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# FXYD Proteins Reverse Inhibition of the $Na^+$-$K^+$ Pump Mediated by Glutathionylation of Its $\beta_1$ Subunit*[S]

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**The seven members of the FXYD protein family associate with the $Na^+$-$K^+$ pump and modulate its activity. We investigated whether conserved cysteines in FXYD proteins are susceptible to glutathionylation and whether such reactivity affects $Na^+$-$K^+$ pump function in cardiac myocytes and *Xenopus* oocytes. Glutathionylation was detected by immunoblotting streptavidin precipitate from biotin-GSH loaded cells or by a GSH antibody. Incubation of myocytes with recombinant FXYD proteins resulted in competitive displacement of native FXYD1. Myocyte and *Xenopus* oocyte pump currents were measured with whole-cell and two-electrode voltage clamp techniques, respectively. Native FXYD1 in myocytes and FXYD1 expressed in oocytes were susceptible to glutathionylation. Mutagenesis identified the specific cysteine in the cytoplasmic terminal that was reactive. Its reactivity was dependent on flanking basic amino acids. We have reported that $Na^+$-$K^+$ pump $\beta_1$ subunit glutathionylation induced by oxidative signals causes pump inhibition in a previous study. In the present study, we found that $\beta_1$ subunit glutathionylation and pump inhibition could be reversed by exposing myocytes to exogenous wild-type FXYD3. A cysteine-free FXYD3 derivative had no effect. Similar results were obtained with wild-type and mutant FXYD proteins expressed in oocytes. Glutathionylation of the $\beta_1$ subunit was increased in myocardium from $FXYD1^{-/-}$ mice. In conclusion, there is a dependence of $Na^+$-$K^+$ pump regulation on reactivity of two specifically identified cysteines on separate components of the multimeric $Na^+$-$K^+$ pump complex. By facilitating deglutathionylation of the $\beta_1$ subunit, FXYD proteins reverse oxidative inhibition of the $Na^+$-$K^+$ pump and play a dynamic role in its regulation.**

The mammalian FXYD proteins are a family of seven small type I membrane proteins named after an invariant FXYD signature sequence (1). They are abundantly expressed and associate with the membrane $Na^+$-$K^+$ pump (2, 3) and with the $Na^+$-$Ca^{2+}$ exchanger (4). Their association with the $Na^+$-$K^+$ pump is highlighted by recently published three-dimensional structures (5–7). Despite the close association demonstrated, FXYD proteins are not an integral functional part of the $Na^+$-$K^+$ pump $\alpha/\beta$ subunit heterodimer, which alone exhibits both catalytic activity and ion transport capacity. However, it is firmly established that they modulate pump function (3, 8). FXYD1, also known as phospholemman, is a major substrate for protein kinase A and PKC in the myocardium (9). Its phosphorylation is implicated in $Na^+$-$K^+$ pump regulation (3, 10), but functional phosphorylation sites have not been identified on the other six mammalian FXYD proteins, indicating there must be additional mechanisms for the role of FXYD proteins in pump regulation.

We have recently identified a mechanism for $Na^+$-$K^+$ pump regulation that preserves a well established role for protein kinases but depends on redox signaling and a reversible oxidative modification as the downstream molecular mechanism. Angiotensin II induced PKC- and NADPH oxidase-dependent glutathionylation of the pump $\beta_1$ subunit in cardiac myocytes. Mutational studies in *Xenopus* oocytes indicated that glutathionylation was causally related to pump inhibition (11). Because two cysteines in the cytoplasmic terminus are conserved among FXYD proteins (1), we examined whether these candidate cysteines are reactive, *i.e.* susceptible to glutathionylation. Studies in *Xenopus* oocytes show one of them is reactive and critical for reversal of $\beta_1$ subunit glutathionylation. Functional studies on *in situ* $Na^+$-$K^+$ pumps in voltage clamped intact myocytes and oocytes under conditions that reasonably resemble those under which the pump normally functions show that reversal of $Na^+$-$K^+$ pump inhibition caused by $\beta_1$ subunit glutathionylation depends on the identified reactive cysteine in FXYD proteins. Although FXYD proteins have been thought to modulate activity of the $Na^+$-$K^+$ pump by their presence or absence according to the needs of the specific tissues, we conclude they have a much broader role in mediating redox-dependent regulation and perhaps in reversing pump inhibition under conditions of oxidative stress.

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## EXPERIMENTAL PROCEDURES

For more details, see supplemental "Methods."

*Cardiac Tissues and Cells*—*S*-glutathionylation was detected in isolated rabbit ventricular myocytes loaded with biotinylated GSH (11). The biotin-tagged glutathionylated subfraction in myocyte lysate was precipitated using streptavidin-Sepharose beads and immunoblotted for $\beta_1$ subunit and FXYD proteins. With an alternative technique, an antibody against a glutathionylated cysteine epitope (anti-GSH antibody) was used in standard co-immunoprecipitation protocols. The GSH antibody technique was also used to detect glutathionylation in myocardial samples.

The whole-cell patch clamp technique was used to measure electrogenic $Na^+$-$K^+$ pump current ($I_p$). The patch pipette solutions perfusing the intracellular compartment included 10 mM $Na^+$, a concentration near physiological intracellular levels. The solution also included L-arginine, paraquat, and recombinant FXYD proteins where indicated. Patch clamped myocytes were exposed to angiotensin II or CL316,243 in the superfusate after the whole cell configuration was established. $I_p$ was measured at 37 °C identified as the ouabain-induced shift in holding currents (12, 13).

*Xenopus Oocytes*—Stage V–VI *Xenopus laevis* oocytes were injected with *Xenopus* $\alpha_1$ and $\beta_1$ $Na^+$-$K^+$ pump subunit cRNAs alone or together with WT or mutant FXYD cRNAs. Two days after cRNA injection, *S*-glutathionylation of $Na^+$-$K^+$ pump $\beta_1$ subunit and FXYD proteins was studied as described previously (11). Glutathionylation was promoted by giving a 50-nl bolus injection of solution containing 1 mM peroxynitrite ($ONOO^-$) into oocytes 15 min before lysis. Assuming an oocyte volume of ~1 μl, the initial concentration is estimated at ~50 μM, but the concentration likely decreases rapidly due to the short half-life of $ONOO^-$.

Measurements of maximal electrogenic $Na^+$-$K^+$ pump current ($I_{max}$) in $Na^+$-loaded oocytes were performed using the two-electrode voltage clamp technique. It was measured at room temperature as the outward current induced by 10 mM $K^+$ after oocytes had been suspended in $K^+$-free superfusate. For the determination of the effect of oxidation on $I_{max}$, oocytes were injected with $ONOO^-$ 15 min before measurements.

## RESULTS

*FXYD Glutathionylation in Heart*—Rabbit ventricular myocytes loaded with biotin-tagged glutathione were lysed, and biotin-tagged, and glutathionylated proteins were precipitated. Myocytes not loaded with biotin-GSH were used as a negative control to confirm specificity of the precipitation step for glutathionylated protein. FXYD1 was detected readily in total cell lysate with an FXYD1 antibody, consistent with its abundant expression in cardiac myocytes. It was also detected in the biotin-tagged, glutathionylated subfraction but not in the negative control (Fig. 1*A*). Exposure of the myocytes to $ONOO^-$ had no effect on expression of FXYD1 in total cell lysate but increased the amount of FXYD1 in the glutathionylated protein subfraction (Fig. 1, *A* and *B*). Glutathionylated FXYD1 was not detected when the lysate was incubated with 1 mM DTT prior to precipitation by streptavidin (Fig. 1*A*), supportive of a mixed disulfide bond between FXYD1 and GSH.

