Published in final edited form as:

Biochem Biophys Res Commun. 2009 June 19; 384(1): 66–70. doi:10.1016/j.bbrc.2009.04.073.

Conformational detection of p53's oligomeric state by FIAsh Fluorescence

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Abstract

The p53 tumor suppressor protein is a critical checkpoint in prevention of tumor formation, and the function of p53 is dependent on proper formation of the active tetramer. *In vitro* studies have shown that p53 binds DNA most efficiently as a tetramer, though inactive p53 is predicted to be monomeric *in vivo*. We demonstrate that FIAsh binding can be used to distinguish between oligomeric states of p53, providing a potential tool to explore p53 oligomerization *in vivo*. The FIAsh tetra-cysteine binding motif has been incorporated along the dimer and tetramer interfaces in the p53 tetramerization domain to create reporters for the dimeric and tetrameric states of p53, though the geometry of the four cysteines is critical for efficient FIAsh binding. Furthermore, we demonstrate that FIAsh binding can be used to monitor tetramer formation in real-time. These results demonstrate the potential for using FIAsh fluorescence to monitor protein-protein interactions *in vivo*.

Keywords

FIAsh; p53; tetramerization; oligomerization; fluorescence

Introduction

The p53 tumor suppressor protein is a 393 amino acid transcription factor that plays a critical role in cellular response to a number of potential cellular stresses, including DNA damage, hypoxia, hypothermia, and oxidative stress [1-3]. Activity of p53 depends on the correct association of the α -helical tetramerization domain, which associates in a dimer of dimers structure (Fig. 1) [4,5]. The DNA-binding domain of p53, which is monomeric, binds DNA with a 10- to 100-fold reduced affinity compared to full-length p53 and tetrameric p53 is more efficient at bending DNA than monomeric p53, highlighting the importance of oligomerization in p53 function [6,7].

The majority of studies on p53 oligomerization have been conducted *in vitro* due to the technical challenges surrounding monitoring of p53's oligomeric state *in vivo*. There are no available antibodies capable of distinguishing between monomeric, dimeric and tetrameric p53 for immuno-imaging. Fluorescence Resonance Energy Transfer (FRET) labeling with variants of green fluorescent protein (FP) is not possible for p53 since the homotetrameric nature of

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p53 would lead to a variety of products upon dimer or tetramer formation, making determination of the oligomeric state impossible via this approach. An alternative to FRET labeling or immunocytochemistry is the fluorescent probe FAsH, a biarsenical-modified fluorescein ligand that binds to a tetra-cysteine (TC) binding motif [8]. FAsH is nonfluorescent until association with the properly formed TC-binding motif, making it a selective reporter of conformational change [8]. Previous studies have utilized FAsH in protein localization studies, for measuring protein stability *in vivo* and as a probe for protein aggregation *in vivo* [9-11]. We now use the p53 tetramerization (p53-tet) domain to demonstrate that the TC-binding motif can be divided between two proteins and FAsH binding can serve as a reporter of oligomeric state changes.

The p53-tet domain packs in a dimer of dimers arrangement, resulting in a natural two-fold symmetry. Specific amino acid contacts occur along the dimer interface and a different set of amino acids form the tetramer interface [12]. The distinct dimer and tetramer interfaces present the possibility of generating reporters that are specific for each interface. In addition, the two-fold symmetry of the tetramerization domain requires only two amino acid substitutions to generate the complete TC-binding motif, making the p53-tet domain an ideal model system for these studies.

The TC-binding motif was incorporated along the dimer and tetramer interfaces of the p53 tetramerization domain to produce reporters that are selective for either the dimeric or tetrameric forms of p53. Our results indicate that proper geometry of the four cysteines composing the TC-binding motif is critical for effective binding and that the kinetics of FAsH binding are sufficient for monitoring changes in protein oligomerization in real-time. These studies demonstrate the potential of using FAsH as a reporter for changes in oligomerization and also the potential of this system to increase understanding of the role of oligomerization in p53 function.

Materials and Methods

Materials

All materials were purchased from major chemical suppliers. Restriction enzymes were purchased from New England Biolabs. DNA polymerase was purchased from Stratagene. Primers were purchased from Integrated DNA Technologies.

p53-Tet domain and TC-Tet domain cloning—The human p53 tetramerization domain coding sequence (amino acids 310 – 360) was cloned into the pRSET A expression vector (Invitrogen). Two cysteines were introduced into the tetramerization domain along either the dimer or tetramer interface using the QuikChange mutagenesis kit (Stratagene, primers shown in Supplementary Table).

