Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3805–3807

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

High-Resolution Assessment of Protein DNA Binding Affinity and Selectivity Utilizing a Fluorescent Intercalator Displacement (FID) Assay

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Accepted 20 June 2003

Abstract—Protein titration displacement of ethidium bromide bound to hairpin deoxyoligonucleotides containing any sequence of interest provides a well-defined titration curve (measuring the loss of fluorescence derived from the DNA bound ethidium bromide) that provides both absolute binding constants (K_a) and stoichiometry of binding. This use of a fluorescent intercalator displacement (FID) assay for establishing protein DNA binding affinity and selectivity is demonstrated with the examination of the LEF-1 HMG domain binding to hairpin deoxyoligonucleotides containing its commonly accepted consensus sequence 5′-CTTTGWW (W = A or T) and those modified (5′-CTNTGWW) to examine sequences implicated in early studies (5′-CTNTG). The effectiveness of the FID assay coupled with its technically non-demanding experimental use makes it an attractive alternative or complement to selection screening, footprinting or affinity cleavage, and electrophoretic mobility shift assays for detecting, characterizing, and quantitating protein DNA binding affinity and selectivity.

The DNA binding affinity and selectivity of proteins are typically assessed by selection screening, footprinting and affinity cleavage, or electrophoretic mobility shift assays (EMSA) and each technique possesses unique strengths.[1] Selection screening[2] provides exhaustive sequence coverage for deducing the preferred binding site(s) of a protein, but does not provide quantitative binding information. Although footprinting and, to a lesser extent, EMSA[3] can and have been used to define, or at least refine, a protein's binding selectivity, their most frequent uses have been to provide qualitative distinctions and quantitative comparisons among candidate binding sites, or those specially constructed to assess single base pair substitutions. Because of the power of the technique,[4] a number of footprinting/affinity cleavage[5] methods have been introduced including DNase I,[6] exonuclease III,[7] MPE-Fe(II),[8] 1,10-phenanthroline-Cu(I),[9] or EDTA-Fe(II)[10] footprinting. Complementary approaches to disrupting binding (interference footprinting) by site specific base[1,11] or phosphate modifications have been developed and used to probe specific base contacts and their location. Herein, we report that a complementary technique, a fluorescent intercalator displacement (FID) assay,[12] may be utilized to qualitatively or quantitatively establish the DNA binding properties of proteins.

Lymphoid enhancer-binding factor[13] (LEF-1, also referred to as TCF-1α[14]) and the closely related T-cell factor 1[15] (TCF-1) are sequence selective DNA binding proteins that play important regulatory roles.[16] The majority of colorectal tumors contain mutations in the tumor suppressor protein, adenomatous polyposis coli (APC), that result in the release and nuclear accumulation of β-catenin. β-Catenin binds to and activates transcription factors including LEF-1 displacing bound corepressors resulting in unregulated and aberrant gene expression key to the development of colon cancer.[17] LEF-1 binds DNA through a high mobility group (HMG) domain recognizing a sequence commonly represented as 5′-CTTTGWW (W = A or T).[13–18] A recent NMR structure[19] of the LEF-1 HMG domain bound to duplex 5′-CACC^{4}CTTTGAA^{11}GCTC revealed that the protein makes extensive and continuous contacts in the DNA minor groove from C4 to A11 encompassing the entire

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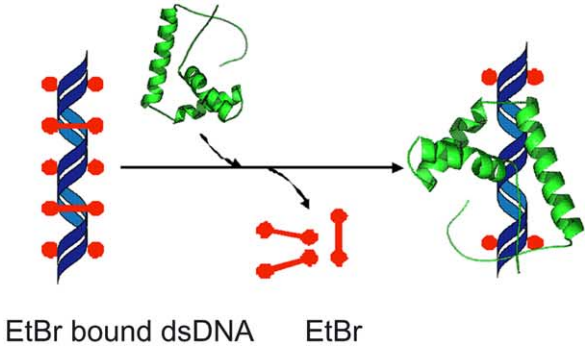
doi:10.1016/j.bmcl.2003.06.002

region implicated in binding by chemical footprinting[13–18] and mutagenesis.