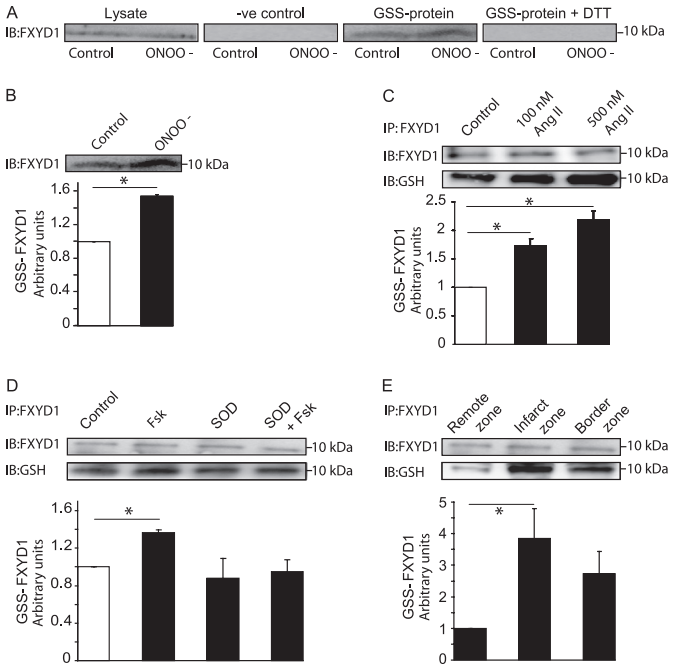


FIGURE 1. **Glutathionylation of FXYD1 in the heart.** *A*, FXYD1 immunoblot (*IB*) of myocyte lysate and glutathionylated proteins (*GSS-protein*) precipitated with streptavidin. Streptavidin precipitate from myocytes not incubated in biotin-GSH was a negative (−*ve*) control. Glutathionylation was sensitive to DTT. *B*, mean densitometry of FXYD1 immunoblots in streptavidin pulldown of control myocytes and myocytes exposed to $ONOO^-$ for 15 min at the nominal concentration of 100 μM. *C*, glutathionylation of FXYD1 detected by GSH antibody technique in myocytes exposed to angiotensin II (*Ang II*) for 15 min. *D*, glutathionylation of FXYD1 in myocytes exposed to 100 nM forskolin (*Fsk*), with and without incubation with 200 international units/ml PEGylated superoxide dismutase (*SOD*). *E*, glutathionylation of FXYD1 from a sheep model of infarction from myocardium remote to the infarct, the peri-infarct zone, and infarct zone. Immunoprecipitations (*IP*) were performed with FXYD1 antibodies and immunoblots with FXYD1 antibodies (*upper panel*) or GSH antibodies (*lower panel*). Densitometry of immunoblots (mean ± S.E.) was normalized against control ($n = 3$ for each experiment). *, $p < 0.05$.

The biotin-GSH technique detects ongoing glutathionylation *in vitro* from the time of loading. The GSH antibody technique was used to assess glutathionylation at the time of cell lysis. As shown in Fig. 1*C*, glutathionylation of FXYD1 was detected at baseline. Exposure of myocytes to angiotensin II for 15 min to activate cardiac NADPH oxidase (12) increased glutathionylation. The adenyl cyclase activator forskolin induces NADPH oxidase-dependent glutathionylation of the $\beta_1$ subunit (13), and forskolin also increased the glutathionylation of FXYD1 (Fig. 1*D*). This increase was abolished by incubating myocytes with PEGylated superoxide dismutase. Because superoxide dismutase catalyzes the dismutation of superoxide to $H_2O_2$, this might be taken to rule out $H_2O_2$ as the species that induces glutathionylation. Such a conclusion is not justified, as reviewed (14), but the effect of superoxide dismutase does support the more limited conclusion that an oxidative stimulus plays a role in forskolin-induced glutathionylation.

We used a sheep model of myocardial infarction (11) to examine whether a pathophysiological increase in oxidative stress *in vivo* induces glutathionylation of FXYD1. Consistent with promotion of protein glutathionylation (15) and inhibition

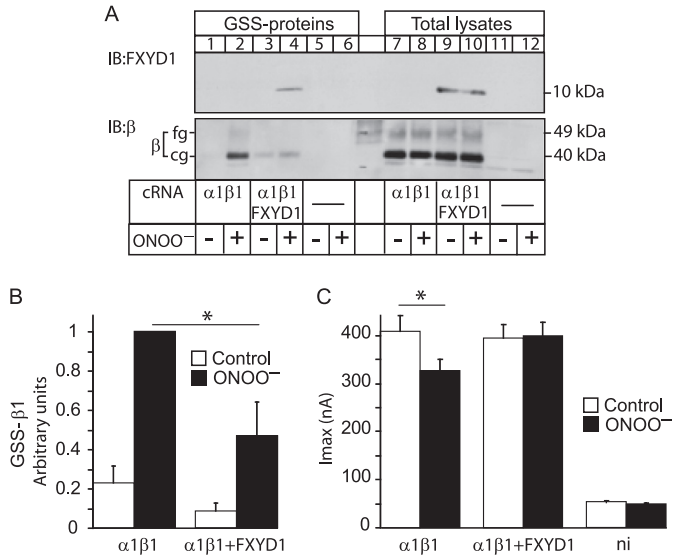


FIGURE 2. **Expression of FXYD1, $\beta_1$ subunit glutathionylation and Na$^+$-K$^+$ pump activity in oocytes.** *A*, FXYD1 (*upper panel*) and $\beta_1$ subunit (*lower panel*) immunoblots of *Xenopus* oocyte microsomes directly loaded on gels (*lanes 7–12*) or precipitated with streptavidin beads (*GSS-proteins*, *lanes 1–6*). Experiments were performed 2 days after injection with Na$^+$-K$^+$ pump subunit and FXYD1 cRNAs as indicated. Injection of ONOO$^-$ 15 min before lysis is indicated. Core glycosylated and fully glycosylated $\beta_1$ subunits are indicated by *cg* and *fg*. *B*, quantification of glutathionylation of Na$^+$-K$^+$ pump $\beta_1$ subunit by densitometry (normalized by total amount of protein expressed, mean ± S.E.) in five independent experiments. *C*, FXYD1 abolishes ONOO$^-$-induced decrease in mean Na$^+$-K$^+$ pump current of 20 oocytes from two different batches. Currents from oocytes not injected with cRNA (*ni*) were not subtracted from currents of injected oocytes. *, $p < 0.05$. *IB*, immunoblot.

of glutaredoxin 1 (Grx1)[7] under hypoxic conditions (16), there was a large increase in the density of the FXYD1 immunoprecipitate immunoblotted with GSH antibody in myocardium in the infarct/peri-infarct zone compared with that in normal myocardium (Fig. 1*E*). A signal was not detected when lysate had been incubated with 1 μM recombinant human Grx1 or 1 mM DTT (supplemental Fig. S1). Because Grx1 is highly selective for glutathionyl mixed disulfide bonds (17), this supports the specificity of the antibody used to detect glutathionylation.

*FXYD Proteins Reduce $\beta_1$ Subunit-mediated Oxidative Inhibition of the Na$^+$-K$^+$ Pump in Xenopus Oocytes*—We overexpressed *Xenopus* $\alpha_1$ and $\beta_1$ Na$^+$-K$^+$ pump subunits with canine FXYD1 in *Xenopus* oocytes. The expressed FXYD1 associates with the Na$^+$-K$^+$ pump as indicated by co-immunoprecipitation experiments (10). FXYD1 was detected in microsomes from cRNA-injected oocytes but not from noninjected oocytes (Fig. 2*A*, *lanes 7–12*). Most $\beta_1$ subunits were core-glycosylated after 2 days of expression representing subunits residing in the endoplasmic reticulum and reflecting their continuous synthesis from injected cRNA (Fig. 2*A*, *lanes 7–12*). However, a population of fully glycosylated $\beta_1$ subunits also appeared, which represents Na$^+$-K$^+$ pumps in transit to or at the plasma membrane. Consistent with its low endogenous expression (18, 19), a signal for $\beta$ subunits in microsomes from noninjected oocytes was not detectable.

Batches of oocytes were injected with biotin-GSH and incubated for 45 min prior to injection with ONOO$^-$. Peroxynitrite induced glutathionylation of FXYD1 and Na$^+$-K$^+$ pump $\beta_1$ subunits (Fig. 2*A*, *lanes 1–6*). However, expression of FXYD1 reduced ONOO$^-$-induced glutathionylation of the $\beta_1$ subunits (Fig. 2*B*). We measured $I_{max}$ in *Xenopus* oocytes overexpressing the $\alpha_1$ and $\beta_1$ subunits of the Na$^+$-K$^+$ pump with or without expression of FXYD1. ONOO$^-$ induced a decrease in $I_{max}$ in oocytes overexpressing $\alpha_1$ and $\beta_1$ subunits alone. We previously have identified Cys$^{46}$ in the $\beta_1$ subunit as the amino acid that mediates the decrease (11), indicating the specific nature of the response to the oxidative signal. Co-expression of FXYD1 had no effect on $I_{max}$ under control conditions but eliminated the decrease in $I_{max}$ induced by ONOO$^-$ (Fig. 2*C*), in parallel with the effect of FXYD1 on $\beta_1$ subunit glutathionylation (Fig. 2, *A* and *B*).

