The study included blood samples from 11 control subjects from the *Etablissement Français du Sang*, 20 PV (8 women and 12 men) and 34 ET (25 women and 9 men) patients treated with low dose aspirin; all PV patients were positive for *JAK2*^{V617F}.

We explored the expression of erythroid membrane proteins by performing a proteomic analysis of red blood cell ghosts from three PV patients and two age-matched controls (CT) using iTRAQ labeling technology.[8] Red blood cell ghosts were prepared by hypotonic lysis of washed cells as described elsewhere,[9] and peptides were prepared from 100 µg of total membrane proteins by trypsin digestion. Peptides from PV samples were labeled with 113, 115 and 117 iTRAQ reagents and peptides from CT samples with 118 and 121. After mixing, peptides were separated by isoelectric focalization and analyzed by liquid chromatography with tandem mass spectrophotometry using an Orbitrap Velos mass spectrometer. Around 400 proteins were identified and quantified. For each protein in each sample we calculated a PV/CT expression ratio and focused on those proteins with a ratio >1.3 in all three PV patients. Eleven proteins, including Lu/BCAM, had a ratio >1.3 (Table 1). The presence of Lu/BCAM in the group of overexpressed proteins confirmed our previous data obtained by flow cytometry with blood samples from 23 PV patients[7] and further validated the proteomic method used in this study. Surprisingly, we found that proteins from the endoplasmic reticulum were highly represented in the overex-

Table 1. iTRAQ ratios for proteins identified as potential biomarkers in PV red blood cell membranes. iTRAQ ratios for proteins upregulated in PV red blood cell membranes compared to iTRAQ ratios in two controls (CT). Proteins from the endoplasmic reticulum are in bold (1, 2, 3, 5, 8, 9 and 11).

			PV / CT						CT / CT
Protein	Gene	#	PV1	PV2	PV3	PV1	PV2	PV3	CT1 / CT2
			CT1			CT2			
Calnexin	**CANX**	**1**	**16.9**	**13.1**	**5.4**	**20.3**	**16.3**	**6.8**	**1.0**
Peroxiredoxin-4	**PRDX4**	**2**	**6.1**	**4.1**	**4.4**	**7.3**	**5.2**	**5.3**	**1.1**
Calreticulin	**CALR**	**3**	**6.0**	**5.9**	**5.0**	**5.9**	**6.2**	**5.5**	**1.0**
Lu/BCAM	BCAM	4	3.8	3.3	3.9	2.9	3.2	3.6	0.9
GRP78	**HSPA5**	**5**	**3.7**	**4.3**	**3.3**	**3.3**	**3.8**	**2.9**	**0.8**
Abhydrolase domain-containing protein 16A	ABHD16A	6	3.6	7.8	2.9	3.1	10.3	3.1	1.0
14-3-3 protein beta/alpha	YWHAB	7	2.7	1.6	2.2	3.1	1.8	2.5	1.1
Protein ERGIC-53	**LMAN1**	**8**	**2.2**	**2.1**	**1.8**	**3.0**	**2.7**	**2.1**	**1.1**
Neutral alpha-glucosidase AB	**GANAB**	**9**	**2.1**	**3.2**	**2.4**	**1.7**	**2.7**	**2.0**	**0.8**
Thioredoxin-related transmembrane protein 1	TMX1	10	1.7	1.9	1.7	2.0	2.2	2.0	1.1
Endoplasmic reticulum resident protein 44	**ERP44**	**11**	**1.4**	**1.3**	**1.5**	**1.4**	**1.3**	**1.5**	**1.0**

ratio

≥ 1.3	≥ 1.7	≥ 2.0	≥ 3.0

pressed proteins, with seven out of the 11 proteins with a ratio >1.3 being endoplasmic reticulum proteins. Among these proteins, we focused on CALR because it is involved in the pathophysiology of myeloproliferative neoplasms.[10]

To confirm CALR overexpression, we performed western blotting with 11 CT and 19 PV red blood cell ghosts (Figure 1A). CALR expression was variable among individuals of both groups and was significantly higher in PV red blood cells than in CT ones, with a 2-fold increase of the median in the PV group (Figure 1B). This result indicated that *JAK2*^{V617F} might affect erythroid protein expression, with a specific impact on endoplasmic reticulum proteins.

