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Altered Phosphorylation of Cytoskeleton Proteins in Sickle Red Blood Cells: The Role of Protein Kinase C, Rac GTPases, and Reactive Oxygen Species

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Abstract

The small Rho GTPases Rac1 and Rac2 regulate actin structures and mediate reactive oxygen species (ROS) production via NADPH oxidase in a variety of cells. We have demonstrated that deficiency of Rac1 and Rac2 GTPases in mice disrupts the normal hexagonal organization of the RBC cytoskeleton and reduces erythrocyte deformability. This is associated with increased phosphorylation of adducin at Ser-724, (corresponding to Ser-726 in human erythrocytes), a domain-target of protein kinase C (PKC). PKC phosphorylates adducin and leads to decreased F-actin capping and dissociation of spectrin from actin, implicating a significant role of such phosphorylation in cytoskeletal remodeling. We evaluated adducin phosphorylation in erythrocytes from patients with sickle cell disease and found it consistently increased at Ser-726. In addition, ROS concentration is elevated in sickle erythrocytes by 150–250% compared to erythrocytes from normal control individuals. Here, we review previous studies demonstrating that altered phosphorylation of erythrocyte cytoskeletal proteins and increased ROS production result in disruption of cytoskeleton stability in healthy and sickle cell erythrocytes. We discuss in particular the known and potential roles of protein kinase C and the Rac GTPases in these two processes.

Introduction

Sickle Cell Disease (SCD) is an inherited disorder of hemoglobin (Hb) affecting millions of people worldwide. It is caused by a single nucleotide mutation resulting in the substitution of valine for glutamic acid at position 6 in the β-globin subunit. Despite being a single-locus genetic disorder, SCD has a broad phenotypic variability, pointing to modifying pleiotropic and epistatic effects [1] that may originate from red blood cell (RBC) components other than Hb and from surrounding tissues and cells [2]. The formation of sickled RBCs in circulation underlies the twin pathogenic mechanisms of intravascular hemolysis and vaso-occlusion that engender the wide range of symptoms and complications in SCD [3]. Although hemoglobin S

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(HbS) polymerization upon deoxygenation is the trigger for the initial sickling deformation of RBCs containing HbS, the subsequent changes in the cytoskeleton and the mechanisms by which reversibly sickled cells become irreversibly malformed have not been fully elucidated. A better understanding of the molecular mechanisms that predispose HbS-containing RBCs to irreversible sickling would potentially identify novel therapeutic targets for SCD.

Analysis of the non-HbS components of sickle RBCs over the past several decades has revealed several alterations specific to HbS-containing RBCs that likely contribute to cellular deformation and structural instability. Among these changes are damage to the lipid bilayer of the erythrocytes, altered membrane permeability to calcium, sodium, and potassium that result in abnormal ion fluxes and cell dehydration, increased reactive oxygen species (ROS) production, depletion of antioxidant defenses with resultant oxidative damage to the cell, and altered phosphorylation of proteins in the cytoskeleton and membrane scaffolding [4]. The relative importance of these different pathogenic alterations in RBC homeostasis continues to be under investigation.

We have demonstrated in genetically-targeted mice that deficiency of Rac1 and Rac2 GTPases alters the erythrocyte cytoskeleton organization, with increased phosphorylation of α-adducin at Ser-724 (corresponding to Ser-726 in human erythrocytes), a domain-target of protein kinase C (PKC) [5]. PKC phosphorylates adducin [6,7] and leads to decreased F-actin capping and dissociation of spectrin from actin in neurons [8] and platelets [9], indicating a significant role of such phosphorylation in cytoskeletal remodeling [10] (Figure 1). Here, we show that adducin phosphorylation at Ser-726 is consistently increased in erythrocytes from patients with sickle cell disease. On the other hand, Rac1 and Rac2 GTPases have been shown to mediate ROS production via NADPH oxidase in a variety of cells [11]. ROS concentration is known to be elevated in sickle erythrocytes; we confirmed by flow cytometry an increase in ROS of 150–250% in HbS RBCs compared to erythrocytes from normal control individuals. Instigated by this data, we discuss here two of the less well-understood subcellular alterations in HbS RBCs: the altered phosphorylation of cytoskeletal proteins and the increased reactive oxygen species production which likely contribute to cellular deformation and structural instability of sickle red blood cells.

