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The integrity of the α-helical domain of intestinal fatty acid binding protein is essential for the collision-mediated transfer of fatty acids to phospholipid membranes

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Abstract

Intestinal FABP (IFABP) and liver FABP (LFABP), homologous proteins expressed at high levels in intestinal absorptive cells, employ markedly different mechanisms of fatty acid transfer to acceptor model membranes. Transfer from IFABP occurs during protein–membrane collisional interactions, while for LFABP transfer occurs by diffusion through the aqueous phase. In addition, transfer from IFABP is markedly faster than from LFABP. The overall goal of this study was to further explore the structural differences between IFABP and LFABP which underlie their large functional differences in ligand transport. In particular, we addressed the role of the αI-helix domain in the unique transport properties of intestinal FABP. A chimeric protein was engineered with the 'body' (ligand binding domain) of IFABP and the αI-helix of LFABP (α(I) LβIFABP), and the fatty acid transfer properties of the chimeric FABP were examined using a fluorescence resonance energy transfer assay. The results showed a significant decrease in the absolute rate of FA transfer from α(I)LβIFABP compared to IFABP. The results indicate that the αI-helix is crucial for IFABP collisional FA transfer, and further indicate the participation of the αII-helix in the formation of a protein–membrane "collisional complex". Photo-crosslinking experiments with a photoactivable reagent demonstrated the direct interaction of IFABP with membranes and further support the importance of the αI helix of IFABP in its physical interaction with membranes.

Keywords: Fatty acid binding protein; Fatty acid; Chimeric protein; Lipid metabolism; Small intestine; Lipid transport

1. Introduction

In vertebrates, tissues like intestine, liver, adipose, and cardiac and skeletal muscles have metabolic pathways that demand a substantial transport of lipids, especially fatty acids (FA), both from other tissues *via* the blood, or from other organelles inside the cell. One of the physiological processes known to involve transport of high amounts of FA is the intestinal absorption of lipids after a meal. Small intestinal enterocytes express high levels of two homologous fatty acid binding proteins (FABPs), liver FABP (LFABP), also named FABP1, and intestinal FABP (IFABP), also named FABP2. It is hypothesized that these FABPs are important in intracellular transport of FA, however their precise functions as well as the reason why a single cell type contains more than one distinct FABP, are only beginning to be understood. Both I- and LFABP bind long chain fatty acids with high affinity, nevertheless, it has been suggested that they are functionally distinct. LFABP is expressed in both small intestine and liver, whereas IFABP is found exclusively in the small intestine [1]. IFABP has a single binding site for long chain FA [2], while LFABP contains at least two FA binding sites [3]. LFABP binds a number of other endogenous

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hydrophobic ligands [4–7], whereas IFABP appears to bind exclusively long chain FA [8]. A functional comparison of these FABPs was made using an *in vitro* fluorescence energy transfer assay in order to examine the rate and mechanism of transfer of fluorescently tagged fatty acids from proteins to phospholipid membranes. These studies have demonstrated that transfer of fatty acids from IFABP to membranes occurs by a collisional mechanism involving a physical contact with membranes, whereas LFABP seems to employ an aqueous diffusion mechanism for ligand transfer [9].

Both I- and LFABP, as well as the other members of the family, share a common tertiary structure consisting of ten antiparallel β-strands that form a β-barrel, which is capped by two short α-helixes arranged as a helix–turn–helix segment. It is believed that this helical region is part of a "dynamic portal" that regulates fatty acid entry and exit from the internal cavity [10,11]. The β-barrel domain contains the ligand binding site. The structural elements underlying collisional transfer of a fatty acid from IFABP to membranes could have important physiological consequences as they may dictate the fatty acid trafficking patterns within the cell. Using a helix-less variant of IFABP [12], we previously demonstrated that the α-helical region of IFABP plays a primary role in the collisional transfer of fatty acid from IFABP to membranes [11,13]. Moreover, analysis of a pair of chimeric proteins generated by exchanging the helix–turn–helix domains between I- and LFABP, showed a significant modification of the absolute rates of fatty acid transfer of the chimeric proteins when compared to the wild types. These results further indicated that the α-helical region of LFABP is responsible for its diffusional mechanism of fatty acid transfer to membranes, as well as the importance of the α-helical region of IFABP in the determination of the collisional fatty acid transfer mechanism [14].

Despite its relatively short length, the 9 residue αI-helix of IFABP would be expected to be membrane interactive, due to its amphipathic character [15]. Amphipathic helixes are well known to be important in the targeting of proteins to membranes, and the charge characteristics of the helix appear to modulate interactions with membranes [16]. Thus, we hypothesized that the charged face of the αI-helix is critical for membrane interactions which lead to the dramatic increase in FA transfer rate to anionic membranes in IFABP but not LFABP [9]. In order to test this hypothesis, in the present studies we engineered a pair of chimeric proteins by exchanging only the αI-helixes from I- and LFABP, thus generating α(I)LβIFABP and α(I)IβLFABP chimeric proteins. Analysis of the structural integrity of the chimeric proteins showed that α(I)LβIFABP folded properly and was able to bind fatty acids. α(I)IβLFABP displayed structural problems that precluded further analysis. Based on this assessment, further functional studies were conducted only with α(I)LβIFABP.