*FXYD Proteins Reduce $\beta_1$ Subunit-mediated Oxidative Inhibition of the Na$^+$-K$^+$ Pump in Myocytes*—We directly exposed freshly isolated myocytes to recombinant FXYD proteins. See supplemental Methods for details and rationale of this approach. Because immunodetection cannot distinguish between the native and recombinant FXYD1 protein, we used FXYD3 (mammary tumor protein 8). Myocytes were incubated with 500 nM recombinant human FXYD3 for 15 min before lysis. The $\alpha_1$ subunit co-immunoprecipitated with FXYD3 (Fig. 3*A*). This association of a FXYD protein, not native to myocytes, with the sarcolemmal Na$^+$-K$^+$ pump, is similar to the observed co-immunoprecipitation of $\alpha$ subunits in kidney membrane fragments exposed to exogenous FXYD10 from the shark rectal gland (20). The $\alpha_1$/FXYD3 co-immunoprecipitation was associated with a decrease in co-immunoprecipitation of the $\alpha_1$ subunit with native FXYD1 (Fig. 3*A*). This is consistent with competitive displacement of the native protein. Glutathionylation of FXYD3 was detected at baseline and was increased in myocytes exposed to ONOO$^-$ (Fig. 3*B*).

We mutated all four cysteines in FXYD3 to serine, thus synthesizing a "Cysless" FXYD3. Mutation of the FXYD3 cysteines in the membrane domain does not affect association with the Na$^+$-K$^+$ pump $\alpha$ subunit (21), supported in the current study by the similar level of Cysless and WT FXYD3 detected in the $\alpha_1$ immunoprecipitate (Fig. 3*A*). WT and Cysless FXYD3 also caused a similar reciprocal decrease in co-immunoprecipitation of the native FXYD1 with the $\alpha_1$ subunit (Fig. 3*A*). Mutation of FXYD3 cysteines abolished the strong band on the GSH antibody immunoblot that represented glutathionylation (Fig. 3*B*). Consistent with the effect of overexpressing FXYD1 in *Xenopus* oocytes (Fig. 2), preincubation of myocytes with WT FXYD3 eliminated the increase in glutathionylation of the $\beta_1$ subunit detected in myocytes exposed to ONOO$^-$. Cysless FXYD3 had no effect (Fig. 3*C*). FXYD3 also eliminated the increase in glutathionylation detected in myocytes exposed to angiotensin II, whereas the Cysless mutant did not (Fig. 3*D*). The effects of recombinant FXYD proteins on $\beta_1$ subunit glutathionylation should be evaluated in the context of the proportion that is glutathionylated as estimated with the biotin-GSH

[7] The abbreviations used are: Grx1, glutaredoxin 1; $\beta_3$ AR, $\beta_3$ adrenergic receptor; C1 and C2, numbering of candidate cysteine residues conserved in many FXYD proteins and corresponding to residues 40 and 42 in FXYD1; Cysless FXYD3, recombinant FXYD3 protein, with all cysteines mutated to serine.

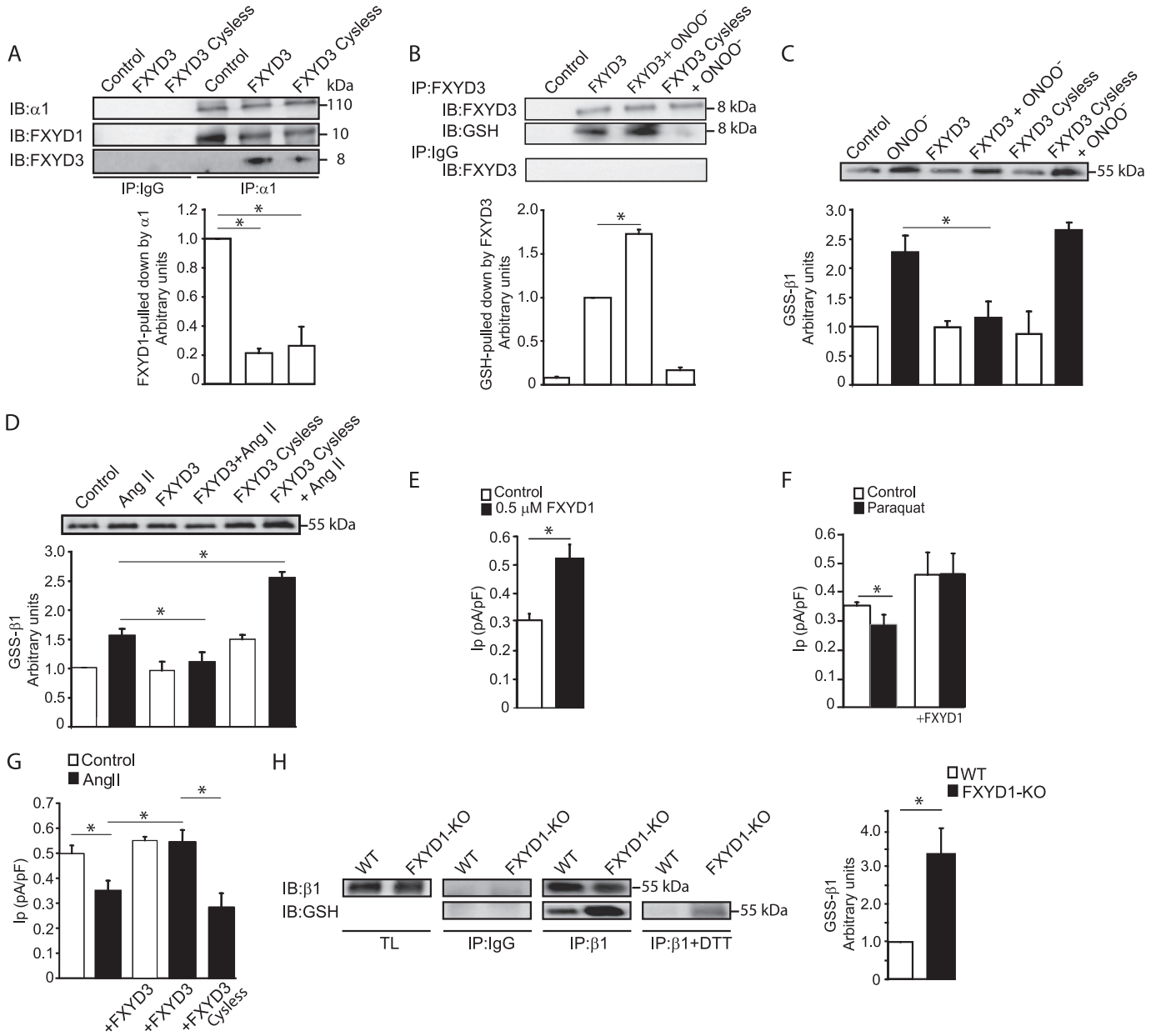


FIGURE 3. **FXYD proteins, $\beta_1$ subunit glutathionylation, and $Na^+$-$K^+$ pump activity in myocytes.** *A*, immunoblot (*IB*) of $\alpha_1$ subunit, FXYD1 and FXYD3 of $\alpha_1$ subunit immunoprecipitate (*IP*) from myocytes incubated with 500 nM recombinant FXYD3, or the mutated Cysless FXYD3 for 15 min. *B*, glutathionylation detected with GSH antibody in FXYD3 immunoprecipitate of lysed myocytes preincubated with recombinant FXYD3 or Cysless FXYD3 before exposure to $ONOO^-$ at a nominal concentration of 100 μM. *C*, glutathionylation of the $\beta_1$ subunit detected with the GSH technique (*GSS-*$\beta_1$) in myocytes preincubated with FXYD3 and Cysless FXYD3 with or without subsequent exposure to $ONOO^-$. *D*, glutathionylation of the $\beta_1$ subunit in myocytes incubated with FXYD3 and Cysless FXYD3 with or without subsequent exposure to 100 nM angiotensin II (*AngII*) for 10 min. *E*, $I_p$ measured with and without 500 nM FXYD1 in pipette solutions ($n \geq 5$ in each group). The superfusate was $Na^+$-free, and the test potential was −40 mV. *F*, $I_p$ measured with or without 100 μM paraquat and 200 nM FXYD1 in the pipette solution ($n \geq 9$ in each group). The superfusate contained $Na^+$, and the test potential was −40 mV. *G*, effect of 500 nM FXYD3 or Cysless FXYD3 on angiotensin II-induced decrease in $I_p$ ($n \geq 5$ in each group). Pipette solutions contained L-arginine, and the superfusate was $Na^+$-free, and the test potential was −14 mV. *H*, $\beta_1$ subunit glutathionylation in myocardium from $FXYD1^{-/-}$ (KO) mice and WT littermates (three mice per group). *TL* indicates total lysate. Histograms show mean ± S.E. *, $p < 0.05$.

technique, which varies in the range of ~15–40% (supplemental Fig. S2).