Post-Translational Modifications of Cytoskeletal Proteins

The erythrocyte cytoskeleton is a complex and dynamic structure, the proper assembly and integrity of which is critical to the survival of RBCs in the high-shear environment of arterial blood flow as well as through the repeated cycles of deformation necessary for transit through capillary networks and subsequent resumption of discoid morphology. It lies under the lipid bilayer of the cell membrane (reviewed in detail by Mohandas and Gallagher [12]) and consists of a highly-ordered hexagonal meshwork of α and β spectrin heterodimers arranged head-to-head and anchored to the lipid bilayer along the spectrin arms by the band 3/ankyrin protein complex, and at the intersection of the arms by a junctional complex containing an actin oligomer and protein 4.1R. The actin oligomer in the junctional complex is capped at the fast-growing (barbed) end by adducin, which, along with protein 4.1R, assembles F-actin with spectrin [13]. Tropomodulin caps the slow-growing (pointed) end of the actin oligomer and tropomyosin may help regulate the length of actin oligomer, binding along its side [14] (Figure 1A). The importance of the various cytoskeletal proteins and their interactions in RBC integrity is highlighted by the discovery that mutations in genes encoding spectrin, ankyrin, band 3, protein 4.2, α and β spectrin, and protein 4.1R result in hemolytic anemias, such as hereditary spherocytosis or elliptocytosis [15,16].

RBCs beyond the reticulocyte stage lack protein synthetic activity, so regulation of RBC structure and function must occur via post-translational modifications of enzymes and

structural proteins. Cytoskeleton proteins can undergo several types of post-translational modification, among them the formation and dissolution of protein complexes, glycosylation, oxidation of amino acid residues, ubiquitination, and phosphorylation and dephosphorylation [17,18]. Of these modifications, the best understood and perhaps the most dynamic is protein phosphorylation and dephosphorylation. RBCs in circulation retain a number of active protein kinases, including protein kinase C (PKC), protein kinase A (PKA), casein kinases I and II, Syk, Lyn, Hck-Fgr, and Fyn, as recently reviewed by Pantaleo et al [19]. Some of the phosphatases identified in RBCs include SHP-1 and SHP-2, which dephosphorylate tyrosine residues in band 3 [20], PTPH1, a tyrosine-phosphatase for protein 4.1R [21], and the protein phosphatases type 1 and type 2A (PP1 and PP2A), which dephosphorylate serine and threonine residues [22]. It appears that in the resting healthy erythrocyte, phosphatase activity exceeds kinase activity as overall protein phosphorylation levels are low [23,24].

Although a number of studies have documented that the phosphorylation status of the erythrocyte cytoskeleton proteins regulates protein-protein interactions in vitro, understanding is limited about the effect of phosphorylation on membrane properties and skeletal stability of the erythrocyte in vivo [17]. Frequently, kinases are rather promiscuous with multiple target sequences in several of the cytoskeleton proteins and it is difficult to elucidate cause and effect between a specific phosphorylation target and altered cellular deformability. Adducin and protein 4.1R are known targets of PKC [8,25] and PKA, while these two proteins as well as spectrin, band 3, ankyrin, and dematin, may also be targets of casein kinases I and II [19].