Employing a fluorescence resonance energy transfer assay, the transfer of anthroyloxy-labeled fatty acids (AOFA) from the chimeric protein to model membranes was analyzed and compared to the wild-type I- and LFABP. Direct protein–membrane interaction was assessed for the α(I)LβIFABP chimera using a photo-crosslinking assay.

The results indicate that the αI-helix of the FABPs plays an important role in determining the rate and, importantly, the mechanism of fatty acid transfer to membranes. For IFABP, the amphipathic character of the αI-helix is critical for collision-mediated FA transfer. Moreover the αI-helix seems to be important in the physical interaction of IFABP with membranes and as a sensor of membrane charge.

2. Materials and methods

2.1. Materials

The mutagenic primers and Pfx polymerase were obtained from Invitrogen (Carlsbad, CA). Restriction enzymes XbaI, BamHI and AgeI, pGEM-T vector and T4 DNA ligase were from Promega (Madison, WI). Fluorescently labeled AOFA, 12-(9-anthroyloxy) oleic acid (12AO) was purchased from Molecular Probes, Inc. (Eugene, OR). Egg phosphatidylcholine (EPC), egg *N*-(7-nitro-2,1,3-benzoxadiaxol-4-yl)-phosphatidylcholine (NBD-PC), brain phosphatidylserine (PS) and bovine heart cardiolipin (CL) were obtained from Avanti Polar Lipids (Alabaster, AL). Lipidex-1000 was purchased from Sigma (Saint Louis, MI). [^{125}I] NaI was from Dupont NEN Products (Boston, MA). All other chemicals were reagent grade or better.

2.2. Construction of chimeric FABPs

Recombinant rat pET11d-IFABP and pET11a-LFABP plasmids were generously provided by Drs. Alan Kleinfeld and Ron Ogata (Medical Biology Institute, La Jolla CA). A unique restriction site (AgeI) was generated in the region between αI and αII in both of the plasmids to allow subsequent separation and exchange of segments. Employing overlapping PCR methodology [17], two point mutations were introduced in positions 104 and 105 of the LFABP cDNA sequence resulting in a substitution of Met for Thr 22; in the IFABP sequence three point mutations were introduced, two of them resulting in a substitution of Met for Thr 19 (positions 77 and 78 of the cDNA sequence) and the third one was a silent mutation in position 81. The sequences of the primers used to construct the restriction site mutations are the following (point mutations underlined): 5′CGGATAACAATTCCCCTCTA3′ and 5′TTCCTTTCGGGCTTTGTTAG3′ as external primers (the same external primers were used for both constructs), 5′CACGTTAATACCGGTTTTCTCCAT3′ and 5′ATGGAGAAAACCGGTATTAACGTG3′ as internal primers for IFABP cDNA, and 5′CTCAGGCAGACCGGTCGCCTTCAT3′ and 5′ATGAAGGCGACCGGTCTGCCTGAG3′ as internal primers for LFABP cDNA. The mutated FABP constructs were verified by sequence analysis. Prior to the treatment with AgeI, the mutant cDNAs were subcloned into pGEM-T vectors by direct ligation of the PCR product. The mutant cDNAs were treated with restriction enzymes AgeI and BamHI in order to separate the αII and β-barrel region from the rest of the construct. The αII and β-barrel of IFABP were ligated to the rest of the construct belonging to LFABP using T4 DNA ligase, generating in this way a chimeric cDNA with βA, αI from LFABP and αII and the remaining β-barrel from IFABP. Similarly, ligation of the αII and β-barrel of LFABP to the rest of the construct belonging to IFABP generated a chimeric cDNA with βA and αI from IFABP, and αII and the remaining β-barrel from LFABP. The chimeric cDNAs were subcloned into pET11d vector by using the XbaI and BamHI restriction sites to construct the expression vectors. The chimeric FABP constructs were verified by sequence analysis.

2.3. Protein expression and purification

The wild-type and chimeric proteins were overexpressed in *Escherichia coli* harboring pET11d-IFABP, pET11a-LFABP, pet11d-α(I)LβIFABP and pet11d-α(I)IβLFABP respectively, as detailed elsewhere [9,11]. The wild-type proteins were purified from *E. coli* as described previously [9]. Neither of the chimeric proteins was expressed as a soluble protein, so it was necessary to purify them from inclusion bodies. The bacterial pellet was therefore dissolved in lysis buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 1 mM; pH=8.0), sonicated on ice for 30 s four times and centrifuged at 10,000 rpm for 10 min. These steps were