Titration displacement of ethidium bromide bound to a hairpin deoxyoligonucleotide containing 5′-CTTT GAAG and the flanking sequence found in the T-cell enhancer with the HMG domain of LEF-1[20] provided incremental losses in fluorescence and a well-defined titration curve (Fig. 1). The intersection of the pre- and post-saturation curves provides the stoichiometry of binding (1:1, Fig. 1a) and Scatchard analysis afforded a K_a of 1.5×10^9 M^{-1} (Fig. 1b), essentially identical to that established by EMSA[18] (saturation-binding) ($K_d = 1\times10^{-9}$ M). Similarly, it bound the hairpin containing 5′-CTTTGTTG with a comparable affinity of 1×10^9 M^{-1} and failed to bind 5′-CCCCGAAG in a discernable or defined manner (Table 1).[21] Thus, the FID titration not only provided a simple approach for the quantitative assessment of the DNA binding, but it concurrently established the stoichiometry of binding.



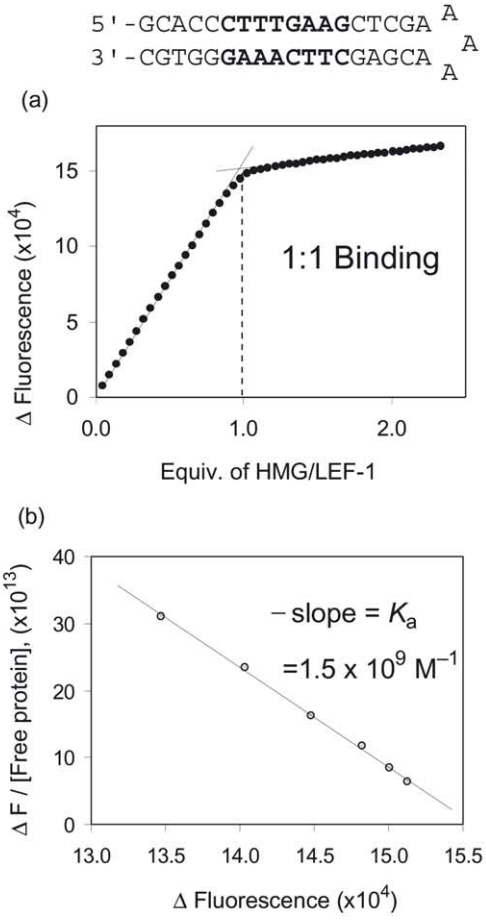


Figure 1. (a) Titration of HMG domain of LEF-1 versus hairpin DNA containing 5′-CTTTGAA-3′; (b) Scatchard plot for the titration of 5′-CTTTGAA-3′, slope = $-K_a$.

Table 1.

5′-GCACC**CTTTGAAG**CTCGA A
3′-CGTGG**GAAACTTC**GAGCA A A

DNA	K_a
CTTTGAAG	1.5×10^9
CTTTGTTG	1.0×10^9
CCCCGAAG	$\leq1.0\times10^8$
CTCTGAAG	9.4×10^8
CTGTGAAG	8.9×10^8
CTATGAAG	8.3×10^8

Having established the viability of the technique, the importance of the third base of the consensus sequence 5′-CTTTGWW was examined. Initial footprinting studies[14] with TCF-1α (LEF-1) indicated that it bound a 5′-CTNTG motif found within the consensus sequence core of the octamer transcription factors. Since such sites were not probed in subsequent footprinting studies and not identified in selection studies,[18] the binding of LEF-1 to such sites has not been systematically examined.[22] Consequently, each base variation at this site was examined and revealed that the C, G, and A substitutions bind the LEF-1 HMG domain effectively and almost indistinguishably exhibiting K_a's less than 2-fold (1.6-fold) lower than that of the commonly accepted consensus sequence (Table 1). As such, the consensus sequence of 5′-CTTTGWW is accurate, but could easily be refined to 5′-CTNTGWW (W = A or T) to reflect this nearly indiscriminant third site.

Thus, the FID assay provides an effective and technically non-demanding method for quantitatively assessing the DNA binding affinity and selectivity of proteins. In addition and although not demonstrated here, the technique is adaptable to high throughput qualitative or quantitative screening[12] amenable to assaying either a library of individual proteins or mutants against a single sequence, or a single protein against a library of DNA sequences providing a binding rank order necessary to establish a consensus sequence and the intrinsic selectivity. Unlike footprinting or affinity cleavage and EMSA, the FID assay is nondestructive providing the opportunity for hairpin immobilization onto reusable supports making the repeated exhaustive screening of all possible sequences realistic.

Acknowledgements

We gratefully acknowledge the financial support of the National Institutes of Health (CA 41986), The Skaggs

Institute for Chemical Biology, and an ACS Medicinal Chemistry Division predoctoral fellowship sponsored by Weyth-Ayerst (WCT, 2002–2003). We especially thank Professors Joel Gottesfeld and Peter E. Wright at The Scripps Research Institute for generously providing the HMG domain of LEF-1 and helpful discussions. W.C.T. is a Skaggs Fellow.

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