

Lipid-binding properties of TRIM72

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TRIM72 is known to play a critical role in skeletal muscle membrane repair. To better understand the molecular mechanisms of this protein, we carried out an *in vitro* binding study with TRIM72. Our study proved that TRIM72 binds various lipids with dissociation constants (K_d) ranging from 88.2 ± 9.9 nM to 550.5 ± 134.5 nM. In addition, the intrinsic fluorescence of TRIM72 exponentially decreased when the protein was diluted with stirring. The time-resolved fluorescence decay occurred in a concentration-independent manner. The fluorescence-decayed TRIM72 remained in its secondary structure, but its binding properties were significantly reduced. The dissociation constants (K_d) of fluorescence-decayed TRIM72 for palmitate and stearate were 159.1 ± 39.9 nM and 355.4 ± 106.0 nM, respectively. This study suggests that TRIM72 can be dynamically converted by various stimuli. The results of this study also provide insight into the role of TRIM72 in the repair of sarcolemma damage. (BMB reports 2012; 45(1): 26-31)

INTRODUCTION

TRIM72, a TRIM family member, is predominantly expressed in cardiac and skeletal muscles (1). Recently, it was reported that TRIM72 is essential for skeletal muscle membrane repair, cardioprotection, and regulation of skeletal muscle differentiation (2-4). In striate muscle cells, TRIM72 was also shown to act as a regulator for membrane budding and exocytosis, which are linked to membrane repair (5). Therefore, known functions of TRIM72 are closely related to membrane-associated functions (2, 5). In addition, TRIM72 was also determined to interact with Caveolin-3 and Dysferin, which play a role in fusion of intracellular vesicles to damaged membranes (6). In a lipid profiling experiment, the recombinant TRIM72 protein appeared to bind phosphatidylserine, indicating that TRIM72 binds to the plasma

membrane (2). However, little is known about the molecular mechanisms behind the interaction between TRIM72 and the plasma membrane.

Using domain homology analysis, TRIM72 was shown to have a variety of functional activities (7). TRIM72 consists of a RING finger domain, a B-box, two coiled coil domains, and a spla and ryanodine receptor (SPRY) domain (8). Among those functional domains, the B-box domain and two coiled coil domains are known to be molecular interacting domains (8). Recently, the structure of the PRY-SPRY domain of TRIM72 was determined by X-ray crystallography (8). From the crystal structure, it was suggested that the PRY-SPRY domain may be responsible for the protein-protein interaction (8). However, no binding partner to TRIM72 has been identified.

In this study, we demonstrated that TRIM72 interacts with fatty acid component of the plasma membrane. The phospholipid/fatty acid-binding activity of TRIM72 provides further insight into the molecular role of TRIM72 in striated muscle membrane repair.

RESULTS

Lipid binding of TRIM72

Extremely limited amounts of TRIM72 was detected in the water-based extraction buffers (data not shown) when TRIM72 was purified from the *E. coli* extract. Therefore, the water solubility of TRIM72 was estimated using a sequence-based protein solubility (PROSO) software (9). This estimation suggested that TRIM72 is a water-insoluble protein (data not shown). To obtain the soluble form of TRIM72, sodium dodecyl sulfate (SDS) fractionation was performed. TRIM72 was determined to be selectively solubilized at 0.3% SDS buffer. After purification, TRIM72 appeared to be in a monomeric state (data not shown). Since TRIM72 has been shown to play an important role in membrane budding and vesicular exocytosis, we then examined whether TRIM72 interacts with various lipid molecules, the major components of the plasma membrane.

TRIM72-lipid binding was tested by measuring changes in its intrinsic fluorescence. TRIM72 possesses five tryptophans in the PRY-SPRY domain. Therefore, the intrinsic fluorescence of tryptophan was used for the *in vitro* binding study, since tryptophan fluorescence is altered by lipid-binding. As shown in Fig. 1, the relative tryptophan fluorescence was significantly enhanced as

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the lipid concentration increased, indicating that a variety of lipids appears to interact with TRIM72. The K_d values for phosphatidylserine and phosphatidylethanolamine were 88.2 ± 9.9 nM and 550.5 ± 134.5 nM, respectively. In addition, the K_d values for palmitic acid, stearic acid and sphingosine were 258.5 ± 58.1 nM, 239.7 ± 56.0 nM and 275.8 ± 51.3 nM, respectively. In regards to the K_d values, phosphatidylserine was determined to be the most tightly bound lipid. This result is consistent with data from the previous lipid profiling experiments (2). In order to better understand the binding activation of the phospholipid

head group for TRIM72, we conducted an additional binding experiment with ethylenediamine, which is a head analog group of phosphatidylethanolamine. However, the addition of ethylenediamine had no effect on the tryptophan fluorescence (Fig. 1). Accordingly, these binding data show that lipids containing fatty acyl groups bind TRIM72, whereas ethylenediamine, which is a head analog group of phosphatidylethanolamine, does not. In conclusion, fatty acyl groups play an important role in the lipid binding activity of TRIM72, and the other parts of phospholipids partially affect the binding affinity.

