FXYD Proteins Reverse Inhibition of the Na\(^+\)-K\(^+\) Pump Mediated by Glutathionylation of Its \(\beta_1\) Subunit*<sup>**</sup>

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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.

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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 mediate 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.
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 β1 subunit and FXD 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 (Ip). The patch pipette solutions perfusing the intracellular compartment included 10 mM Na⁺, a concentration near physiological intracellular levels. The solution also included 1-arginine, paraquat, and recombinant FXD proteins where indicated. Patch clamped myocytes were exposed to angiotensin II or CL316,243 in the superfusate after the whole cell configuration was established. Ip 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 α, and β1 Na⁺-K⁺ pump subunit cRNAs alone or together with WT or mutant FXD cRNAs. Two days after cRNA injection, S-glutathionylation of Na⁺-K⁺ pump β1 subunit and FXD 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 (Ip(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 Ip(max), oocytes were injected with ONOO⁻ 15 min before measurements.

RESULTS

FXD 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. FXD1 was detected readily in total cell lysate with an FXD1 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. 1A). Exposure of the myocytes to ONOO⁻ had no effect on expression of FXD1 in total cell lysate but increased the amount of FXD1 in the glutathionylated protein subfraction (Fig. 1, A and B). Glutathionylated FXD1 was not detected when the lysate was incubated with 1 mM DTT prior to precipitation by streptavidin (Fig. 1A), supportive of a mixed disulfide bond between FXD1 and GSH.

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. 1C, glutathionylation of FXD1 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 β1 subunit (13), and forskolin also increased the glutathionylation of FXD1 (Fig. 1D). This increase was abolished by incubating myocytes with PEGylated superoxide dismutase (GOD). Glutathionylation of FXD1 from a sheep model of infarction from myocardium remote to the infarct, the peri-infarct zone, and infarct zone. Immunoprecipitations (IP) were performed with FXD1 antibodies and immunoblots with FXD1 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.

![Figure 1. Glutathionylation of FXD1 in the heart.](image)

Glutathionylation of FXD1 in the heart. A, FXD1 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 FXD1 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 FXD1 detected by GSH antibody technique in myocytes exposed to angiotensin II (Ang II) for 15 min. D, glutathionylation of FXD1 in myocytes exposed to 100 nM forskolin (Fsk), with and without incubation with 200 international units/ml PEGylated superoxide dismutase (GOD). E, glutathionylation of FXD1 from a sheep model of infarction from myocardium remote to the infarct, the peri-infarct zone, and infarct zone. Immunoprecipitations (IP) were performed with FXD1 antibodies and immunoblots with FXD1 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.
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. 1E). A signal was not detected when lysate had been incubated with 1 \(\mu\)M 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 \(\beta\), 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. 2A, 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. 2A, 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. 2A, lanes 1–6). However, expression of FXYD1 reduced ONOO\(^-\)-induced glutathionylation of the \(\beta_1\) subunits (Fig. 2B). We measured \(I_{\text{max}}\) 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 \(I_{\text{max}}\) in oocytes overexpressing \(\alpha_1\) and \(\beta_1\) subunits alone. We previously have identified Cys\(^{\text{46}}\) 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 \(I_{\text{max}}\) under control conditions but eliminated the decrease in \(I_{\text{max}}\) induced by ONOO\(^-\) (Fig. 2C), in parallel with the effect of FXYD1 on \(\beta_1\) subunit glutathionylation (Fig. 2, A and B).

**FXYD Proteins Reduce \(\beta\), 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. 3A). 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. 3A). 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. 3B).