Expression and Purification of p53-Tet clones—Expression of p53-Tet was induced by IPTG at 37 °C overnight in BL21(DE3)pLysS cells. Expressed cells were collected by centrifugation and resuspended in 35 mL of Wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 7.5) with 5 mL 10% Triton X-100 (v/v), 5 µg/ml DNase, and 1 µg/ml MgCl₂. Cells were lysed by a combination of freeze/thaw and sonication. Insoluble protein was removed by centrifugation and expressed p53-Tet was purified using a HiTrap™ Chelating HP column containing Ni²⁺ as a cation (GE Healthcare) and a 250 mM imidazole step gradient. Eluted protein was further purified using a Superdex™ 75 Prep column and Wash Buffer as the mobile phase. Final protein purity was assessed by Coomassie staining as >98% pure. Protein concentration was determined using a conversion factor of A₂₈₀ 1 = 2.23 mg/ml, as determined by Bradford and purified protein was stored at 4° C.

Size-Exclusion Chromatography (SEC) Characterization

p53-Tet and TC-Tet proteins were separated using an analytical Superdex™ 75 column with Wash buffer as a mobile phase. Protein elution was monitored by UV at 280 nm.

FIAsH binding

Purified p53-Tet or TC-Tet protein was diluted to 10 μ M in FIAsH binding buffer (10 mM HEPES, 1 mM TCEP, 1 mM BME). 0.4 μ M FIAsH (final concentration) was added to each sample followed by a 2-3 hour incubation at room temperature in the dark. Emission spectra were measured from 500 nm to 650 nm (excitation = 490 nm) using a Fluoromax-3 spectrofluorimeter (Jobin-Yvon, Edison, NJ).

Tetramerization domain Refolding

Purified protein was diluted to 10 μ M in 50 mM Tris, 150 mM NaCl, 8 M urea, pH 7.5 and allowed to unfold for 1 hour. Refolding was initiated by dilution of 150 μ l of denatured protein in to 1.350 mL of 50 mM Tris, 150 mM NaCl, pH 7.5 in the presence of 40 nM FIAsH. Tryptophan fluorescence was measured over time by excitation at 280 nm and emission at 340 nm. FIAsH fluorescence was measured over time by excitation at 490 nm and emission at 530 nm.

Results

The predicted ideal geometry of the TC-binding motif is a parallelogram-like geometry with distances of 4-5 Å between cysteines on one set of parallel faces and 6-8 Å between cysteines on the other set of parallel faces [13,14]. We identified potential TC-binding sites along both the dimer (Fig 1a) and tetramer (Fig 1b) interfaces that exhibited ideal distances and geometry. However, previous studies had shown that mutation of amino acid 350 destabilizes the tetramerization domain [12]. Therefore, a second site was identified along the tetramer interface (Fig 1c) albeit with a non-ideal geometry but also without any predicted negative effects on tetramer stability.

The TC-binding motif was incorporated into all three identified sites (Tet-329, Tet-350, and Tet-351), and a mutation known to inhibit tetramer formation (L344A) [15] was incorporated into the Tet-350 and Tet-351 mutants (Tet-5044 and Tet-5144 respectively). Each protein was expressed and purified under identical conditions as the native p53-tet protein. Gel filtration chromatography was used to verify that the insertion of the TC-binding motif did not affect the overall structure of the p53-tet domain (Fig 2b). Tetrameric p53-tet domain elutes with a retention time of 9.3 minutes, while dimeric p53-tet protein (Tet-344) elutes with a retention time of 10.2 minutes. The Tet-329, Tet-350 and Tet-351 proteins all elute with similar retention times as the native p53-tet protein while the Tet-5044 and Tet-5144 proteins eluted with a similar retention time as the Tet-344 protein (Tet-329 and Tet-5144 data not shown). More concentrated protein samples elute with slightly faster retention times, consistent with the known concentration dependence associated with this protein. These results indicate that the insertion of the TC-binding motif has not altered the oligomeric state of the TC-tet proteins.