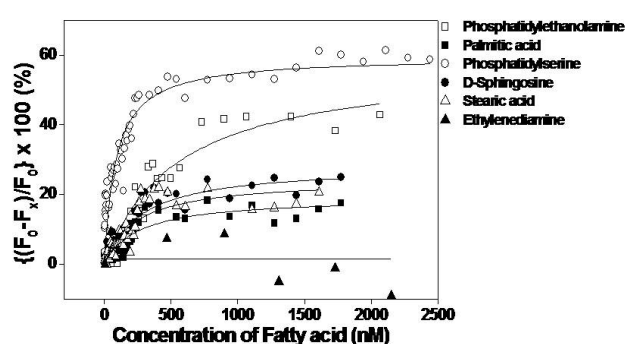


Fig. 1. Lipid-binding properties of TRIM72 were detected based on the intrinsic tryptophan fluorescence at various lipid concentrations. The F values were calculated using equation ($F = F_x - F_0$), where F_0 and F_x represent the fluorescence intensities of vehicle only and TRIM72 at various lipid concentrations. Data were plotted as a function of ligand concentration and the data fits were obtained as described in the Methods and Materials.

Structural conversion of TRIM72

Time-resolved intrinsic fluorescence of TRIM72 exponentially decreased upon dilution (Fig. 2A). After adding chloroform and carbon tetrachloride, the time-resolved fluorescence was shown to decrease more rapidly, indicating that the non-polar solvent enhances allosteric changes of TRIM72. To determine if the concentration dependency of this allosteric change is concentration-dependent, the time-resolved fluorescence was measured at three different TRIM72 concentrations (50, 100, or 250 nM). It was determined that the allosteric conversion of TRIM72 occurs in a concentration-independent manner (Fig. 2B). This finding also suggests that these allosteric changes monitored by fluorescence did not originate from changes in the multimeric state of TRIM72, because the process of multimerization occurs in a concentration-dependent manner. Accordingly, this conversion was probably caused either by dissociation of multimeric TRIM72 or by intramolecular structural changes. To determine which one was the major cause for the time-resolved decay, we measured changes in oligomerization using cross-linking experiments.