To further address the effects of FYXD proteins, we exposed myocytes to recombinant FXYD proteins by including them in the filling solutions of patch pipettes in voltage clamp experiments. Baseline control conditions varied for the reasons described in the supplemental Methods. FXYD1 increased $I_p$ (Fig. 3*E*), consistent with the increase in $Na^+$-$K^+$ ATPase activity with the exposure of shark rectal gland membrane fragments to a molar excess of exogenous purified FXYD10 that we reported previously (22). The decrease in $I_p$ with an oxidant signal induced by paraquat (23) was not observed when FXYD1 was included in patch pipette solutions (Fig. 3*F*). Similarly, an angiotensin II-induced decrease in $I_p$ was eliminated by FXYD3, but not by Cysless FXYD3 (Fig. 3*G*). These results indicate that functional effects of angiotensin II can be eliminated by exogenous FXYD3 shown to associate with the $Na^+$-$K^+$ pump (Fig. 3*A*) and that functional effects of the protein and its

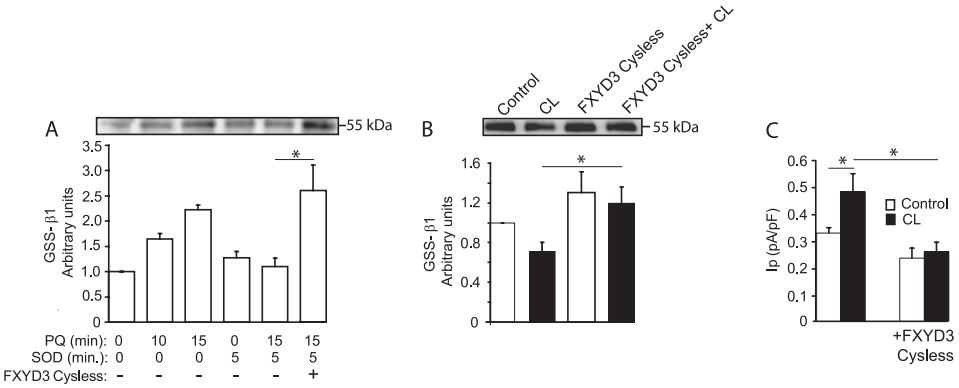


FIGURE 4. **Role of native FXYD1 in reversal of oxidative $Na^+$-$K^+$ pump modification.** *A*, myocytes were preincubated with Cysless FXYD3 or control solutions and exposed to 100 $\mu$M paraquat for 0, 10, or 15 min. This oxidant signal was quenched with superoxide dismutase (*SOD*) 10 min after paraquat (*PQ*) exposure, as indicated, and myocytes were lysed after an additional 5 min. $\beta_1$ subunit glutathionylation was assessed using the biotin-GSH technique. *B*, myocytes preincubated with Cysless FXYD3 were exposed to 1 $\mu$M CL316,243 (*CL*) prior to lysis. *C*, effect of Cysless FXYD3 on the increase in $I_p$ induced by 10 nM CL316,243. The superfusate was $Na^+$-free, and the test potential was −14 mV. Histograms show mean densitometry ± S.E. of immunoblots normalized against control (*A* and *B*; $n = 3$ for each experiment), or mean $I_p$ (*C*; $n = 6$–9 in each group). *, $p < 0.05$.

Cysless mutant are consistent with their effects on glutathionylation of the $\beta_1$ pump subunit (Fig. 3*D*). Thus, FXYD1 made by biosynthetic mechanisms (Fig. 2) and FXYD3 introduced as an exogenous protein reduced glutathionylation of the $\beta_1$ subunit and had similar effects on the pump inhibition induced by oxidative stimuli. In both cases, immunoprecipitation showed association of the FXYD proteins with the pump.

We used FXYD1$^{-/-}$ mice to examine *in vivo* effects of the FXYD protein on the $\beta_1$ subunit. We determined $\beta_1$ subunit glutathionylation in the myocardium of FXYD1$^{-/-}$ mice and their WT littermates. FXYD1 was detected in WT but not the FXYD$^{-/-}$ mice (data not shown). Consistent with our *in vitro* findings, the level of $\beta_1$ subunit glutathionylation was much lower in WT than FXYD1$^{-/-}$ myocardium (Fig. 3*H*).

*FXYD Proteins Reverse $Na^+$-$K^+$ Pump $\beta_1$ Subunit Glutathionylation*—The $\beta_1$ subunit is glutathionylated under baseline conditions in cardiac myocytes, reflecting an equilibrium that is determined by rates of glutathionylation and the reverse, "deglutathionylation" (24). The observation that FXYD proteins decrease oxidant-induced $\beta_1$ subunit glutathionylation may result from their ability to prevent glutathionylation or to facilitate deglutathionylation. We preincubated myocytes in control solutions or solutions containing Cysless FXYD3 with the aim of displacing native FXYD1. They were then exposed to paraquat for 0, 10, or 15 min. The oxidant signal was quenched 10 min after the onset of exposure to paraquat by the addition of PEGylated superoxide dismutase (11), and myocytes were lysed after an additional 5 min. We have shown that paraquat increases $\beta_1$ subunit glutathionylation and that addition of superoxide dismutase reduces glutathionylation compared with either the 10 or 15 min control experiments with exposure to paraquat only (11). This suggests that spontaneous deglutathionylation occurs. A decrease in $\beta_1$ subunit glutathionylation did not occur in myocytes incubated with Cysless FXYD3 (Fig. 4*A*), suggesting that displacement of native FXYD1 (Fig. 3*A*) with the recombinant protein disrupted the cellular pathways involved in deglutathionylation.

With a second approach, we avoided an oxidant signal and hence any increase in the rate of glutathionylation that might be prevented by native FXYD1. Activation of the $\beta_3$ adrenergic receptor ($\beta_3$ AR) decreases glutathionylation of the $\beta_1$ subunit from baseline in the absence of a preceding oxidant signal and stimulates the $Na^+$-$K^+$ pump (25). We hypothesized that Cysless FXYD3, by displacing FXYD1, would eliminate $\beta_1$ subunit deglutathionylation and hence pump stimulation. Preincubation of myocytes with Cysless FXYD3 eliminated a decrease in glutathionylation of the $\beta_1$ subunit induced by the $\beta_3$ AR agonist CL316,243 (Fig. 4*B*) and an increase in $I_p$ when included in patch pipette solutions (Fig. 4*C*). This suggests that the presence of a FXYD protein with a reactive cysteine is obligatory for $\beta_1$ subunit deglutathionylation and pump stimulation and that FXYD proteins, here FXYD1, are acting while associated with the $Na^+$-$K^+$ pump. Unassociated excess Cysless FXYD3 would not have been expected to have a nonspecific effect on $\beta_1$ subunit deglutathionylation. The data also provide additional independent evidence indicating exogenous FXYD competes with bound FXYD for association with the pump.

*Identification of Reactive FXYD1 Cysteine*—Two cysteines in the cytoplasmic domain were likely candidates for glutathionylation because they are conserved across the FXYD family and have adjacent basic amino acids. We refer to the cysteines as C1 and C2, which correspond to residues 40 and 42 in FXYD1 (Fig. 5*A*). We expressed WT FXYD1 and the Cys→Ala mutants FXYD1(C1A), FXYD1(C2A), and FXYD1(C1AC2A), in *Xenopus* oocytes. WT FXYD1 (*lane 4*) and the FXYD1(C1A) mutant (*lane 6*) were glutathionylated in oocytes injected with $ONOO^-$ as indicated by their immunodetection in the biotin-tagged glutathionylated fraction. Glutathionylation of FXYD1(C2A) or FXYD1(C1AC2A) mutants was not detected (Fig. 5, *B* and *C*). The effect of WT FXYD1 to reduce net $ONOO^-$-induced $\beta_1$ subunit glutathionylation (also shown in Fig. 2*A*) was preserved for the FXYD1(C1A) mutant but eliminated for the FXYD1(C2A) and FXYD1(C1AC2A) mutants (Fig. 5, *D* and *E*).