PKC in erythrocytes

PKC isozymes comprise a family of serine/threonine protein kinases, which differ in cofactor requirement, substrate specificity, subcellular localization, and tissue expression [26,27]. The conventional PKCα (requiring a phospholipid (PL), diacylglycerol (DAG) or phorbol ester, and calcium (Ca^{2+}) for optimal activation) and the atypical isozymes PKCζ and ι, (requiring PL but neither DAG, nor Ca^{2+}) have been detected in the cytosolic fraction of mature human erythrocytes [28]. Upon stimulation with the DAG-synthetic analog phorbol 12-myristate 13-acetate (PMA), PKCα is activated and translocates to the RBC membrane [28,29]. It could thus mediate several of the reported PMA-regulated membrane modifications. Interestingly, it has been demonstrated in neutrophils that although PKCζ activation does not require DAG, PMA induces translocation of PKCζ to the membrane where it is activated by phospholipids [30]. Multiple effects of PKC have been reported in erythrocytes, including phosphorylation of the glucose transporter to stimulate glucose uptake [31], modulation of the activity of the calcium channel [32] and the Na^+/H^+ exchange [33,34], and phosphatidylserine exposure at the cell surface [35]. Subcellular localization of PKC was shown to correlate with age, as shown in biotin-labeled rabbit RBCs of progressively increasing age, demonstrating a gradual decline in cytoplasmic activity and increase in membrane-associated activity as the erythrocytes age [36].

PKC phosphorylates protein 4.1R at Ser-312, α-adducin at Ser-726, and β-adducin at Ser-713 [25] in the erythrocyte cytoskeleton. Protein 4.1R is also phosphorylated by PKA at Ser-331. Phosphorylation by either kinase in vitro results in significant reduction in the ability of 4.1R to promote spectrin binding to F-actin [37]. Adducin phosphorylation by PKC leads to decreased F-actin capping and dissociation of spectrin from actin in neurons [8] and platelets [9]. RBC membrane mechanical stability declines little following adducin phosphorylation by PKC, while it is markedly decreased by the additional phosphorylation of 4.1R [25]. However, adducin phosphorylation may be essential for protein 4.1R phosphorylation-induced changes in membrane stability, since adducin phosphorylation precedes protein 4.1R phosphorylation upon PMA stimulation [25].

Cytoskeleton phosphorylation and PKC activity in sickle cells

Dzandu and Johnson [38] showed that the phosphorylation of membrane proteins in sickle cells is altered in comparison to HbA-RBCs, using autoradiographs of RBC ghosts after incubation of erythrocytes with $^{32}P_i$. The relative incorporation of $^{32}P_i$ in β-spectrin is low in irreversibly sickled cells (ISC), supporting the idea that some of the abnormalities of the ISC membrane are associated with altered cytoskeleton phosphorylation [38]. Fathallah et al [39] demonstrated that deoxygenation induces dephosphorylation of membrane proteins both in HbS and HbA cells, while PMA and the serine/threonine phosphatase-inhibitor okadaic acid prevent deoxygenation-induced cytoskeleton dephosphorylation. However, this study, also using $^{32}P_i$ autoradiographs, could not give specific information on the target sequences of RBC proteins altered by deoxygenation. The altered phosphorylation of sickle cells was not seen in young normal RBCs or in reticulocytes from patients with thalassemia, suggesting that this altered phosphorylation is not an artifact of cell age [40]. Calcium and ionophore A23187 was shown to induce phosphorylation in normal erythrocytes in a pattern analogous to the sickle membrane phosphorylation pattern [40]. Phorbol esters were also found to induce phosphorylation of the cytoskeleton proteins most significantly phosphorylated in sickle cells: protein 4.1R, protein 4.9 (dematin), and an ~100–110kDa protein, likely corresponding to α-adducin [41]. Membrane PKC content in HbS cells has been found to be about 6-times higher than in HbA cells, by [^{3}H]-phorbol ester binding [42]. The difference is less pronounced when PKC activity is determined by histone phosphorylation. PKC activity was found to be increased in HbS-RBC membranes (by 40% in unfractionated cells and by 56% in the light fraction) but decreased in the cytosol compared to the activity in HbA-RBC membranes and cytosol, respectively [42]. In addition to PKC, phorbol esters bind and activate several other proteins that also contain regulatory C1 domains [43]. Although, it is likely that some phorbol ester-induced effects are mediated via proteins other than PKC, the effects of PKC inhibitors in various cell types indicate that PKC isoforms have significant roles in regulating cytoskeleton driven processes [10].