We next investigated the selectivity of FIAsH binding by incubating purified p53-tet, Tet-350 and Tet-351 protein with 0.4 μ M FIAsH and measuring the fluorescence emission spectrum from 500 to 650 nm. FIAsH binding to the Tet-350 protein exhibits a 3-fold increase in fluorescence intensity over native p53-tet protein (Fig 3a), while the Tet-351 protein only showed 1.5-fold increase in signal over the unlabeled p53-Tet. The decreased signal of Tet-351 relative to Tet-350 suggests that the geometry of the tetra-cysteine binding motif is less than ideal in the Tet-351 reporter, as indicated by our modeling of the binding site. The Tet-329 protein binds FIAsH with similar efficiency as the Tet-350 protein (supplemental data), as

would be predicted by our modeling. It should be noted that there is significant non-specific binding of FAsH to the p53-tet protein due to interaction of FAsH with the His-tag on the p53-tet protein. Removal of the His-tag decreases FAsH non-specific binding (supplemental data) but also decreases the stability of the Tet-domain. Due to this, all measurements were made in the presence of the his-tag for these studies.

A similar binding experiment was conducted using the Tet-351 and Tet-5144 to determine if the Tet-351 was truly specific for the tetrameric form of p53. Introduction of the L344A mutation results in a dimeric form of p53-tet and no specific binding of FAsH is expected (Fig. 3b). Equivalent molar concentrations of p53-tet, Tet-351 and Tet-5144 were treated with FAsH for 2-3 hours and the fluorescence spectrum of each sample was measured. As shown in Fig. 3b, the fluorescence intensity of Tet-5144 was equivalent to the intensity of p53-Tet (~50% of the intensity of Tet-351), consistent with only non-specific binding of FAsH. Comparison of Tet-350 to Tet-5044 yielded similar results (supplemental data). This demonstrates that FAsH binding to both the Tet-350 and Tet-351 proteins is specific for the tetrameric state.

The ability to monitor the oligomeric state of a protein using FAsH creates the possibility of monitoring oligomerization in real time. In order for this system to be useful for most biological studies, FAsH binding would have to occur on a realistic timescale. To test the feasibility of using either the Tet-350 or Tet-329 protein as a reporter of oligomeric state, we monitored refolding of the tetramerization domain by both intrinsic fluorescence and FAsH fluorescence. A single tryptophan residue exists in the p53-tet proteins that can be used to monitor folding of the protein over time by intrinsic fluorescence as a control for refolding. Purified Tet-350 was denatured in 8 M urea and refolding was initiated by dilution. Tryptophan fluorescence was monitored for 1 hour at 340 nm, with maximum fluorescence occurring in the unfolded state and fluorescence intensity decreasing over time (Fig. 4). In contrast, FAsH fluorescence increases over time during formation of the p53-tet tetramer (Fig. 4). Intrinsic fluorescence time scans of p53-tet and Tet-350 overlap (data not shown), indicating that the presence of the TC-binding motif does not alter the refolding of the p53-tet domain.

Two observations are apparent upon comparing intrinsic fluorescence time scans with FAsH time scans. First, although the initial change in FAsH fluorescence plateaus at 6.5 minutes, the tryptophan signal reaches a brief plateau at approximately 11 minutes, with a continued signal decrease throughout the remainder of the experiment. The continual decrease in tryptophan signal over time suggests photobleaching or conformational changes unrelated to folding and oligomerization might be occurring. Our results demonstrate that not only does FAsH fluorescence report on conformational changes in real time, it is actually a more accurate reporter folding and assembly than intrinsic fluorescence for the p53-tet domain. Second, the intensity change of FAsH fluorescence is approximately twice the change measured by intrinsic fluorescence, which would allow future measurements to be made at more dilute protein concentrations. Taken together, this data demonstrates the feasibility of using FAsH as a probe to measure oligomerization events in real-time.

Discussion

Previous studies have highlighted the importance of proper oligomerization in p53 function. Li-Fraumeni syndrome, a genetically linked disorder that results in early onset tumor formation, is the result of two mutations in the p53 oligomerization domain [16,17]. Centrifugation studies have established that dimeric p53 binds DNA with 6-fold lower affinity than tetrameric p53 [18]. *In vitro*, oligomerization of the p53 tetramerization domain is concentration dependent, with a 10 μ M solution of a peptide consisting of amino acids 303-393 existing as 83% tetramer at 20° C, while a 1 μ M solution of the same peptide exists as 28%

tetramer and 0.1% tetramer at 0.1 μM concentration [19]. This concentration dependence of oligomerization has resulted in the following model of activation of p53. The physiological concentration of p53 is maintained at nanomolar concentrations in healthy cells, suggesting that unactivated p53 is monomeric under these conditions. Upon activation, the concentration of p53 increases due to disruption of the interaction between Mdm2 (an E3 ubiquitin ligase) and p53 resulting in the formation of active, tetrameric p53 complexes that are transiently assembled from pools of monomeric p53 and then rapidly dissociated once active p53 is no longer required. This model explains the formation of heterozygous p53 tetramers (a mixture of native and mutant p53), which are often isolated from tumor cells [20], since mutant and wild-type p53 have an equal probability of forming into tetramers if both exist in the pool of monomeric protein.