As several endoplasmic reticulum proteins were overexpressed in PV patients we checked whether this was due to a high reticulocyte count. We determined the percentage of reticulocytes by flow cytometry after labeling with thiazole orange dye and found no difference between the PV and the CT groups (Figure 1C). In addition, there was no correlation between this percentage and CALR expression values determined by western blotting (Figure 1D), indicating that abnormal CALR expression was independent of the reticulocyte count in PV patients and most probably takes place in both young and mature red blood cells. To further explore this conclusion we isolated reticulocytes from three PV blood

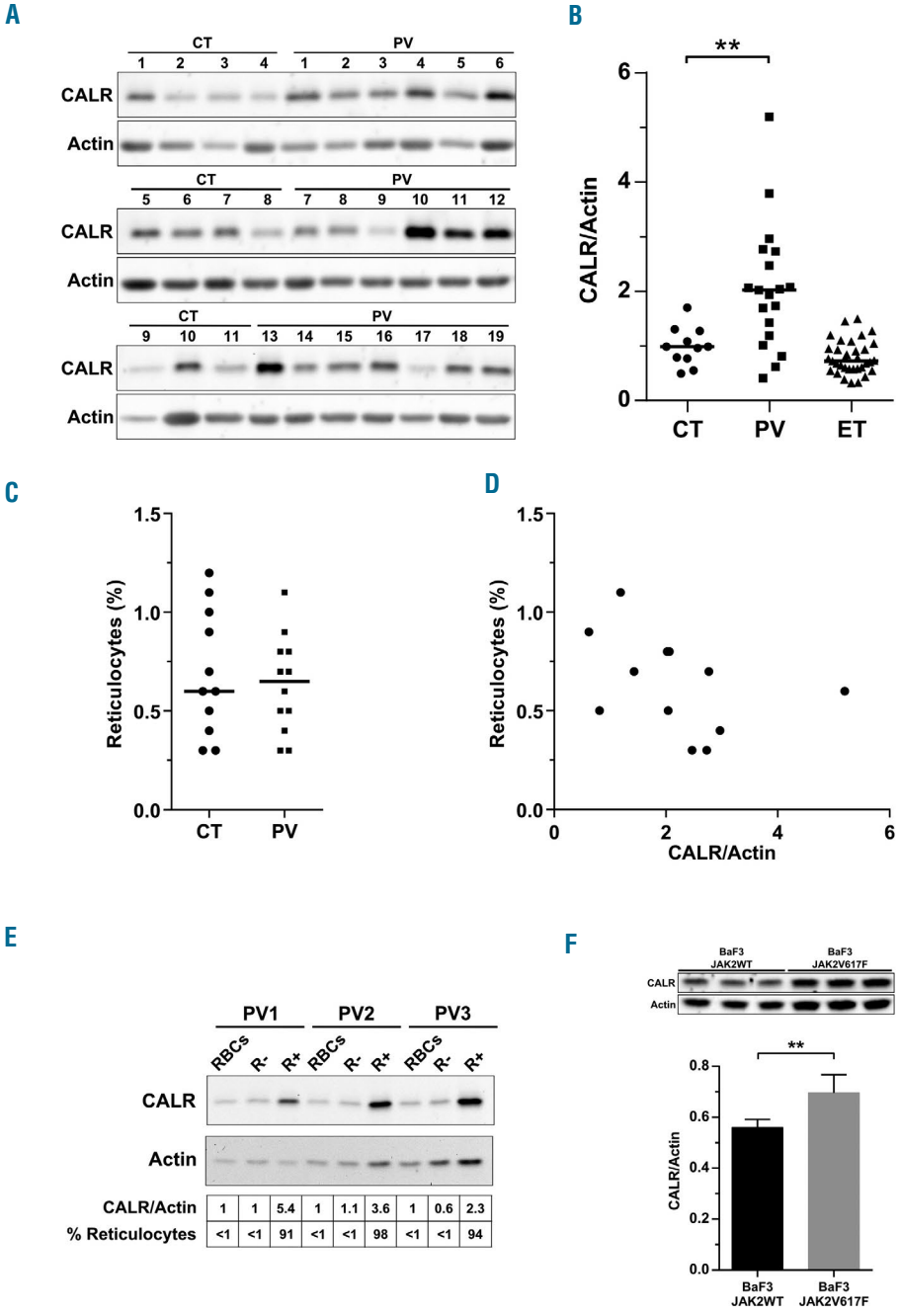

Figure 1. CALR expression in red blood cell membranes of patients with polycythemia vera and in BaF3 cell lines. (A) Western-blot analysis of CALR expression in control (CT, n=11) and PV (n=19) erythrocyte membranes. β-actin (Actin) is used as a loading control. (B) Quantification of CALR expression normalized by β-actin from the 11 CT and 19 PV patients in addition to 34 ET patients (see Figure 2). Horizontal lines represent medians (0.9883, 2.023 and 0.7290, respectively). Mann-Whitney test, ***P*=0.0024. (C) Percentage of reticulocytes in 11 CT and 12 PV red blood cell samples, as determined by flow cytometry using thiazole orange dye (medians=0.6 and 0.65, respectively; Mann-Whitney test *P*=0.7489). (D) Absence of correlation between the percentage of reticulocytes in PV red blood cells and CALR expression values (Spearman correlation r=-0.5247, *P*=0.0781). (E) Western-blot analysis of CALR expression in membranes obtained from total red blood cells (RBCs), and reticulocyte-depleted (R-) or reticulocyte-enriched (R+) fractions from three PV patients. The percentage of reticulocytes in the three populations was determined by flow cytometry using thiazole orange dye. (F) Upper panel: western-blot analysis of CALR expression in whole lysates of BaF3-*JAK2*^{WT} and -*JAK2*^{V617F} cell lines; a triplicate is shown for each cell line. Lower panel: quantification of CALR expression normalized by β-actin from BaF3-*JAK2*^{WT} and -*JAK2*^{V617F} cell lines (n=6); Mann-Whitney test, ***P*=0.0022.

samples using anti-CD71 beads and performed western-blot experiments with ghosts using the anti-CALR antibody. CALR expression was higher in the reticulocyte-enriched than the reticulocyte-depleted fraction (Figure 1E), indicating that young reticulocytes express higher amounts of CALR than mature red blood cells and that CALR was partially lost during reticulocyte maturation. However, the fold-increase of CALR expression in the reticulocyte-enriched fraction (2.3-5.4) was much lower than the fold-enrichment in reticulocytes (91-98) (Figure 1E) indicating that the observed CALR overexpression in total PV red blood cells does not only result from its higher expression in reticulocytes.