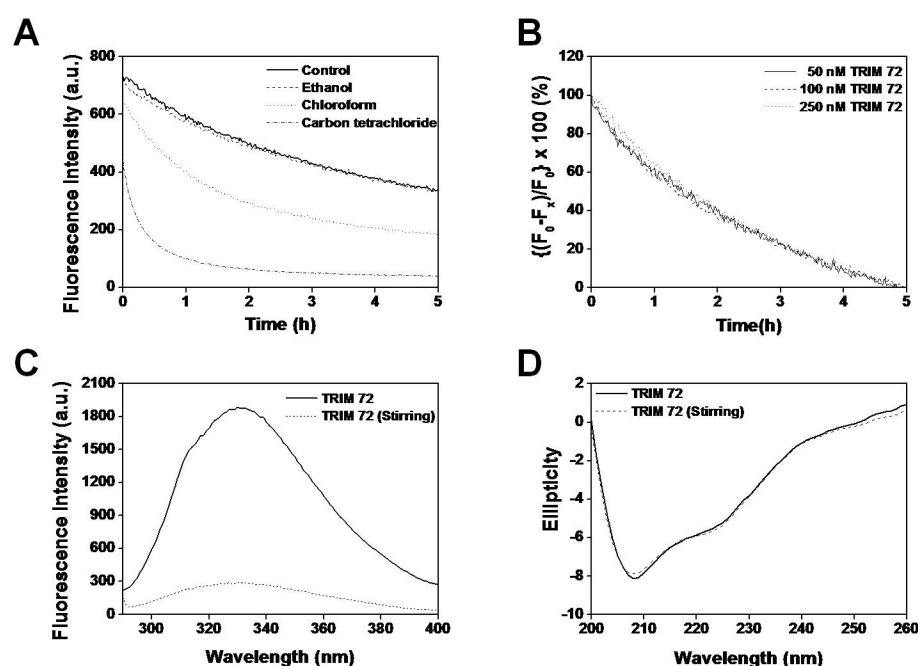


Fig. 2. Time-resolved fluorescence decay. (A) Time-resolved fluorescence values were monitored after dilution/stirring or addition of chloroform or carbon tetrachloride. (B) Time-resolved fluorescence was obtained at various TRIM72 concentrations. (C) Fluorescence spectra of an intact TRIM72 protein and a fluorescence-decayed TRIM72 after 10 h of stirring. (D) CD spectra for the intact protein and the fluorescence-decayed TRIM72.

From the cross-linking analysis, TRIM72 was determined to be monomeric (data not shown). Accordingly, it was estimated that the time-resolved decay may be caused by intramolecular structural changes. We then tested whether the secondary structure was altered during the time-resolved decay. CD spectra were acquired for high and low fluorescent protein samples to monitor change in the secondary structure (Fig. 2C, D). The fluorescence-decayed TRIM72 protein sample was obtained by stirring a high fluorescent protein sample for 10 h at room temperature. The CD spectrum for the fluorescence-decayed protein was the same as that for the high fluorescent sample (Fig. 2D). This data suggests that the allosteric conversion monitored by fluorescence was not due to secondary structural changes, but rather was caused by local environment changes in the tryptophan residues in intramolecular TRIM72.

Lipid binding properties of two forms of TRIM72

Because TRIM72 is converted to the fluorescence-decayed form by dilution and stirring for 10 h at room temperature, we determined if the fluorescence-decayed form of TRIM72 possessed lipid-binding activity. As shown in Fig. 3A, the fluorescence-decayed form of the protein was able to bind palmitic acid and stearic acid with K_d values of 159.1 ± 39.9 nM and 355.4 ± 106.0 nM, respectively. However, sphingosine and phosphatidylethanolamine did not bind to the fluorescence-decayed form of TRIM72, indicating that the lipid-binding properties of the fluorescence-decayed form were significantly altered when compared with those of the high fluorescent form. These results suggest that the conversion monitored by fluorescence was mainly

caused by an intrastructural change, which lead to a decrease in its binding properties. Our findings suggest that the structure and binding activity of TRIM72 is highly sensitive to the molecular environment, such as concentration, hydrophobicity, and physical forces.

Fatty acids, such as palmitate and stearate still interact with the fluorescence-decayed form of TRIM72, which indicates that the fatty acid binding activity of the protein is retained during the time-resolved fluorescence decay. To which factor is more important for binding between carboxylic acid and alkyl chains of fatty acid, we measured binding affinities for alkyl amines. Alkyl amines contain an alkyl group, but they do not have a carboxylic acid. In an experiment using the high fluorescent form of TRIM72, a variety of alkyl amines were observed to be associated with TRIM72. However, alkyl amines did not interact with the fluorescence-decayed form (Fig. 3B, C). Thus, the binding patterns of alkyl amines for the high fluorescent form were different from those of fatty acids. The K_d values for butyl, hexyl and octyl amines were 30.7 ± 4.1 mM, 19.4 ± 0.9 mM, and 29.0 ± 3.5 mM, respectively. The number of binding sites for butyl, hexyl and octyl amines were 0.9 ± 0.1 , 1.2 ± 0.1 , and 1.7 ± 0.2 , respectively.

Time-resolved fluorescence decay for fatty acid-binding

We also evaluated the kinetics of the dilution/stirring-induced fluorescence decay of TRIM72. The kinetic data were fit to a single exponential equation for fatty-acid free TRIM72 (Fig. 4A). However, when fatty acids were added to the TRIM72 protein, the fluorescence decay trends could not be fit to a single ex-