We evaluated the phosphorylation profile of erythrocyte ghost proteins from SCD patients and normal controls by immunoblotting with anti-phosphoserine/threonine antibody (BD Biosciences, San Jose, CA). As shown in Figure 2A we found increased phosphorylation of proteins at the molecular weights of adducin (~100kDa), protein 4.1R (80kDa), and dematin (48kDa), similarly with what has been previously shown by incubation of erythrocytes with $^{32}P_i$ and autoradiography [38]. Using a phosphospecific antibody for adducin (Ser-726), we demonstrated that RBCs from patients with SCD have increased phosphorylation of adducin at Ser-726 (a PKC-target) compared with control subjects with HbA (Figure 2B).

Reactive Oxygen Species in Sickle Cell Disease

ROS generation in sickle red cells has been shown to be approximately twofold greater compared with HbA RBCs [44,45]. In agreement, we found that ROS generation was consistently elevated in sickle vs. normal RBCs by 50–150%, using flow cytometry with 5-(and 6)- chloromethyl- 2′,7′, dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Invitrogen, Carlsbad, CA). As a consequence of ROS generation, HbS RBCs demonstrate increased membrane rigidity and mechanical instability [46]. Exposure of normal erythrocytes to phenazine methosulphate, which induces superoxide generation within the cells, demonstrated that ROS dose-dependently induce membrane rigidity, reduce RBC elasticity, and oxidize membrane-associated hemoglobin as well as sulfhydryl-groups of cytoskeleton and transmembrane proteins [47,48]. Conversely, treatment of sickle RBCs with the antioxidants N-acetylcysteine (NAC) or dithiothreitol can prevent irreversible sickling induced by hypoxia in vitro [49].

Increased ROS generation in SCD is due to activation of enzymatic (NADPH oxidase, xanthine oxidase) as well as non-enzymatic (HbS auto-oxidation) sources [50]. NADPH oxidase, the major superoxide-producing enzyme in leucocytes, is also expressed in human reticulocytes [51]. The family of NADPH oxidases plays an important role in the production of ROS in response to growth factors or inflammatory cytokines that signal through Rac1 or Rac2 GTPases [11]. Sequentially, ROS act as signal transduction mediators, by oxidative modification of nucleic acids, sugars, lipids, and proteins, in part leading to altered phosphatase-kinase activity balance [52]. Increased ROS production in murine sickle RBCs has been proposed as a possible cause of the excess exposure of phosphatidylserine on the surface of these cells [53], while endothelial cell NADPH oxidase-derived superoxide was demonstrated to mediate increased adhesion of leucocytes and platelets in cerebral venules of SCD mice [54].

These results suggest that the increased ROS generation in SCD, both in erythrocytes and from other sources, contributes to SCD pathology by activating endothelial expression of adhesion molecules and increasing endothelium-leukocyte and platelet adhesion, and by reducing RBC deformability and making them more prone to hemolysis. Thus, ROS generation plays a part in both vaso-occlusion and hemolysis, the twin pathophysiologic mechanisms of SCD.

PKC, Rac GTPases, and NADPH oxidase

Rac GTPases are intracellular signaling proteins, members of the Rho family and Ras superfamily, which switch between the inactive GDP-bound form and the active GTP-bound form in order to regulate multiple pathways involved in cell acto-myosin organization, adhesion and proliferation. They have been shown to play unique and overlapping roles in hematopoietic and blood cells (recently reviewed by Mulloy et al [55]). Inactive GDP-bound Rac proteins are sequestered in the cytosol by Rho GDP-dissociation inhibitors (RhoGDIs) which bind and mask the hydrophobic C-terminal region of Rac, the same region that is responsible for targeting Rac to the plasma membrane (Figure 3).