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 \(\alpha_1\) subunit (21), supported in the current study by the similar level of Cysless and WT FXYD3 detected in the \(\alpha_1\) immunoprecipitate (Fig. 3A). WT and Cysless FXYD3 also caused a similar reciprocal decrease in co-immunoprecipitation of the native FXYD1 with the \(\alpha_1\) subunit (Fig. 3A). Mutation of FXYD3 cysteines abolished the strong band on the GSH antibody immunoblot that represented glutathionylation (Fig. 3B). 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. 3C). FXYD3 also eliminated the increase in glutathionylation detected in myocytes exposed to angiotensin II, whereas the Cysless mutant did not (Fig. 3D). 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
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 FYXD 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. FYXD1 increased \( I_p \) (Fig. 3E), consistent with the increase in \( \text{Na}^+/-\text{K}^+ \) ATPase activity with the exposure of shark rectal gland membrane fragments to a molar excess of exogenous purified FYXD10 that we reported previously (22). The decrease in \( I_p \) with an oxidant signal induced by paraquat (23) was not observed when FYXD1 was included in patch pipette solutions (Fig. 3F). Similarly, an angiotensin II-induced decrease in \( I_p \) was eliminated by FYXD3, but not by Cysless FYXD3 (Fig. 3G). These results indicate that functional effects of angiotensin II can be eliminated by exogenous FYXD3 shown to associate with the \( \text{Na}^+/-\text{K}^+ \) pump (Fig. 3A) and that functional effects of the protein and its
Cysless mutant are consistent with their effects on glutathionylation of the \( \beta \) subunit subunit (Fig. 3D). Thus, FXYD1 made by biosynthetic mechanisms (Fig. 2) and FXYD3 introduced as an exogenous protein reduced glutathionylation of the \( \beta \) 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 \( \beta \) subunit. We determined \( \beta \) subunit glutathionylation in the myocardium of FXYD1
\(^{-/-}\) mice and their WT littermates. FXYD1 was detected in WT but not the FXYD1
\(^{-/-}\) mice (data not shown). Consistent with our in vitro findings, the level of \( \beta \) subunit glutathionylation was much lower in WT than FXYD1
\(^{-/-}\) myocardium (Fig. 3H).

FXYD Proteins Reverse Na\(^{+}\)-K\(^{+}\) Pump \( \beta \) Subunit Glutathionylation—The \( \beta \) 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 \( \beta \) 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 \( \beta \) 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 \( \beta \) subunit glutathionylation did not occur in myocytes incubated with Cysless FXYD3 (Fig. 4A), suggesting that displacement of native FXYD1 (Fig. 3A) 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 \( \beta \) subunit adrenergic receptor (\( \beta \)AR) decreases glutathionylation of the \( \beta \) 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 \( \beta \) subunit deglutathionylation and hence pump stimulation. Preincubation of myocytes with Cysless FXYD3 eliminated a decrease in glutathionylation of the \( \beta \) subunit induced by the \( \beta \)AR agonist CL316,243 (Fig. 4B) and an increase in \( I_p \) when included in patch pipette solutions (Fig. 4C). This suggests that the presence of a FXYD protein with a reactive cysteine is obligatory for \( \beta \) 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 \( \beta \) 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. 5A). We expressed WT FXYD1 and the Cys→Ala mutants FXYD1(C1A), FXYD1(C2A), and FXYD1(C1A2A), 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(C1A2A) mutants was not detected (Fig. 5, B and C). The effect of WT FXYD1 to reduce net ONOO\(^{-}\)-induced \( \beta \) subunit glutathionylation (also shown in Fig. 2A) was preserved for the FXYD1(C1A) mutant but eliminated for the FXYD1(C2A) and FXYD1(C1A2A) mutants (Fig. 5, D and E).

Expression of the FXYD1(C1A) mutant eliminated the decrease in \( I_{\text{max}} \) 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(C1A2A) double mutant had no effect on the decrease in \( I_{\text{max}} \) (Fig. 5F). As described previously (11), ONOO\(^{-}\) did not affect the number of
functional Na\(^{+}\)-K\(^{+}\) pumps at the cell surface (\(^{3}H\)ouabain binding studies on intact oocytes; supplemental Fig. S3A), 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 FXYD1(C1AC2A) mutants, is due to a decrease in Na\(^{+}\)-K\(^{+}\) pump turnover (supplemental Fig. S3B).

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.

![ 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 \(\alpha\), \(\beta\), and \(\beta\) 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, \(\beta\) subunit immunoblot of oocyte microsomes (upper panel) or glutathionylated proteins (GSS protein; lower panel). E, mean densitometry of GSS-\(\beta\) immuno-blots normalized to the total amount of proteins from four independent experiments. Data from oocytes expressing \(\alpha\), \(\beta_{1}\), 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.](https://doi.org/10.1074/jbc.M111.286513)
glutathionylation of Na\(^+\)-K\(^+\) pump β subunits (Fig. 6D, lanes 5 and 6, and E). We mutated Lys adjacent to Cys in FXYD1 to the neutral Gly, present in FXYD2. This FXYD1 RCK→RCG mutant was resistant to ONOO\(^-\) -induced glutathionylation (Fig. 6C). The effect of WT FXYD1 to abolish an ONOO\(^-\) -induced decrease in \(I_{\text{max}}\) (Fig. 6B) and increase β₁ subunit glutathionylation was not observed with the mutant (Fig. 6D, lanes 3 and 4, and E).