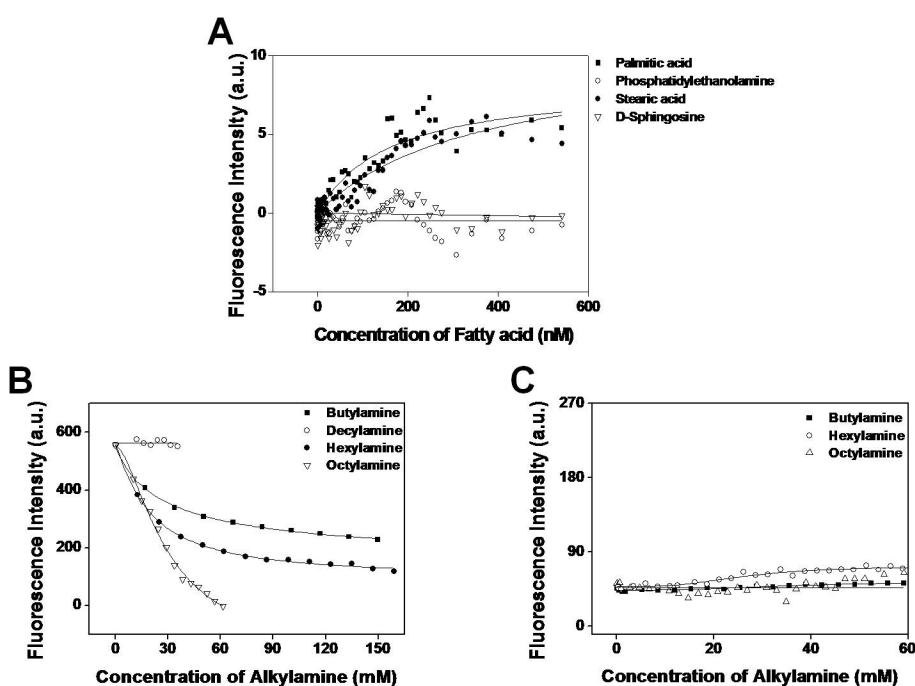


Fig. 3. Alkylamine-binding properties of both the high fluorescent TRIM72 and the fluorescence-decayed TRIM72. (A) Binding of the fluorescence-decayed form of TRIM72 to lipids was obtained at various ligand concentrations. Fatty acids were shown to interact with the fluorescence-decayed protein. (B) Binding profiles of the high fluorescent TRIM72 to alkylamines were also monitored as shown in panel A. Data fits were carried out as described in the Methods and Materials. (C) Binding of the fluorescence-decayed form of TRIM72 was detected as shown in panel B. Alkylamines did not bind to the fluorescence-decayed protein.

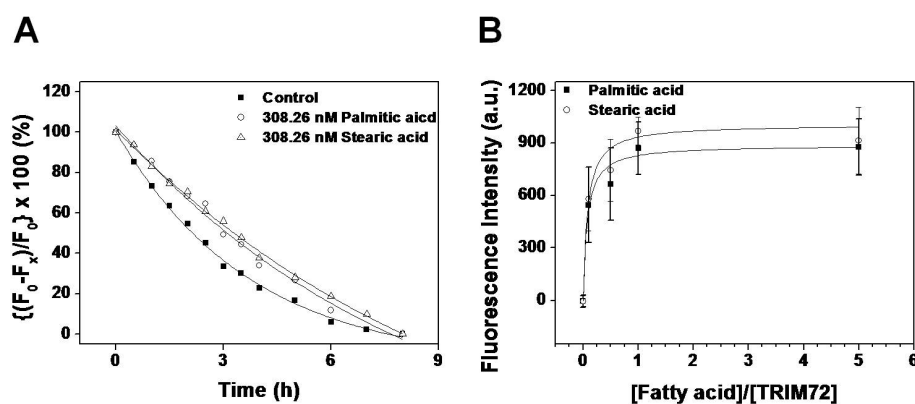


Fig. 4. Time-resolved fluorescence of ligand-free or fatty-acid saturated TRIM72. (A) Time-resolved fluorescence of TRIM72 (308 nM) was carried out as shown in Fig. 1. The data fits were obtained using equations (4) and (5) as described in the Methods and Materials. (B) Additional time-resolved fluorescence data were obtained at various fatty acid concentrations. These data were fit to equation (5), which provided F_2 . The F_2 values were then plotted as a function of molar ratio (fatty acids to protein).

ponential equation. Therefore, we hypothesized that fluorescence of fatty acid-bound TRIM72 may be differentially decayed. The time-resolved fluorescence data for fatty acid-bound TRIM72 were fit to an equation derived from a model that included both bound- and unbound-species (see Materials and Methods). Our model was feasible because fluorescence changes due to fatty acid binding were 10-fold less than the fluorescence difference between from the high fluorescent form and the fluorescence-decayed form of TRIM72. Fitting the data to a two species model produced a parameter (F_2) that represents the amount of fatty acid-bound form of TRIM72. Subsequently, we replotted F_2 versus molar ratios of fatty acids to protein (Fig. 4B). The result from this replotting confirmed that fatty acid binds TRIM72 at a 1 : 1 molar ratio.

