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Cardiac Tissue-Specific Binding Agent of Troponin I

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Abstract

A fluorescently labeled, biphenylalanine-rich peptide was identified as a cTnI-specific binding agent with preferential staining affinity to myocardium tissues and extremely low staining to other stromal components. Fluorescence images demonstrate that this new peptide is an excellent contrast agent useful for examining troponin I structural distribution and expression density within heart tissue sections. It provides clear contrast between myocardial cells and the surrounding collagen matrix.

Keywords

Troponin; biphenylalanine; contrast agent; staining; myocyte

The successful management of heart disease requires the ability to accurately perceive, assess, and monitor structural and functional changes within the myocardium. In acute pathologies such as myocardial infarct, prognosis and treatment are greatly affected by the ability to reliably assess the extent of damage to the myocardium resulting from the insult. Several clinical biomarkers have been used to assess heart damage after acute myocardial diseases^{1, 2}. Cardiac Troponin I (cTnI) is a cardiomyocyte-specific protein that plays a key role in regulating myocardial contraction^{3, 4}. It immobilized on the cardiac muscle tissues through cross-bridging the interaction between the thin and thick myofilaments within the cardiomyocyte. The adaptive remodeling process during myocardial infarction typically associated with cTnI release from the ischemic myocardium and damaged cardiac cells along with development of excess fibrous connective tissues that mainly composed of type I collagen. The tissue loss of cTnI can be seen in the infarcted areas within 30 minutes even before histological evidence of necrosis is apparent. Due the specific expression of the cTnI isoform within viable myocardium and not within infarcted tissue, it has become the biomarker of choice for the early diagnosis of cardiac muscle tissue damage and necrosis after infarction. Largely because of this unique myocardial tissue specificity and confined expression^{5, 6}, the variation of cTnI expression within the myocardial tissue is essential to evaluate the integrity of cardiac muscle^{7–9}.

Identifying selective binding ligands for each biomarker has relied extensively on the screening of phage-displayed combinatorial libraries^{10, 11}. Although normal phage libraries cannot incorporate unnatural amino acids, many conformationally constrained and unnatural residues have been incorporated into identified constructs in order to impart specific features and enhance their durability against biological degradation¹². Phenylalanine-like analogs

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Supporting Information Available: Experimental details of targeting peptides synthesis, protein electrophoresis analysis using SDS-PAGE, and tissue immunohistochemical and histofluorescence staining are available.

have been incorporated into several structures to investigate various targets^{13–16}. For example, we recently demonstrated the potential of a biphenylalanine-rich peptide labeled with fluorescein isothiocyanate (FITC) as a fluorescent stain specific for viable myocardium¹⁷. Because this compound had distinct capabilities allowing it to recognize viable cardiomyocytes and unveil the dynamic alternations of the myocardial matrix, it was designated as myocyte targeting peptide (MTP) (Figure 1A)¹⁷. The observed superb staining of viable myocardial tissue and live cells, where cTnI expression is uninterrupted, occurred in parallel with genuine loss of staining in the infarcted myocardial tissue sections and dead myocytes wherein the cTnI experience massive degradation and release. The precise mapping of the viable and ischemic myocardium and its associated ultrastructure loss of cTnI underlying the essential role that cTnI might play in myocyte binding. Therefore, we hypothesized that cTnI could be the potential biological target for MTP staining agent. In this current study, we aimed to understand the biological target of MTP and how the biphenylalanine residues affect the binding affinity.

To identify the potential target of MTP, we immunohistochemically stained mouse heart tissue sections for cTnI and the extracellular matrix type I collagen basic proteins. After a 1-h incubation in a biphenylalanine-rich MTP solution (10 μ M), the tissue sections were imaged with a fluorescent microscope (Figure 1B, panel a). Mouse heart tissue sections stained using antibodies specific for troponin I or type I collagen are shown in Figure 1B, panels b–c. The tissue sections stained with fluorescent MTP show a fluorescent signal within cardiomyocytes but not in the surrounding connective tissue (Figure 1B, panel a). Similarly, tissue sections stained with a troponin I-specific antibody show a staining pattern inside the viable cardiac cells, consistent with the cytoplasmic expression of troponin I (Figure 1B, panel b). In contrast, strong stromal expression of type I collagen is evident as dark brown zones within the extracellular matrix, bordering the cardiac cells (Figure 1B, panel c). This evidence supports the hypothesis that the superb cardiomyocyte selectivity of the fluorescent MTP probe can be attributed to the preferential binding of the probe to troponin I protein within the myocardial tissue.