Expression of the FXYD1(C1A) mutant eliminated the decrease in $I_{max}$ after injection of $ONOO^-$, an effect that is similar to the effect of WT FXYD1. However, expression of the FXYD1(C2A) mutant and the FXYD1(C1AC2A) double mutant had no effect on the decrease in $I_{max}$ (Fig. 5*F*). As described previously (11), $ONOO^-$ did not affect the number of

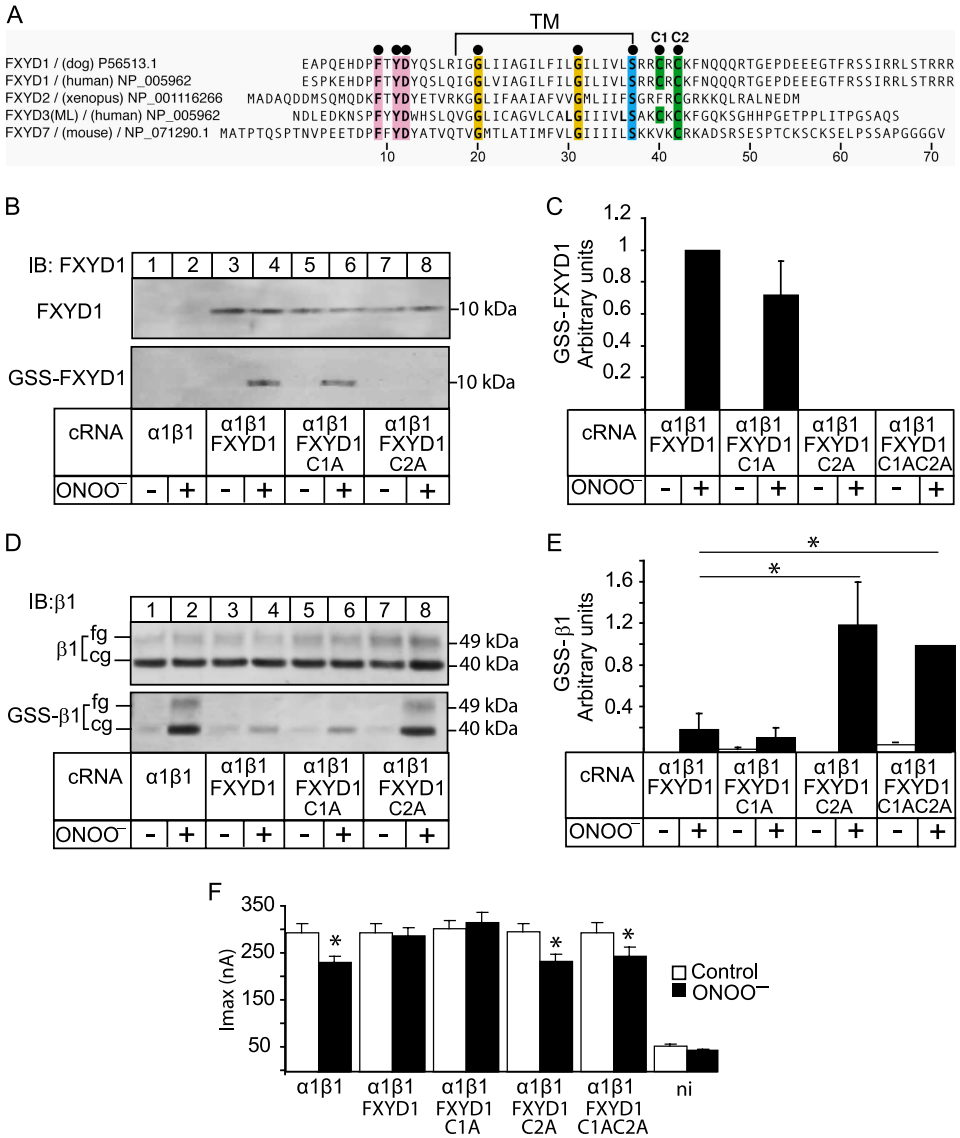


FIGURE 5. **Reactive cysteine residue in FXYD1, $\beta_1$ subunit glutathionylation and Na$^+$-K$^+$ pump current.** *A*, sequence alignment of FXYD proteins. *Numbering below* corresponds to the sequence of FXYD1 and begins at 1 after the signal peptide (not shown). Conserved residues are marked with *filled circles*. Transmembrane domain is indicated by *TM*. *B–E*, *Xenopus* oocytes expressing *Xenopus* $\alpha_1$ and $\beta_1$ pump subunits alone or with WT or mutated FXYD1 (FXYD1C1A, FXYD1C2A, or FXYD1C1AC2A) were injected with ONOO$^-$ as indicated. *B*, FXYD1 immunoblot (*IB*) of oocyte microsomes directly loaded on gels (*upper panel*) or immunoprecipitated with streptavidin beads (GSS-FXYD1, *lower panel*). *C*, mean densitometry ± S.E. of GSS-FXYD1 immunoblot normalized against control from four independent experiments. *D*, $\beta_1$ subunit immunoblot of oocyte microsomes (*upper panel*) or glutathionylated proteins (GSS protein; *lower panel*). *E*, mean densitometry of GSS-$\beta_1$ immunoblots normalized to the total amount of proteins from four independent experiments. Data from oocytes expressing $\alpha_1$, $\beta_1$, and FXYD1C1C2 subunits were arbitrarily set to 1. *F*, Na$^+$-K$^+$ pump currents of 20 oocytes from four different batches. Values of oocytes not injected with cRNAs (*ni*) were not subtracted from values of injected oocytes. *, $p < 0.05$ *versus* control. Core glycosylated and fully glycosylated $\beta_1$ subunits are indicated by *cg* and *fg*.

functional Na$^+$-K$^+$ pumps at the cell surface ([$^3$H]ouabain binding studies on intact oocytes; supplemental Fig. S3*A*), indicating that inhibition of the pump induced by ONOO$^-$ when $\alpha_1/\beta_1$ subunits were expressed alone or co-expressed with the FXYD1(C2A) or FXYD(C1AC2A) mutants, is due to a decrease in Na$^+$-K$^+$ pump turnover number (supplemental Fig. S3*B*). This supports a role for C2 of FXYD1 in modulating glutathionylation of the $\beta_1$ subunit and redox-dependent pump regulation.

*Basic Amino Acids Flanking Cysteines Are Essential for FXYD Protein Glutathionylation*—Because adjacent basic amino acids, positively charged at a physiological pH, facilitate glutathionylation of protein cysteines (26), we investigated whether the relationship of C1 and C2 with surrounding amino acids explain their differential susceptibility to glutathionylation. Fig. 6*A* shows a model of the predicted spatial relationship between C1 and C2 and the $\alpha_1$ subunit derived from the crystal structure of shark rectal gland Na$^+$-K$^+$ ATPase (7) and from cross-linking experiments (27). The model suggests that C2 comes into close proximity to basic amino acids of helix 4 of the $\alpha$ subunit. In contrast, C1 faces away from the positively charged environment.

C2 is flanked by two basic amino acids in FXYD1 but only one in FXYD2 (Fig. 5*A*). FXYD2, when expressed in *Xenopus* oocytes, did not eliminate an ONOO$^-$-induced decrease in $I_{max}$ (Fig. 6*B*), Na$^+$-K$^+$ pump turnover (supplemental Fig. S4), or