The Rac GTPases are a critical component of NADPH oxidase complex, and their recruitment from the cytoplasm into the membrane-bound complex is necessary for NADPH oxidase activity [56]. The phagocyte NADPH oxidase was the first identified and best-studied member of the NOX family, contributing to microbial killing. Nox2 (previously called gp91^{phox}) along with the p22^{phox} subunit localizes in the membrane of intracellular vesicles in resting neutrophils. Upon activation, Rac GTPases mediate the association of p67^{phox} with the catalytic subunit Nox2 to form the NADPH oxidase complex within the plasma or phagosomal membrane, which functions as a transmembrane redox chain that connects the electron donor (NADPH) in the cytosol with the electron acceptor (molecular O_2) on the other side of the membrane (extracellular or intravesicular) to produce superoxide radicals [56]. Several studies provide evidence that PKC activation induces NADPH oxidase-mediated ROS production. In aortic endothelial and renal mesangial cells, it has been shown that high glucose levels stimulate ROS production via a PKC-dependent activation of Rac1 and NADPH oxidase, and that this activation is inhibited by PKC inhibitors [57]. In epithelial cell lines, activation of PKC results in increased phosphorylation of RhoGDIα which dissociate to allow Rac to translocate to the membrane and interact with membrane-associated activators [58]. In neutrophils, PKC has been shown to activate NADPH oxidase by phosphorylation of Nox2 [59] or phosphorylation of p47^{phox} [60] (Figure 3). Whether a similar mechanism is at work to generate ROS in erythroid cells, and more intriguingly, if abnormality of such a ROS producing molecular complex is associated with sickle cell disease phenotype, awaits future investigation.

Conclusion

In summary, sickle cells have altered phosphorylation of the cytoskeleton proteins that is dynamically affected by deoxygenation, increased PKC content and activity, and increased ROS generation. Rac GTPases mediate ROS production in a variety of cells via NADPH oxidase. We have demonstrated that Rac-deficient mouse RBCs have decreased deformability and increased phosphorylation of adducin-Ser724 [5] (corresponding to Ser-726 in human ⟨-adducin), a PKC-target. Phosphorylated adducin-Ser726 leads to dissociation of spectrin from F-actin. We have found that adducing-Ser726 is consistently more phosphorylated in sickle cells, in agreement with the long-known finding of increased PKC activity.

The activation status as well the localization of PKC and Rac GTPases determines their function. It would be interesting to speculate that increased PKC activity in sickle RBC membranes may redirect Rac GTPases towards activation of NADPH oxidase and increased ROS production, while it also phosphorylates adducin at Ser-726. Thus, aberrant PKC and Rac GTPase function in sickle RBCs may affect membrane mechanics, aggravating the rigidity and fragility of sickle cells, while it may also increase ROS production playing a role in both vaso-occlusion and hemolysis. Analyzing further the pathways leading to increased ROS production and altered cytoskeleton phosphorylation in sickle cells has the potential to reveal therapeutic targets in SCD, amenable to disease modification treatment.