To determine the potential role of *JAK2*^{V617F} in the overexpression of CALR, we used the murine cellular model BaF3 expressing human *JAK2*^{V617F} or wild-type *JAK2* (*JAK2*^{WT}).[1] We studied the expression of the protein by western blot and found that CALR was overexpressed in BaF3-*JAK2*^{V617F} cells by 1.24-fold as compared to its level in BaF3-*JAK2*^{WT} cells (Figure 1F), suggesting a link between *JAK2*^{V617F} and increased levels of CALR. We investigated this increase at the transcriptional level by performing quantitative reverse transcriptase polymerase chain reaction experiments. We found no difference between CALR mRNA levels of the two cell lines, indicating that the *JAK2*^{V617F}-mediated increase of CALR was most probably due to post-translational modifications, at least in the BaF3 cellular model.

To address the link between *JAK2*^{V617F} and CALR overexpression *in vivo* we explored CALR expression in red blood cells from ET patients and found no difference between these patients and controls (Figure 1B). Although no difference was observed, this group of patients offers the possibility of studying erythroid CALR expression in the presence or absence of *JAK2*^{V617F}. We analyzed CALR expression in three ET subgroups, depending on the mutational status of the patients: with a *JAK2*^{V617F} mutation (n=16), with a *CALR* mutation (n=10), or triple-negative (n=8) (Figure 2A). There was no significant difference of CALR expression between the CT and the *JAK2*^{V617F}-positive groups, but surprisingly CALR expression was significantly lower in the ET group with *CALR* mutations than in the CT and the *JAK2*^{V617F}-positive groups (Figure 2B). This lower expression is most probably due to instability of CALR mutants as recently reported.[11] On the other hand, the similar expression levels of CALR observed in the *JAK2*^{V617F}-positive and the CT groups might result from the absence or the low number of erythroid clones harboring the *JAK2*^{V617F} mutation in the ET patients included in this study. As a matter of fact, the *JAK2*^{V617F} mutation is detected by quantitative polymerase chain reaction in DNA extracted from circulating blood cells, which does not reflect the mutational status of erythroid clones in these patients. In addition, the incidence of the *JAK2*^{V617F} mutation in the erythroid lineage of ET patients is supposed to be much lower than that of PV patients, as ET patients have normal hematocrit values.

Our study shows that *JAK2*^{V617F} leads to abnormal expression of numerous proteins at the membrane of circulating PV red blood cells, with overexpression of CALR and persistence of CANX. This abnormal presence of endoplasmic reticulum proteins at the red blood cell membrane could result from overexpression of these proteins during erythroid differentiation, or from defects in organelle degradation and erythroid maturation, or both. These findings raise the question about the potential implication of JAK2 signaling in terminal erythroid differentiation and maturation.