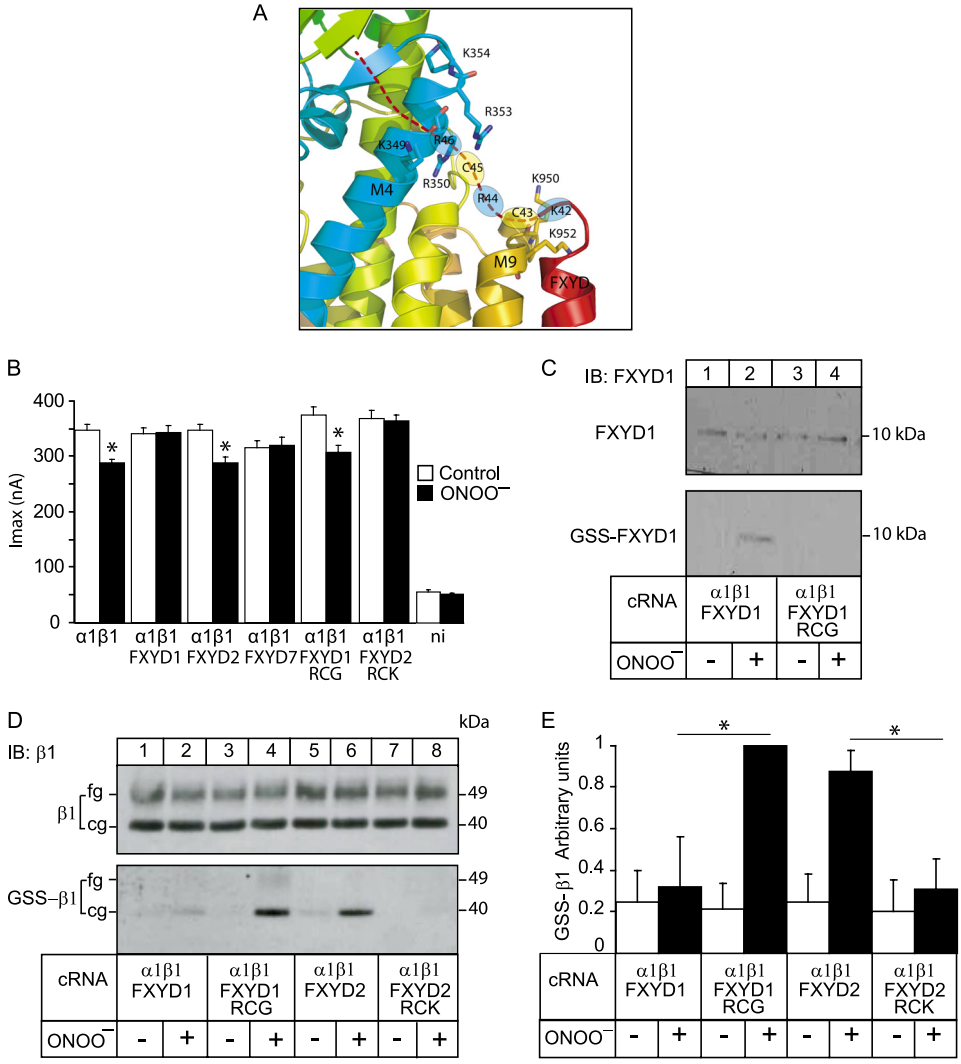


FIGURE 6. **Adjacent basic amino acids and reactivity of FXYD C2-equivalent cysteine.** *A*, projected three-dimensional structure, around the membrane/cytoplasmic interface of FXYD10. The extrapolated model is depicted using PyMOL. The last amino acid residue firmly detected in the crystal structure is $Lys^{42}$ just before the C1 equivalent ($Cys^{43}$). The putative trajectory of the FXYD cytoplasmic domain is shown by a *dotted red line*. *M* indicates transmembrane helix. *B*, $Na^+$-$K^+$ pump currents of oocytes expressing WT and mutant FXYD proteins. RCG and RCK refer to C2 (Fig. 5*A*) and its adjacent residues. Oocytes were injected with $ONOO^-$ as indicated. Results from 15 oocytes from three different batches are shown. $I_{max}$ from oocytes not injected with cRNAs (*ni*) were not subtracted from currents of injected oocytes. *C*, immunoblots (*IB*) of WT and mutant FXYD1. Oocyte microsomes were directly loaded on gel (*upper panel*) and glutathionylated subfraction precipitated by streptavidin (GSS-FXYD1, *lower panel*). *D*, $\beta_1$ $Na^+$-$K^+$ pump subunit immunoblots of oocyte microsomes directly loaded on gel (*upper panel*); and glutathionylated subfraction precipitated by streptavidin (GSS-$\beta_1$). *E*, mean densitometry of GSS-$\beta_1$ immunoblots from three independent experiments (as shown in *D*) in arbitrary units normalized by the total amount of proteins expressed. Data from oocytes expressing $\alpha_1$, $\beta_1$, and FXYD1 RCG were arbitrarily set to 1. *, $p < 0.05$. Core glycosylated and fully glycosylated $\beta_1$ subunits are indicated by *cg* and *fg*. Histograms show mean ± S.E.

glutathionylation of $Na^+$-$K^+$ pump $\beta_1$ subunits (Fig. 6*D*, *lanes 5* and *6*, and *E*). We mutated Lys adjacent to C2 to the neutral Gly, present in FXYD2. This FXYD1 RCK→RCG mutant was resistant to $ONOO^-$-induced glutathionylation (Fig. 6*C*). The effect of WT FXYD1 to abolish an $ONOO^-$-induced decrease in $I_{max}$ (Fig. 6*B*) and increase $\beta_1$ subunit glutathionylation was not observed with the mutant (Fig. 6*D*, *lanes 3* and *4*, and *E*).

Mutation of Gly adjacent to the C2 in FXYD2 to the basic Lys (FXYD2 RCG→RCK) was a "gain-of-function" mutation, with the mutant FXYD2 RCK reproducing the effect of WT FXYD1 in eliminating both $Na^+$-$K^+$ pump inhibition (Fig. 6*B*) and glutathionylation of the $\beta_1$ subunit of the pump (Fig. 6*D*, *lanes 7* and *8*, and *E*). The "loss-of-function" and gain-of-function mutations of FXYD1 and FXYD2 indicate the importance of flanking basic amino acids in reactivity of C2 in FXYD proteins.

C2 in FXYD1 and FXYD7 are flanked by Arg and Lys in the reverse order, and Arg promotes glutathionylation more than Lys (28). We examined whether the difference in sequence is functionally important. As was the case for FXYD1, expression of FXYD7 abolished an $ONOO^-$-induced decrease in $I_{max}$ (Fig. 6*B*) and pump turnover (supplemental Fig. S4*B*).

*Oxidative Stimuli Affect FXYD/$\beta_1$ and FXYD/$\alpha_1$ Interaction*—Effects of FXYD proteins on the $Na^+$-$K^+$ pump are usually attributed to their association with the $\alpha$ subunit. However, the three-dimensional structure of shark rectal gland $Na^+$-$K^+$ ATPase indicates that FXYD proteins also interact with the $\beta$ subunit (7). We examined whether oxidative stimuli alter the

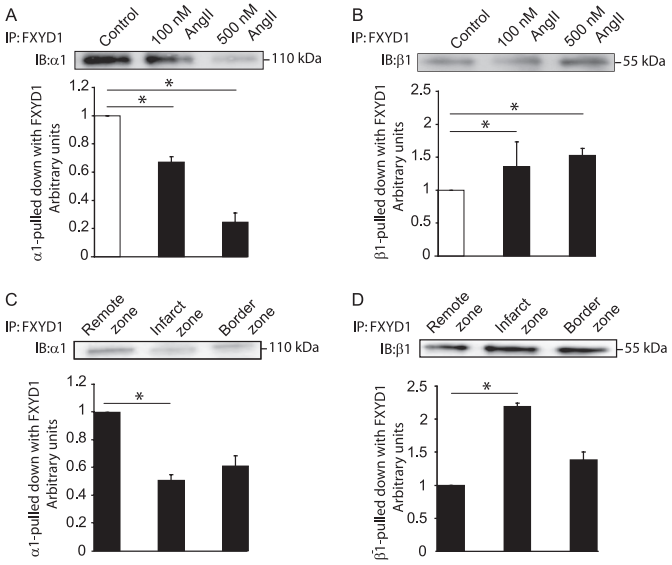


FIGURE 7. **Oxidative stimuli and cardiac FXYD1/$\alpha_1/\beta_1$ $Na^+$-$K^+$ pump subunit interaction.** *A*, $\alpha_1$ subunit immunoblot (*IB*) of FXYD1 immunoprecipitate (*IP*) in myocytes exposed to angiotensin II (*AngII*) or control. *B*, $\beta_1$ subunit immunoblot of FXYD1 immunoprecipitate in myocytes exposed to angiotensin II or control. *C*, $\alpha_1$ subunit immunoblot of FXYD1 immunoprecipitate in sheep myocardial samples taken from regions in and around a zone of myocardial infarction. *D*, $\beta_1$ subunit immunoblot of FXYD1 immunoprecipitate in myocardial samples from regions in and around a zone of myocardial infarction. Histograms show mean densitometry ± S.E. of immunoblots normalized against control ($n = 3$ for each experiment). *, $p < 0.05$.

interaction of FXYD1 with $Na^+$-$K^+$ pump subunits, as reflected by co-immunoprecipitation. Exposing myocytes to angiotensin II decreased the co-immunoprecipitation of FXYD1 with the $Na^+$-$K^+$ pump $\alpha_1$ subunits (Fig. 7*A*) but increased its co-immunoprecipitation with the $\beta_1$ subunits (Fig. 7*B*). In a similar manner, the oxidative stress of myocardial infarction decreased the co-immunoprecipitation of FXYD1 with the $\alpha_1$ subunits in infarct and border zones of the myocardium (Fig. 7*C*) but increased its co-immunoprecipitation with the $\beta_1$ subunits (Fig. 7*D*). We did not detect an effect of myocardial infarction on subunit expression levels.