DISCUSSION

The cellular functions of TRIM72 have been suggested to be associated with exocytosis, membrane budding and skeletal muscle membrane repair (5). These suggested cellular functions imply that TRIM72 may interact with the plasma membrane. TRIM72 has been shown to bind membrane proteins, e.g., Caveolin-3 (6). In addition, Cai *et al.* found that a recombinant his-tagged TRIM72 protein interacts with phosphatidylserine (2). These findings confirm that TRIM72 can be localized and act as a regulator of membrane biology. However, the biochemical features of molecules that bind to TRIM72 have not been explicitly determined through *in vitro* binding experiments. To date, *in vitro* analyses have been carried out using only water-soluble constructs such as tagged TRIM72 proteins or deletion mutants (2, 8). In the current study, the binding properties of untagged/undeleted and intact TRIM72 were evaluated. To solubilize the water-insoluble TRIM72, SDS fractionation was performed. SDS fractionation probably hinders the functionally active 3-D structure of the cellular form of TRIM72. However, given that TRIM72 is a membrane-associated protein (a biological detergent-surrounded protein), TRIM72 prepared by our method is more likely to be in the cellular form, because detergents are mimics of membrane lipids. The prepared TRIM72 was shown to bind tightly to phosphatidylserine, which

is consistent with a previous report (2). This result suggests that the TRIM72 protein retained its biological activity.

Our *in vitro* binding studies can be linked to the *in vivo* functions of TRIM72. The *in vitro* binding data demonstrated that TRIM72 differentially binds lipids in the plasma membrane and the level of binding depends on the lipid concentrations. At low concentration, TRIM72 can selectively interact with phosphatidylserine, because the K_d for phosphatidylserine is lower than the other lipids that we measured (Fig. 1). As lipid concentration increases, TRIM72 was shown to bind to a variety of other membrane lipid components including palmitate, stearate, and sphingosine. This binding behavior may explain how TRIM72 functions during the period of skeletal muscle repair. In the initial stage of membrane repair, membrane damage induces the inner leaflet, which contains phosphatidylserine, to flip onto the outer leaflet and under these conditions TRIM72 can be recruited to phosphatidylserine in the wound lesion. This recruitment is likely to elevate its local concentration. Elevated levels of TRIM72 in the local membrane lesion have been suggested to be assembled and surrounded by other non-polar lipids. Our findings further suggest that TRIM72 can be dissociated with lipid molecules in the non-polar environment (Fig. 3). This dissociation indicates that TRIM72 can be delocalized from the repaired membrane regions after the wounded membrane is repaired. Take together, our *in vitro* data provides insight into a potential *in vivo* model of the skeletal muscle repair process.

However, there are some discrepancies between these *in vitro* experiments and its potential *in vivo* functions. For example, cellular TRIM72 was shown to form a dimer under certain conditions, whereas TRIM72 obtained by SDS fractionation was monomeric. The homology domain-based conjecture suggests that TRIM72 is oligomeric, because two coiled coil domains are known to be responsible for oligomerization (10). In addition, Cai *et al.* reported that TRIM72 was a dimer under oxidized conditions (2). Therefore, our *in vitro* data may have some limitations in regards to its *in vivo* function. Our monomeric TRIM72 results demonstrate one notable biochemical feature, the stoichiometric number. The kinetic data from the time-resolved fluorescence decay experiments (Fig. 4) showed

that TRIM72 binds fatty acids with 1 : 1 molar ratio. This stoichiometric number provides further information on the structural features of full-length TRIM72. Since the crystal structure of full-length TRIM72 has not yet been determined, our stoichiometric data provides a valid structural feature that may explain a mechanistically unknown functionality. It is still unknown how TRIM72 can bind lipids in a multimeric form.

In summary, our present study identified new TRIM72-binding molecules and their binding behaviors, thereby providing further insight into the detailed mechanisms of TRIM72 activity in regards to membrane budding, vesicular trafficking and muscle repair.