Erythroid CALR is known to play a role in the phagocytosis of red blood cells by splenic macrophages in the absence of a functional CD47-SIRPα interaction.[12] CANX was shown to be lost during late erythroid differentiation,[13] so its presence in the membrane of circulating PV red blood cells is potentially interesting as it might affect physiological functions. Indeed, little is known about the role of CALR and CANX as calcium-binding chaperones

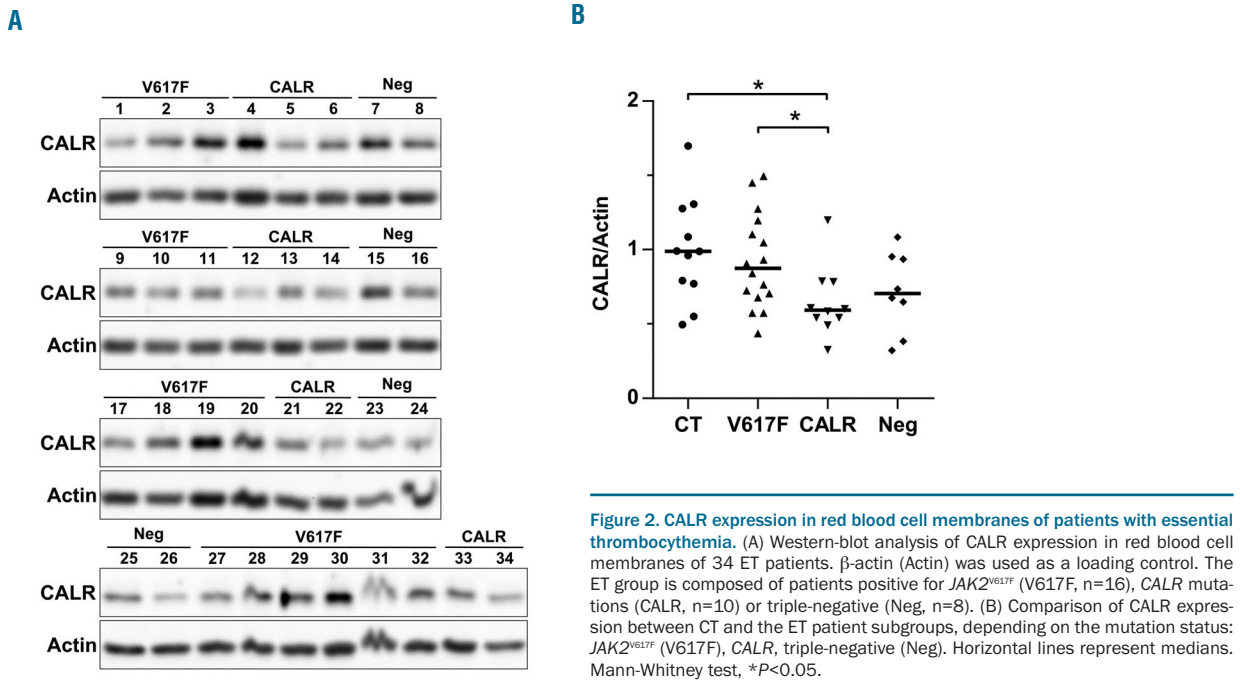

Figure 2. CALR expression in red blood cell membranes of patients with essential thrombocythemia. (A) Western-blot analysis of CALR expression in red blood cell membranes of 34 ET patients. β-actin (Actin) was used as a loading control. The ET group is composed of patients positive for *JAK2*^{V617F} (V617F, n=16), *CALR* mutations (CALR, n=10) or triple-negative (Neg, n=8). (B) Comparison of CALR expression between CT and the ET patient subgroups, depending on the mutation status: *JAK2*^{V617F} (V617F), *CALR*, triple-negative (Neg). Horizontal lines represent medians. Mann-Whitney test, *P<0.05.

in red blood cells. Considering the important role of calcium in regulating erythrocyte functions,[14] we believe that overexpression of CALR and CANX might directly affect intracellular calcium levels and subsequent cellular properties such as volume, rheological properties and other physiological parameters. Four decades ago, Scharff *et al.* reported decreased Ca^{2+}-stimulated ATPase activity in PV erythrocyte membranes;[15] in the light of our findings this decrease might result from low intracellular Ca^{2+} concentrations because of Ca^{2+} binding to CALR and CANX.

Our study opens a new field of investigations to address the role of the abnormal expression of CANX and CALR in calcium homeostasis of PV red blood cells. These new erythroid markers could be of interest in PV pathophysiology as they may alter normal red blood cell functions, similarly to what was reported for activated markers of leukocytes, platelets and endothelial cells which promote a prothrombotic state in this disease.[16]

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