Glutathionylation of the $\beta_1$ subunit of the $Na^+$-$K^+$ pump is associated with a decrease in its co-immunoprecipitation with the $\alpha_1$ subunit (11). Because FXYD proteins stabilize functional $Na^+$-$K^+$ pumps (29, 30) and decrease glutathionylation of the $\beta_1$ subunit (Figs. 2 and 3), we examined whether they affect $\alpha_1/\beta_1$ subunit co-immunoprecipitation. We overexpressed $\alpha_1/\beta_1$ subunits in oocytes with or without FXYD1 or the FXYD1(C2A) mutant. $ONOO^-$-induced $\beta_1$ subunit glutathionylation was associated with a decrease in $\alpha_1/\beta_1$ co-immunoprecipitation, which was reversed by co-expression of WT FXYD1 but not by co-expression of FXYD1(C2A) mutant (Fig. 8, *A* and *B*). $ONOO^-$ also decreased $\alpha_1/\beta_1$ subunit co-immunoprecipitation in cardiac myocytes. Addition of recombinant Cysless FXYD3 protein had no effect on the decrease, but WT FXYD3 prevented it (Fig. 8*C*). As for other experiments, concordant results were obtained whether FXYD proteins were expressed in oocytes or added exogenously to myocytes. Oxidant stress associated with myocardial infarction also decreased $\alpha_1/\beta_1$ subunit co-immunoprecipitation in infarct and peri-infarct zones of the myocardium (Fig. 8*D*).

## DISCUSSION

We show that a conserved cysteine residue in members of the FXYD protein family is susceptible to glutathionylation. We also show that this susceptibility is critical for a role they have in counteracting glutathionylation of the $\beta_1$ subunit of the $Na^+$-$K^+$ pump and the pump inhibition caused by subunit glutathionylation.

*FXYD-mediated Prevention or Reversal of $\beta_1$ Subunit Glutathionylation*—Co-expression of WT FXYD1 decreased glutathionylation of the $\beta_1$ pump subunit and eliminated pump inhibition in *Xenopus* oocytes injected with $ONOO^-$. We are not aware of mechanisms by which FXYD1 might prevent glutathionylation. Nonspecific antioxidant buffering is unlikely to contribute significantly because buffering by FXYD protein sulfhydryl groups would be negligible in comparison with the effect of abundant free sulfhydryl groups intrinsic to all cells (31). Consistent with this, the location and neighboring basic amino acids, but not the number, of FXYD1 cysteines was critical for effects on $\beta_1$ subunit glutathionylation and pump function.

As an alternative to prevention of $\beta_1$ subunit glutathionylation, FXYD1 might facilitate its reversal, or deglutathionylation, during the 15-min period after the bolus injection of $ONOO^-$, before we can measure glutathionylation or $I_{max}$. The oxidant signal would fade rapidly reflecting the short half-life of $ONOO^-$ (seconds). This might allow deglutathionylation to occur in the 15-min time period. Consistent with FXYD-mediated reversal of glutathionylation, Fig. 4*A* shows that reversal in myocytes did not occur when Cysless FXYD3 displaced WT FXYD1 (Fig. 3*A*). A role for WT FXYD1 to mediate deglutathionylation was supported independently by the effect of Cysless FXYD3 to eliminate a receptor-mediated decrease in glutathionylation from baseline (Fig. 4*B*) and an increase in $I_p$ (Fig. 4*C*) in the absence of a preceding oxidant signal.

*Reactivity of Cysteines in FXYD Proteins and $\beta_1$ Subunit*—One but not the other of the two most comprehensively studied FXYD proteins was susceptible to glutathionylation; the susceptible FXYD1 has two cytoplasmic cysteines adjacent to the membrane in a C1-R-C2 motif, whereas the corresponding FXYD2 motif (F-R-C2) has no C1-equivalent cysteine. Although this data suggested that C1 may be the reactive cysteine residue, mutation of C2 but not C1 abolished susceptibility to glutathionylation of FXYD1 identifying C2 as the reactive residue and indicating that C1 is not critical for reactivity. This was also supported by reactivity of the wild-type FXYD7 protein despite its lack of a C1-equivalent cysteine.

Glutathionylation is facilitated if basic amino acids flank cysteines in the amino acid sequence of proteins (26). Consistent with this, the RCG→RCK mutation of FXYD2 to flank C2 with basic amino acids rendered the derivative protein reactive. Conversely, the RCK→RCG mutation, which removed one of the basic amino acids flanking C2 in FXYD1, eliminated reactivity. Of the basic amino acids, Arg in particular facilitates glutathionylation of adjacent cysteines due to the distribution of its positive charge over a large volume (28). Despite this, C1 in FXYD1 with its R-C1-R motif (Fig. 4*A*) was not reactive. However, proximity of cysteines to basic amino acids in the

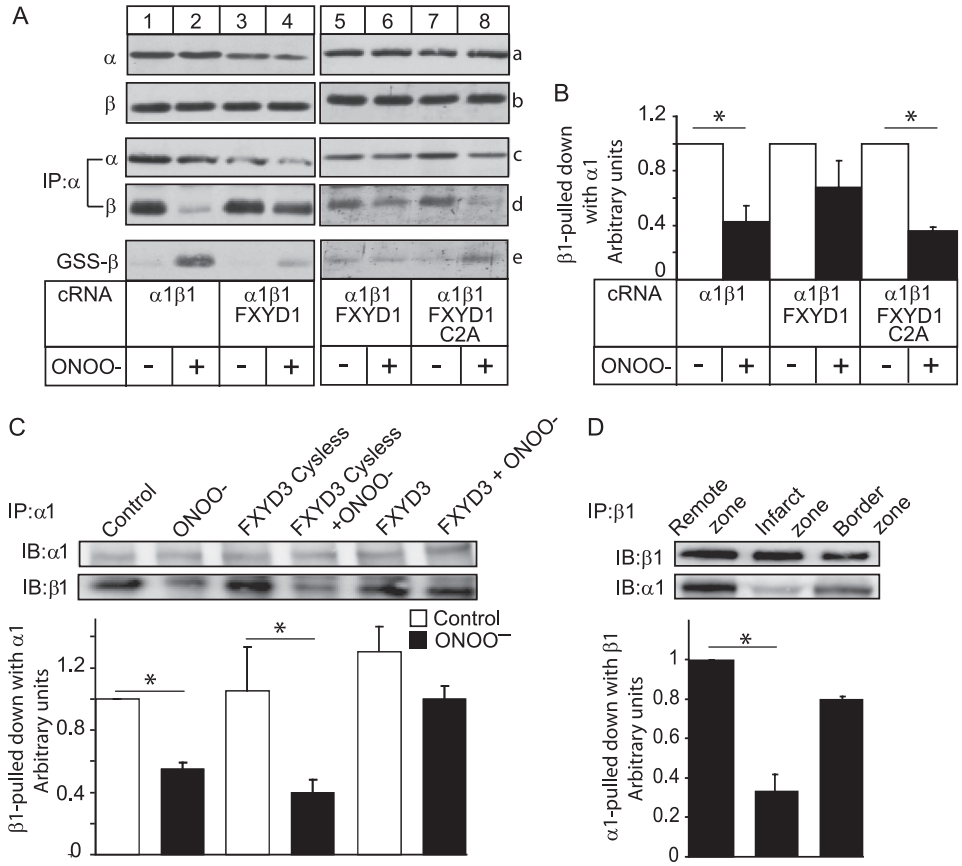


FIGURE 8. **Reactivity of FXYD protein and $\alpha_1/\beta_1$ subunit co-immunoprecipitation.** *A*, oocytes expressing $\alpha_1$ and $\beta_1$ pump subunit cRNAs with or without WT or mutant FXYD1 (FXYD1C2) were injected with $ONOO^-$ as indicated. Oocyte microsomes were directly loaded on gels (*a* and *b*), or immunoprecipitated (*IP*) with an $\alpha_1$ subunit antibody (*c* and *d*) or with streptavidin beads (GSS-*β*, *e*). *a* and *c* show $\alpha_1$, and *b*, *d*, and *e* show $\beta_1$ subunit immunoblots (*IB*). *B*, densitometry of $\beta_1$ subunit immunoblot after $\alpha_1$ subunit immunoprecipitation in three independent experiments. Data are normalized by the total amount of protein and by $\alpha_1$ subunit immunoprecipitated, expressed as a ratio compared with control oocyte. *C*, myocytes preincubated with recombinant FXYD3 or Cysless FXYD3 were exposed to $ONOO^-$ as indicated, and $\alpha_1/\beta_1$ subunit co-immunoprecipitation was detected in cell lysate. Densitometry of immunoblots from three independent experiments normalized against control are summarized. *D*, effect of oxidant stress of sheep myocardial infarction on $\alpha_1/\beta_1$ subunit interaction. Densitometry normalized against control in three experiments are summarized. Histograms show mean ± S.E. *, $p < 0.05$ *versus* control.

three-dimensional structure of a macromolecular complex is also important (28), and the model extrapolated from data previously published (Fig. 5*A*) suggests that the C1 equivalent (C43) in FXYD10 is pointing away from the two basic residues on transmembrane helix 9 ($Lys^{950}$ and $Lys^{952}$). In contrast, basic residues on transmembrane helix 4 ($Lys^{349}$, $Arg^{350}$, and $Arg^{353}$) are expected to add to the effect of the positive charges of the basic residues flanking C2 ($Cys^{45}$ in Fig. 5*A*) with the resulting milieu promoting glutathionylation of C2. This is also supported by models based on the three-dimensional structures of FXYD1 (5) and $Na^+$-$K^+$ ATPase (7).