MATERIALS AND METHODS

Purification of TRIM72

TRIM72 cDNA was obtained by PCR amplification with using a forward primer (5'catgccatggctatgtcggctgcacccgg3'), a reverse primer (5'ccgctcagtcaggcctgttcc 3'), and a previously cloned template DNA containing *trim72* gene (4). Then the PCR product was inserted into a pET-28a plasmid by cutting with Nco I and Xho I and ligation. The fusion plasmid designed to express untagged TRIM72 was subsequently transformed into *Escherichia coli* BL21. The transformed *E. coli* strains were grown overnight in 5 ml Luria-Bertani (LB) medium with 50 µg/ml kanamycin at 37°C. The *E. coli* cells were subcultured in 2.5 L LB medium with 50 µg/ml kanamycin at 37°C until the optical density (600 nm) was between 0.4 and 0.6. Protein expression was then induced via the addition of 0.1 mM IPTG for 4 h at 37°C. The cells were harvested by centrifugation. The cell pellet was resuspended in ice-cold ST buffer (7.5 mM Tris-Cl (pH 8.0), 150 mM NaCl) in the presence of 1 mM PMSF, 5 mM DTT and lysozyme (100 µg/ml) and subsequently disrupted by ultrasonication. The lysed cells were separated by centrifugation. The debris was dissolved and fractionated by adding 0 to 0.33% SDS in ice-cold ST buffer (see supplementary data). The debris fractions were then analyzed by 10% SDS-PAGE and visualized with Coomassie Brilliant Blue staining.

Fluorescence spectroscopy

Fluorescence spectra for TRIM72 recombinant protein were obtained using a Shimadzu RF-5301PC spectro-fluorometer (Kyoto, Japan). Protein samples were excited at 280 nm, and emission spectra were obtained from 290 to 400 nm. In the binding assays with TRIM72, 0.25 µM protein was placed in a cuvette, and a small volume of concentrated lipid solution was added to the cuvette in a stepwise manner. The fluorescence intensity (F) for the mixture was obtained at 340 nm, and F was calculated according to Equation 1,

$$F = F_x - F_0 \quad (1),$$

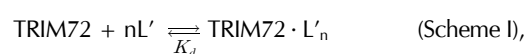
where F_0 and F_x represent the fluorescence intensities of vehicle only and TRIM72 at various lipid concentrations. A non-

linear fitting procedure was conducted using the following equation (Equation 2) for binding of protein (TRIM72) to lipids (L) to calculate the dissociation constant (K_d),

$$F = (F_{\max} \times [L]) / (K_d + [L]) \quad (2),$$

where F_{\max} is the F value at lipid saturation. Data were fitted using Origin software (Microcal, Northampton, MA, USA).

For nonlinear fits for binding of protein (TRIM72) to alkylamine (L'), we built a model (Scheme 1) describing multiple binding events,



where the dissociation constant (K_d) equals (TRIM72) (L')ⁿ / (TRIM72 · L'_n). We were then able to derive the following equation (Equation 3),

$$F = \{(F_{\min} \times K_d) + (F_{\max} \times [L']^n)\} / (K_d + [L']^n) \quad (3),$$

where n is the number of ligand-binding sites. Data were fitted using Origin software (Microcal, Northampton, MA, USA).

Time-resolved fluorescence

Time-resolved binding data were obtained by measuring fluorescence intensities monitored at 340 nm (excitation at 280 nm). We added 2.31 µl of 200 µM palmitate to 1.5 ml of 308.26 nM protein with stirring at 25°C. Fluorescence intensity was detected and recorded at 1.2 min intervals. Experimental data for ligand-free TRIM72 were obtained using the fluorescence intensities for time-resolved fluorescence and then the data was fit to a single exponential equation,

$$F(t) = F_1 e^{-k_1 t} \quad (4),$$

where F(t) and F_1 are values of the bound protein fraction at $t = t$ and $t = 0$, respectively. Data were fit using Origin software (Microcal, Northampton, MA, USA).

For binding of TRIM72 to fatty acids, experimental data were fit to two independent exponential equations,

$$F(t) = F_1 e^{-k_1 t} + F_2 e^{-k_2 t} \quad (5),$$

where F_1 and F_2 represent fluorescent intensities for ligand-free TRIM72 and fatty acid-bound TRIM72, respectively.

Circular dichroism spectroscopy

Circular Dichroism (CD) spectra of TRIM72 protein and its fluorescence-decayed form were obtained in ST buffer at room temperature using a Circular Dichroism Detector (Leatherhead KT22, United Kingdom). The cell pathlength was 1 mm. Spectra were acquired in duplicate for each sample and then an averaged spectrum was calculated. A buffer baseline scan was then subtracted from

the average protein scan. The CD data are described as the mean residue ellipticity as previously described (11).

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