To establish the preferential binding profile of MTP, cTnI, cTnT isoforms, and actin protein were mixed with MTP and then analyzed using SDS-PAGE. A separate gel with the same proteins was stained with Coomassie blue in parallel for verification (Figure 2A). The in-gel protein electrophoresis shows a fluorescent signal associated with troponin I protein band at 24 kDa (Figure 2B). No fluorescent signal was evident at either 38 kDa or 42 kDa, reflecting the lack of staining proclivity of MTP for either actin or troponin T, respectively. The binding selectivity of MTP for extracellular matrix collagen I and collagen III, other abundant cellular proteins, was also assessed (Figure 2C). Once more, the in-gel protein electrophoresis shows only a fluorescent signal associated with troponin I at 24 kDa and not with others (Figure 2D). For further confirmation, the change in the absorbance spectra of MTP was assessed upon association with cTnI and cTnT proteins in the UV/Vis region. MTP showed a prominent absorption peak maximum ($\lambda_{\text{abs.}}_{\text{max}}$) at 440 nm, whereas cTnI and cTnT proteins shows no signs of absorbance at the observed spectrum (Figure 2E). Upon interaction, the MTP-cTnI bound form shows characteristic change in the spectral shape and significant red shift to 490 nm. The change in the spectral shape and the shift in the absorption maximum indicate the efficient association of MTP ligand and cTnI protein. On the contrary, no change was observed in the spectral shape or the absorption maximum of MTP upon interaction with cTnT, indicating inefficient interaction. This evidence strongly supports the hypothesis that MTP specifically binds to cTnI and explains the unique myocardial tissue staining and distribution of the probe.

To understand the critical contribution of the biphenylalanine residues, these residues were sequentially replaced within structurally similar peptide constructs, and the ability of cTnI

protein to bind to these peptides was assessed. There are a total of three biphenylalanine residues in MTP (Bip3). We reduced this number to two and one within the compounds Bip2 and Bip1, respectively (Figure 3A). In each of these peptide constructs, multiple glycine residues were employed as a template containing two lysine residues at positions 4 and 7 for biphenylalanine conjugation (see Methods). The biphenylalanine peptide derivatives were prepared by the selective removal of the Mtt protecting groups followed by coupling with Fmoc-biphenylalanine to the free ϵ -amino group of the Lys residues using HBTU/HOBT. The fluorescent tag, FITC, was coupled to the last *N*-terminal amino acid, Beta Alanine (β Ala) during the peptide chain elongation. Bip1-Bip3 peptides were purified by HPLC.

The differential staining profiles of fluorescent targeting peptides Bip1-Bip3 for cTnI protein were determined by SDS-PAGE. The fluorescent images show an intense fluorescent signal associated with Bip3-stained troponin I protein band at 24 kDa (Figure 3B). The intensity of the fluorescent signal decreases progressively with the reduction of biphenylalanine residues in Bip2 and Bip1. In-gel Bip3 staining of cTnI showed a 37% higher fluorescence signal than that with Bip1. This evidence indicates that the binding efficiency of the peptide for troponin I increase with the number of biphenylalanine residues present in the probe. It is known that the binding affinity can be increased by incorporating multiple copies of interacting motifs^{7, 18}. Given that the hydropathy index value of biphenylalanine is 4.5 (significantly higher than other used amino acids), these data suggest that hydrophobic interactions are critical for the binding interaction with troponin I. This finding is consistent with other molecular recognition studies^{7, 19}.

To date, histological analysis remains the most accepted and sensitive method for examining the pathological alterations of heart tissues for which troponin I has unique cardio-specific expression³. To validate our findings and corroborate the histological localization of troponin I, we compared mouse heart and skeletal muscle tissue sections stained with Bip3. Tissue sections were subjected to hematoxylin and eosin (H&E) staining, in addition to immunohistochemical staining for cTnI (Figure 4A–F). H&E staining of the cardiac and skeletal muscle tissue sections showed honest localization of the pink-stain to the cardiac and skeletal muscle cells but not to the adjacent collagen-rich connecting matrixes (Figure 4A, B). Strong, brown staining corresponding to high troponin I expression was only observed in the cardiac muscle (Figure 4C). In contrast, skeletal tissues failed to show appreciable troponin I-dependent staining, strongly suggesting the lack of troponin I expression in skeleton tissues (Figure 4D). Importantly, Bip3-dependent staining was pronounced in the troponin I-rich cardiomyocytes with a concurrent lack of staining present in the surrounding collagen I-rich matrix (Figure 4E). As expected, Bip3 failed to show a significant fluorescent signal within the mouse skeletomyofibrils (Figure 4F). These results are consistent with the results from gel electrophoresis, which showed a high staining tendency of Bip3 for cTnI. Therefore, the histopathological staining with Bip3 not only distinguished the myocytes from the surrounding extracellular matrix, but it also was able to reveal the histological details that mark the cardiac distribution of troponin I isoform and eventually be useful for mapping damaged vs. viable regions within myocardial tissue sections¹⁷. Comparing to antibodies, this peptide-based probe is a small synthetic molecule which is easy to be prepared, cost-effective, and further chemical modifications are feasible and often straightforward. Moreover, antibodies can only work under physiological conditions, thus their ranges of applications are restricted. Peptides in general are more stable than antibodies at various conditions so that Bip3 probe would have a longer shelf life and broader applications.