Glutathionylation of the $Cys^{46}$ of the $\beta_1$ subunit seems unlikely in view of its location in the transmembrane domain and the absence of neighboring basic amino acids as seen in the crystal structure of the $Na^+$-$K^+$ pump in its $E_2$ conformation (7). However, the transmembrane domain of the $\beta_1$ subunit is somewhat detached from the transmembrane domains of the $\alpha$ subunit with the interaction stabilized by a single hydrogen bond only (7). Movements of $\alpha/\beta$ subunits relative to each other during the change to the $E_1$ conformation (7, 32) may disrupt the interaction and shift $Cys^{46}$ into a domain accessible to the aqueous environment of the cytosol where glutathionylation can occur. It is consistent with this that $\alpha_1/\beta_1$ subunit co-immunoprecipitation is markedly decreased in the $E_1$ relative to the $E_2$ conformation and that glutathionylation of the $\beta_1$ subunit is strongly dependent on $E_1$.[8] The low $pK_a$ required for reactivity of $Cys^{46}$ might be achieved if it is translocated into a milieu of basic amino acids in the three-dimensional structure of the $E_1$ conformation. Other determinants of cysteine $pK_a$ are also important, as reviewed (17, 33). Identifying location and orientation of $Cys^{46}$ in the crystal structure of the $E_1$ conformation of the $Na^+$-$K^+$ pump may explain its reactivity.

*Physiological and Pathophysiological Implications*—Physiological, receptor-coupled pathways can induce glutathionylation of the $\beta_1$ subunit and $Na^+$-$K^+$ pump inhibition by activating NADPH oxidase (11–13). Reversal of glutathionylation and pump inhibition is equally important, and we show that the reversal depends on FXYD proteins. We directly implicate FXYD1 in the pump stimulation and reversal of glutathionylation induced by $\beta_3$ AR activation *in vitro*. A similar increase in $\beta_1$ subunit glutathionylation in the myocardium from $FXYD1^{-/-}$ (Fig. 3*H*) and $\beta_3$ $AR^{-/-}$ (25) mice suggests this is of

[8] C.-C. Liu, A. Garcia, K. Karimi, N. A. S. Fry, E. J. Hamilton, R. J. Clarke, F. Cornelius, G. Figtree, and H. H. Rasmussen, unpublished studies on myocytes and pig kidney $Na^+$-$K^+$ ATPase.

*in vivo* relevance; similarities beween the cardiac phenotype of the mice (35, 36) is consistent with a shared downstream effect.

The physiological implications of FXYD1 glutathionylation may extend beyond $Na^+$-$K^+$ pump regulation; $Na^+$-$Ca^{2+}$ exchange activity is also redox-sensitive (37); but, to our knowledge, an oxidative molecular modification of the $Na^+$-$Ca^{2+}$ exchanger has not been identified. Because the exchanger is regulated by FXYD1 (4), we speculate that FXYD1 may confer redox sensitivity to its activity.

There may be pathophysiological implications of FXYD-dependent redox regulation of membrane transport. Raised levels of neurohormones that activate redox signaling (12, 13, 25), increased myocardial oxidative stress, and dysregulation of cytosolic $Na^+$ and $Ca^{2+}$ handling contribute to the pathophysiology of heart failure (38). Decreased FXYD1 expression (39) may accentuate such abnormalities. $Na^+$ and $Ca^{2+}$ dysregulation, known to occur with ischemia and infarction (40), may also be modified by glutathionylation of FXYD1 and $\beta_1$ subunits.

FXYD-dependent redox regulation may be important in the pathophysiology of some cancers. Malignant cells have a high oxidative load and strong intrinsic antioxidant defenses (41). Some tumors, notably from breast and prostate, overexpress FXYD3. Down-regulation of FXYD3 by siRNA techniques (42) or $Na^+$-$K^+$ pump inhibition with cardiac glycosides (43) impairs growth of prostate cancer cells, and clinical data suggest cardiac glycosides increase survival from breast cancer (44). Increased expression of FXYD3 may act to reduce oxidative inhibition of the $Na^+$-$K^+$ pump in cancer cells and promote their survival.

*Unresolved Issues*—This study did not examine whether reactivity of C2 in FXYD proteins is a determinant of $Na^+$-$K^+$ pump function independent of changes in $\beta_1$ subunit glutathionylation. Because $\beta_2$ or $\beta_3$ subunits do not have reactive cysteines (11), dependence of their glutathionylation status on FXYD cannot account for regulation of $\alpha/\beta$ pump heterodimers with these $\beta$ subunit isoforms. In view of the relationship between C2 and the $\alpha$ subunit in the three-dimensional structure (Fig. 6*A*) glutathionylation may nevertheless contribute to regulation, for example by influencing the PEGL motif further down transmembrane helix 4 of the $\alpha$ subunit that is critical for $Na^+$ binding (34). Other effects of glutathionylation on FXYD/$\alpha$ interaction may also contribute. The interaction identified in the known three-dimensional structure is mediated by van der Waals contacts and a single hydrogen bond. A charged 305-Da GSH adduct to C2 may disrupt the interaction, consistent with the decrease in FXYD/$\alpha$ coimmunoprecipitation with oxidative stress (Fig. 7). This may be important for regulation of activity since *in vitro* studies indicate FXYD1 stabilizes a functional $\alpha/\beta$ heterodimer (29, 30).

We do not identify the mechanism of interaction between C2 in FXYD proteins and $Cys^{46}$ in the $\beta_1$ subunit; it is of interest to briefly consider how a reactive C2 may facilitate deglutathionylation of $Cys^{46}$. In view of the large distance between them in the three-dimensional structure (7), any functional interaction may require greater disruption of the structure of the *FXYD*-$\alpha$-$\beta$ complex than what can be expected with $E_2 \rightarrow E_1$ conformational change alone. Relatively weak bonds of the $\alpha$ subunit to $\beta$ subunits and FXYD proteins identified in the $E_2$ conformation may be further weakened due to a combination of conformational changes and glutathionylation of C2. However, a network of multiple hydrogen bonds between FXYD and the $\beta$ subunit in the extracellular domain (7) is not expected to be affected by glutathionylation, speculations consistent with the effect of oxidative stress on the co-immunoprecipitation pattern of the *FXYD*-$\alpha$-$\beta$ complex (Fig. 7). Weakening of bonds to the $\alpha$ subunit may allow the *FXYD*-$\alpha$-$\beta$ complex or parts thereof to move to a domain that facilitates deglutathionylation. With waning of an oxidant signal, a functional FXYD/$\alpha/\beta_1$ complex may be restored.

Although it is established that Grx mediates deglutathionylation (24), the mechanism of Grx activation is unknown. Gallogly *et al.* (17) proposed that Grx may be bound to proteins or to components of multimeric protein complexes and that conformational changes facilitate deglutathionylation by bringing it into proximity of target mixed disulfide bonds. This would effectively allow regulation of activity. Consistent with such a scheme, Grx1 co-immunoprecipitates with FXYD1 and the $\beta_1$ subunit in cardiac myocytes lysate (supplemental Fig. S6). Because deglutathionylation is an "encounter reaction" (17), an intermediate enzyme-substrate complex does not exist. Thus, the co-immunoprecipitation of Grx1 with the $\beta_1$ subunit or FXYD1 likely reflects interactions at sites other than the reactive cysteines. Reactivity of C2 may be important for deglutathionylation of $Cys^{46}$ in $\beta_1$ subunit if C2 glutathionylation changes the three-dimensional structure of the C terminus and brings Grx1 bound to FXYD into proximity of binding sites near $Cys^{46}$ on the $\beta_1$ subunit. Because Grx1 possibly also facilitates the reverse reaction (17), a similar scenario could contribute to glutathionylation of the $\beta_1$ subunit under conditions of increased oxidative stress.

In conclusion, we show that reactivity of a conserved FXYD protein cysteine is important for physiologically relevant, receptor-coupled redox signaling, Our study also highlights remaining questions about mechanistic details. Some of these may be shared with other multimeric protein complexes.

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