One key advantage of the p53 tetramerization domain is its natural two-fold symmetry of this region. This symmetry allows the TC-binding motif to be generated with minimal modifications to the protein's amino acid sequence, thereby minimizing potential destabilizing effects on the protein. In addition, the unique dimer and tetramer interfaces allow for the development of reporters that distinguish between various oligomeric states. Potentially, this approach could be adapted to other homo-oligomeric proteins or to the study of hetero-association events between proteins if suitable binding sites can be identified. However, the difference in fluorescent intensity between the Tet-350 and Tet-351 proteins serves as a measure of the importance of the geometry of the FAsH binding site. The Tet-350 protein has predicted distances that range between 4-8 Å between the four cysteines, while the Tet-351 protein has predicted distances of over 9 Å between three of the cysteines, resulting in an unsymmetrical geometry. These differences result in a 50% decrease in fluorescent intensity between the Tet-350 and Tet-351 proteins. Future applications of this approach will certainly require accurate molecular knowledge of the interaction to be monitored to ensure maximum binding of FAsH.

While the function of the p53 tetramerization domain has been well characterized *in vitro*, the role of oligomerization in p53 function within the cellular environment has been more challenging to characterize, leaving *in vivo* characterization of the p53 activation model largely unexplored. Using FAsH as a reporter for various oligomeric states provides us with a previously unavailable approach to study the relationship between p53 oligomerization and function *in vivo*. In recent years, FAsH has been used for *in vivo* studies of protein localization [21], protein dynamics [22], and protein folding and stability [9]. The results presented here demonstrate that *in vivo* FAsH studies can potentially also be extended to monitoring of protein-protein interactions as well.

In this report, we have successfully demonstrated the potential of FAsH to act as a reporter of oligomeric state. Using the p53 tetramerization domain as a model system, we have shown that the FAsH tetra-cysteine binding motif can be introduced to the tetramerization domain so that FAsH fluorescence can be used to distinguish between dimeric and tetrameric p53. In addition, FAsH fluorescence provides an accurate measure of folding and assembly kinetics, highlighting the potential of FAsH to act as a real-time reporter of protein-protein association events. This provides a potential approach for monitoring oligomerization events where FRET is not feasible and further highlights the potential of FAsH as a probe for changes in protein conformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by in part by NIH grant U54 CA096320-04 and by Northern Arizona University's Growing Biotechnology Initiative, with funding derived from the state of Arizona's Technology Research Innovation Fund.

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Fig. 1.

Predicted distances between cysteines comprising the FLAsH binding motif. Structure of the p53 tetramerization domain showing the cysteine substitutions at (A) amino acids 329 and 331, (B) amino acids 347 and 350, and (C) amino acids 347 and 351. The side chains of the four cysteines comprising the tetracysteine motif expanded in the inset with the distances between each sulfur atom shown. Numbering based on full-length p53 protein.

Fig. 2. Gel filtration analysis of Tet domain proteins. Separation of p53-Tet, Tet-350, Tet-351, Tet-344 and Tet-5044 protein using a Superdex™ 75 gel filtration column demonstrated that p53-Tet, Tet-350 and Tet-351 migrated as tetramers while Tet-344 and Tet-5044 migrated as dimers.

Fig. 3.

Binding of FAsH to the p53-Tet, Tet-350, Tet-351 and Tet-5144 proteins. (A) Fluorescence spectra of 0.4 μ M FAsH bound to 10 μ M purified Tet domain proteins. The Tet-351 reporter exhibits about half of the signal of the Tet-350 protein. (B) Comparison of fluorescence intensity of FAsH bound to either Tet-351 or Tet-5144. Addition of the L344A mutation results in a FAsH fluorescence

Fig. 4. Refolding and assembly of the Tet-domain as measured by FAsH fluorescence and intrinsic tryptophan fluorescence. Refolding was initiated by dilution into refolding buffer (50 mM Tris, pH 7.4, 150 mM NaCl) containing 0.3 μ M FAsH. Intrinsic fluorescence was monitored by emission at 340 nm (Ex: 280 nm). FAsH fluorescence was monitored at 530 nm (Ex: 495 nm).