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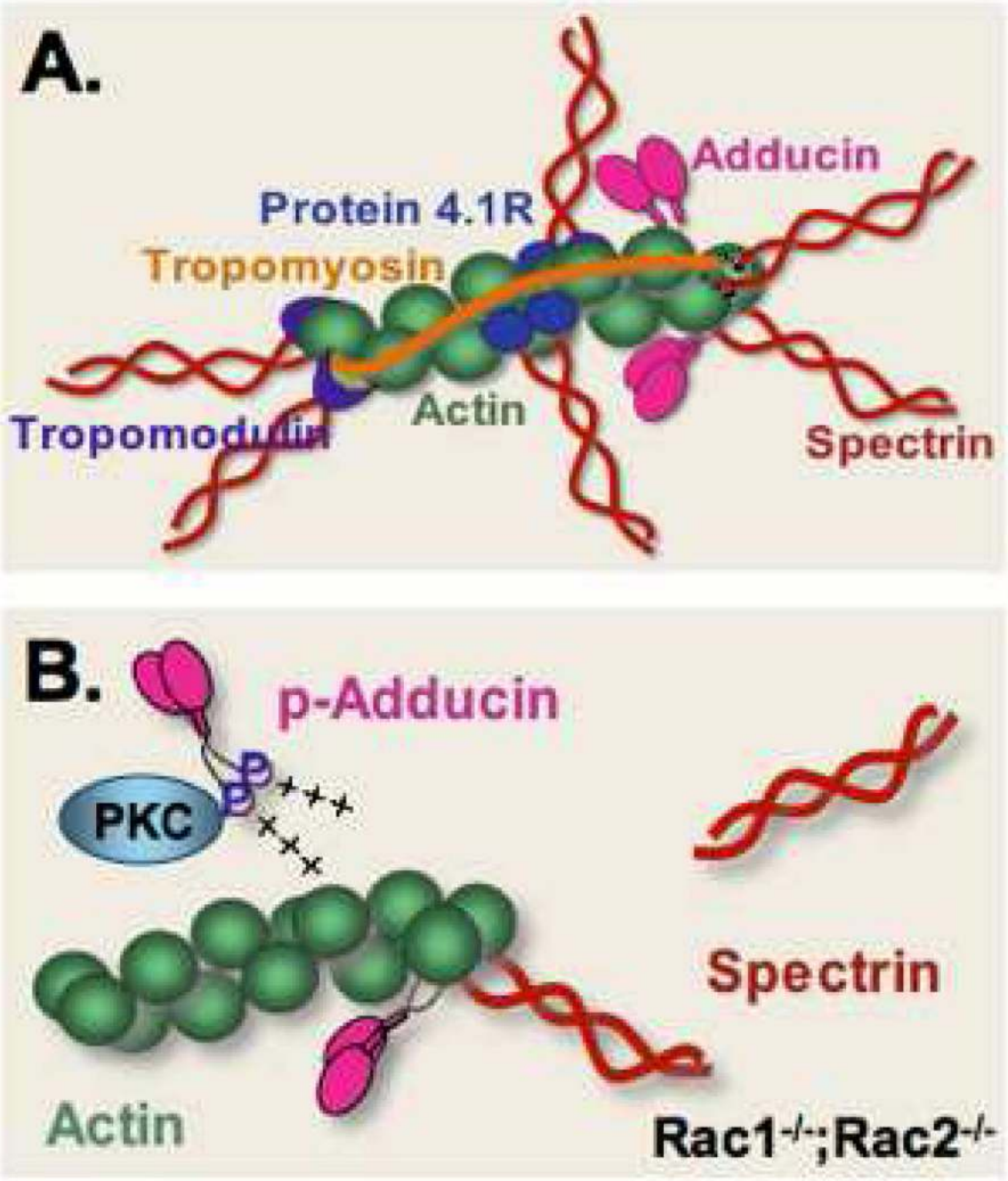

Figure 1.

A. A working model for the junctional complex of the RBC cytoskeleton, demonstrating the capping actions of tropomodulin and adducin. Tropomodulin, protein 4.1R, and adducin also mediate spectrin-actin association. **B.** PKC phosphorylates α-adducin at Ser-726 leading to decreased F-actin capping and dissociation of spectrin from actin.