Mutation of Gly adjacent to the Cys in FXYD2 to the basic Lys (FXYD2 RCG→RCK) was a “gain-of-function” mutation, with the mutant FXYD2 RCG reproducing the effect of WT FXYD1 in eliminating both Na\(^+\)-K\(^+\) pump inhibition (Fig. 6B) and glutathionylation of the β₁ subunit of the pump (Fig. 6D, 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 \(I_{\text{max}}\) (Fig. 6B) and pump turnover (supplemental Fig. S4B).

Oxidative Stimuli Affect FXYD/β\(_1\) and FXYD/α\(_3\) Interaction—

Effects of FXYD proteins on the Na\(^+\)-K\(^+\) pump are usually attributed to their association with the α subunit (7). We examined whether oxidative stimuli alter the
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. 7A) but increased its co-immunoprecipitation with the \(\beta_1\) subunits (Fig. 7B). 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. 7C) but increased its co-immunoprecipitation with the \(\beta_1\) subunits (Fig. 7D). 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 \(\beta_1\) subunits (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. 8A 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. 8C). 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. 8D).

**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 \(I_{\text{max}}\). 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. 4A shows that reversal in myocytes did not occur when Cysless FXYD3 displaced WT FXYD1 (Fig. 3A). 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. 4C) and an increase in \(I_p\) (Fig. 4C) 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\(--\rightarrow\text{RCK}\) mutation of FXYD2 to flank C2 with basic amino acids rendered the derivative protein reactive. Conversely, the RCK\(--\rightarrow\text{RCK}\) 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. 4A) was not reactive. However, proximity of cysteines to basic amino acids in the
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three-dimensional structure of a macromolecular complex is also important (28), and the model extrapolated from data previously published (Fig. 5A) 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. 5A). The C1 equivalent in the crystal structure of the E1 conformation of FXYD10 is shown previously published (Fig. 5A) suggests that the C1 equivalent of the E1 conformation of FXYD1subunit is strongly dependent on E1.8 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 E1 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 E1 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. 3H) and \(\beta_3\) AR \(^{-/-}\) (25) mice suggests this is of

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**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-\(\beta_1\), e) 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 densitometry normalized against control in three experiments are summarized. Histograms show mean \pm S.E. *, \(p < 0.05\) versus control.
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in vivo relevance; similarities between 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⁺-Ca²⁺ exchange activity is also redox-sensitive (37); but, to our knowledge, an oxidative molecular modification of the Na⁺-Ca²⁺ 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 Ca²⁺ handling contribute to the pathophysiology of heart failure (38). Decreased FXYD1 expression (39) may accentuate such abnormalities. Na⁺ and Ca²⁺ dysregulation, known to occur with ischemia and infarction (40), may also be modified by glutathionylation of FXYD1 and β₁ 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 β₁ subunit glutathionylation. Because β₉ or β₁ subunits do not have reactive cysteines (11), dependence of their glutathionylation status on FXYD cannot account for regulation of α/β pump heterodimers with these β subunit isoforms. In view of the relationship between C2 and the α subunit in the three-dimensional structure (Fig. 6A) glutathionylation may nevertheless contribute to regulation, for example by influencing the PEGL motif further down transmembrane helix 4 of the α subunit that is critical for Na⁺ binding (34). Other effects of glutathionylation on FXYD/α 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/α coimmunoprecipitation with oxidative stress (Fig. 7). This may be important for regulation of activity since in vitro studies indicate FXYD1 stabilizes a functional α/β heterodimer (29, 30).

We do not identify the mechanism of interaction between C2 in FXYD proteins and Cys⁴⁶ in the β₁ subunit; it is of interest to briefly consider how a reactive C2 may facilitate deglutathionylation of Cys⁴⁶. 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-α-β complex than what can be expected with E₂ → E₁ conformational change alone. Relatively weak bonds of the α subunit to β subunits and FXYD proteins identified in the E₂ 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 β 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-α-β complex (Fig. 7). Weakening of bonds to the α subunit may allow the FXYD-α-β complex or parts thereof to move to a domain that facilitates deglutathionylation. With waning of an oxidant signal, a functional FXYD/α/β₁ 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 β₁ 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 β₁ subunit or FXYD1 likely reflects interactions at sites other than the reactive cysteines. Reactivity of C2 may be important for deglutathionylation of Cys⁴⁶ in the β₁ 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 Cys⁴⁶ on the β₁ subunit. Because Grx1 possibly also facilitates the reverse reaction (17), a similar scenario could contribute to glutathionylation of the β₁ 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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