In conclusion, we have developed a novel fluorescent contrast agent that preferentially binds to cardiomyocyte-specific troponin I. This agent allows for myocardial tissue mapping using

fluorescent microscopy. Our studies indicate that histofluorescence staining for cTnI using Bip3 holds great potential for recognizing early alternations in the myocardium at the protein level before morphological remodeling becomes apparent. Although the exact binding mechanism of the biphenylalanine-rich peptides remains to be delineated, Bip3 shows considerable promise as a new tool for studying myocyte viability in cardiac tissue. Previously, we have used Bip3 fluorescent staining to study myocardial infarction¹⁷. In that study, we demonstrated a lack of Bip3-dependent staining in injured myocytes post-myocardial infarct. Here we demonstrate that this lack of staining is likely a direct reflection of the deficiency of cardiac-troponin I expression in these cells, as Bip3 selectively binds troponin I. Further studies are underway to investigate the *in vivo* fluorescent properties, biodistribution, and pharmacokinetics of biphenylalanine-rich Bip3 probe in animal models of heart-related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) General structure of the fluorescent myocyte targeting peptide (MTP). (B) Representative images of mouse heart tissue sections with various stains, (a) fluorescent MTP (Bip3), (b) troponin I-specific antibody, and (c) type I collagen-specific antibody. Images show localized fluorescent staining and strong cytoplasmic expression of troponin I in cardiomyocytes, whereas strong staining of type I collagen was seen within the extracellular matrix.

Figure 2.

Protein electrophoresis in SDS-PAGE gels. **(A, C)** Coomassie blue staining of cardiac actin, troponin T, troponin I, collagen III, collagen I, and actin proteins. **(B, D)** In-gel differential staining profiles of fluorescent targeting peptide Bip3 (1 μ M) to human cTnI, actin, troponin T, collagen III, collagen I, and actin proteins (2 μ g each). **(E)** Absorption spectra of MTP (blue solid line), MTP-cTnT (red dotted line), MTP-cTnI (green dashed line), cTnI (green solid line), and cTnT (red solid line) in the UV/Vis region at 21°C showing the change in the spectral shape of MTP and the associated red shift in absorbance peak maximum upon interaction with cTnI but not cTnT

A

Peptide	Amino Acid Sequence
Bip1	FITC-βAla ¹ -Gly ² -Gly ³ -Lys ⁴ -Gly ⁵ -Gly ⁶ -Lys ⁷ -Gly ⁸ -Gly ⁹ -Bip ¹⁰ .
Bip2	FITC-βAla ¹ -Gly ² -Gly ³ -Lys(Bip) ⁴ -Gly ⁵ -Gly ⁶ -Lys ⁷ -Gly ⁸ -Gly ⁹ -Bip ¹⁰ .
Bip3 (MTP)	FITC-βAla ¹ -Gly ² -Gly ³ -Lys(Bip) ⁴ -Gly ⁵ -Gly ⁶ -Lys(Bip) ⁷ -Gly ⁸ -Gly ⁹ -Bip ¹⁰ .

B **Figure 3.**

(A) CTnI-targeting peptides: structures of the developed fluorescent troponin I-targeting peptides Bip1, Bip2, and Bip3. (B) Protein electrophoresis in SDS-PAGE gel: In-gel differential staining profiles of fluorescent targeting peptides, Bip1- Bip3 (1 μM), to human cTnI (2 μg).

Figure 4.

Representative images of mouse heart and skeletal muscle tissue sections stained with H & E (**A, B**), troponin I antibody (**C, D**), and fluorescent Bip3 contrast agent (**E, F**). Images show co-localization of the fluorescent stain with troponin I in mouse cardiomyocytes but not within the skeletal muscle cells.