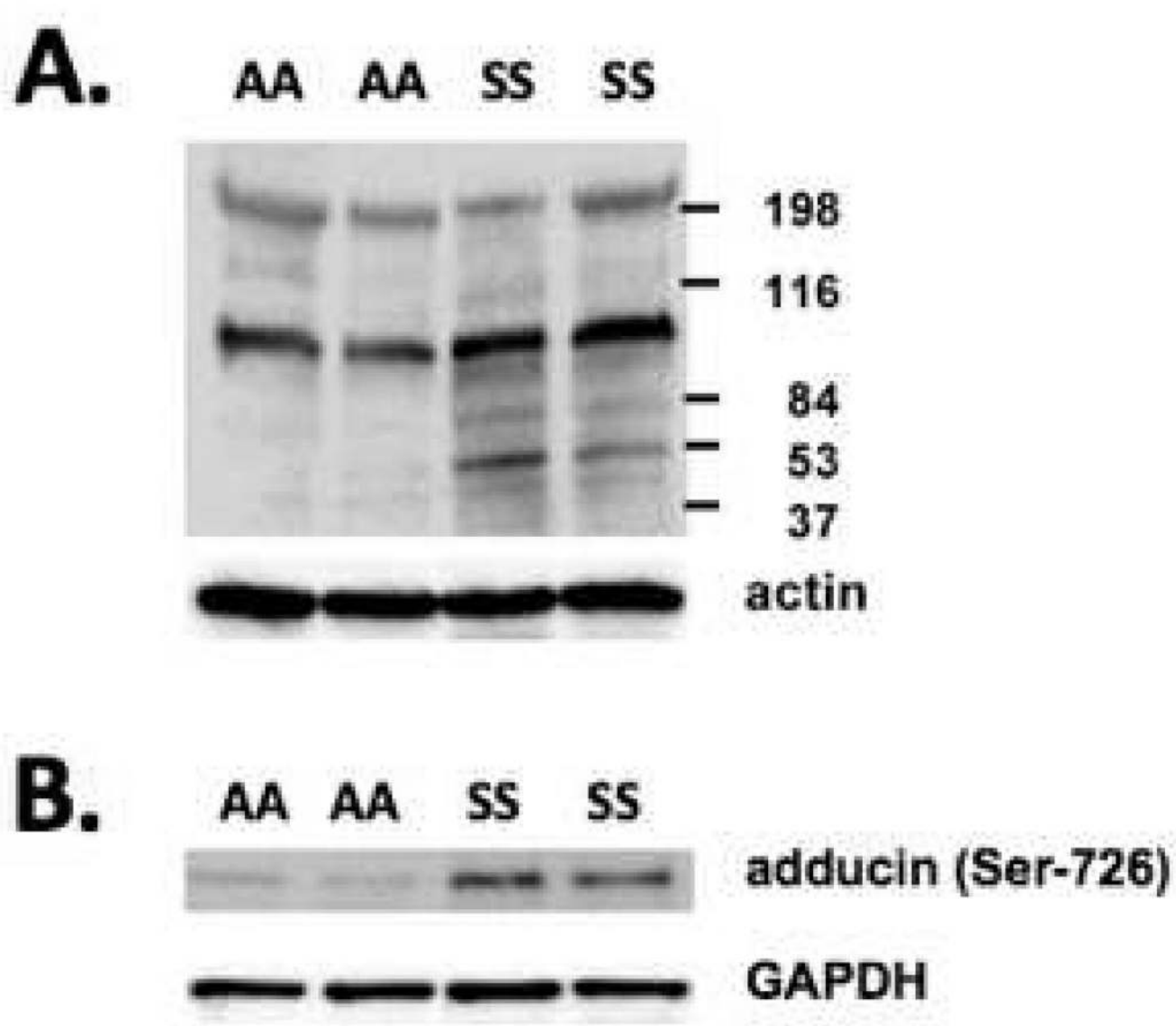

Figure 2.

Phosphorylation profile of RBC cytoskeleton proteins in two different representative patients with HbS (SS) and two controls with HbA (AA), as depicted by immunoblotting with **A.** anti-phosphoserine/threonine antibody, and **B.** phosphospecific antibody for adducin (Ser-726). Samples representative of more than ten patients per Hb genotype. Actin and GAPDH are shown as loading controls.

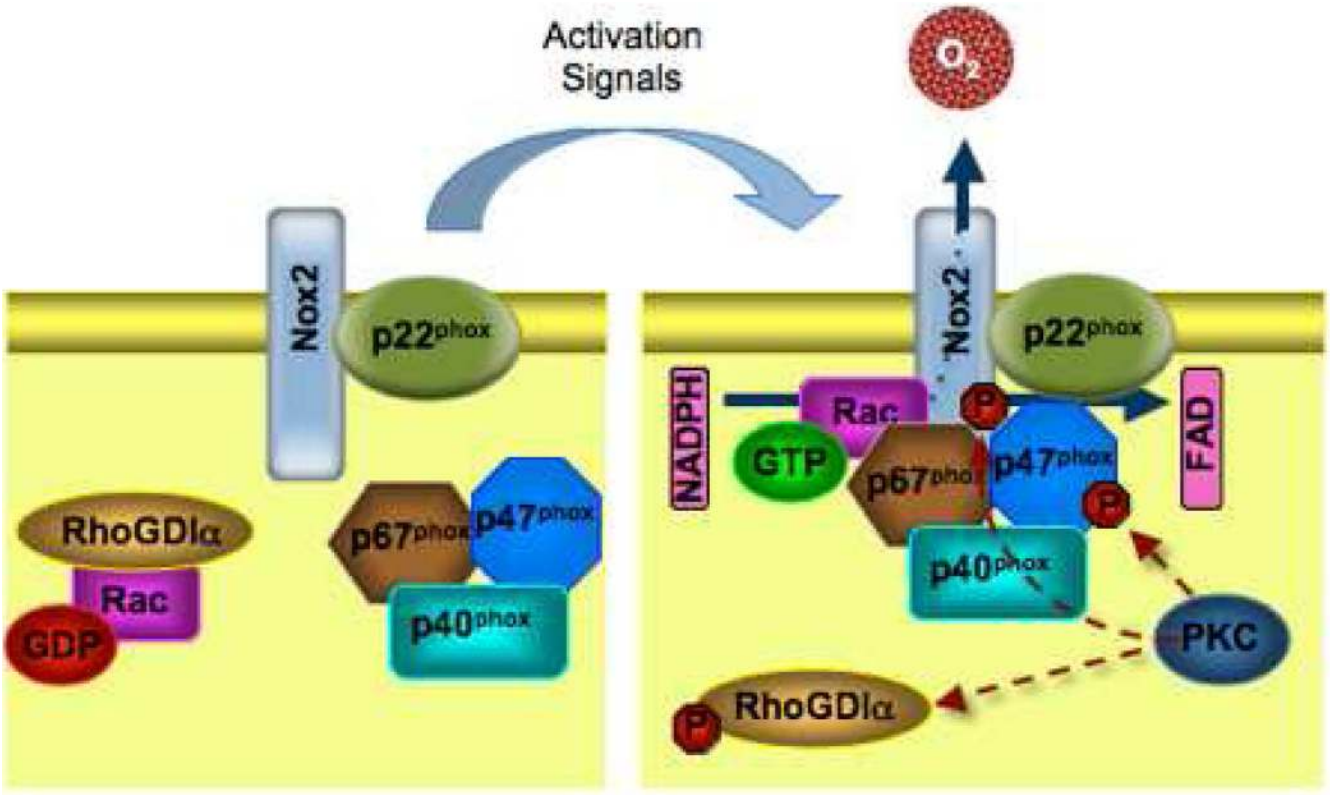

Figure 3.

Model of NADPH oxidase activation. In the inactive state, gp91^{phox} (Nox2, the Nox isoform found in neutrophils) and p22^{phox} exist in a membrane-associated complex, in intracellular vesicles. The p40^{phox}, p47^{phox}, and p67^{phox} subunits are located in the cytoplasm, as is inactive Rac, associated with GDP and RhoGDI. Activation signals result in translocation of p40^{phox}, p47^{phox}, p67^{phox}, and active Rac-GTP to the cell or phagosomal membrane to form the active NADPH oxidase complex with Nox2 and p22^{phox}. The active complex transfers electrons from NADPH to molecular oxygen to form superoxide radicals. Part of the activation process is postulated to occur by PKC phosphorylation of p47^{phox} and p67^{phox}. PKC phosphorylation of RhoGDI may also mediate its dissociation from Rac and the transition from inactive RacGDP to active RacGTP. The basic process of NADPH oxidase activation is similar in other cell types but may involve other members of the Nox, p40^{phox}, p47^{phox}, and p67^{phox} families of proteins.