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Inhibition of HMGA2 binding to DNA by netropsin:

a biosensor-surface plasmon resonance assay

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Abstract

The design of small synthetic molecules that can be used to affect gene expression is an area of active interest for development of agents in therapeutic and biotechnology applications. Many compounds that target the minor groove in AT sequences in DNA are well characterized and are promising reagents for use as modulators of protein-DNA complexes. The mammalian high mobility group transcriptional factor, HMGA2, also targets the DNA minor groove and plays critical roles in disease processes from cancer to obesity. Biosensor-surface plasmon resonance methods were used to monitor HMGA2 binding to target sites on immobilized DNA and a competition assay for inhibition of the HMGA2-DNA complex was designed. HMGA2 binds strongly to the DNA through AT hook domains with K_D values of 20 - 30 nM depending on the DNA sequence. The well-characterized minor groove binder, netropsin, was used to develop and test the assay. The compound has two binding sites in the protein-DNA interaction sequence and this provides an advantage for inhibition. An equation for analysis of results when the inhibitor has two binding sites in the biopolymer recognition surface is presented with the results. The assay provides a platform for discovery of HMGA2 inhibitors.

Keywords

Biacore; Biosensor-surface plasmon resonance; SPR; Netropsin; DNA; Minor groove binding affinity; protein-DNA

Transcription factors are essential for the regulation of eukaryotic gene expression and for normal cellular function [1]. For this reason, design of small synthetic molecules that can be used to inhibit or activate transcription factor - DNA interactions and, therefore, affect gene expression is an area of active research interest [2-5]. The transcription factors that have been targeted to date have primarily been DNA major groove binding proteins but there are also important minor groove binding transcription factors [6-9]. Because many small molecules that target AT sequences in DNA are well characterized [10-15], study of inhibition of minor-groove transcription factor interactions with DNA by these molecules should provide valuable insight into design of new types of therapeutic agents.

The mammalian high mobility group protein HMGA2 is a transcriptional factor that targets the DNA minor groove and it is a member of the HMGA family of non-histone chromosomal

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DNA-binding proteins [6-9,16-20]. HMGA proteins act as architectural transcription factors and play critical roles in many cellular processes such as regulating the transcription of cellular and viral genes, modeling chromosome structure and enhancing cell transformation and differentiation [16-23]. Importantly, HMGA protein expression is barely detected in normal adult somatic cells in contrast to their high expression during the early stages of embryonic development [7,16,24]. High levels of expression of HMGA proteins, however, have been observed in a number of tumor cells including breast, lung, pancreatic and other cancers [25-29]. A number of studies have shown that the over-expression of HMGA associated with transformation and metastatic progression of neoplastic cells and the expression level exhibits a correlation with the degree of malignant transformation. Therefore, HMGA proteins are promising biomarkers for cancer detection as well as potential targets for development of new anticancer drugs. The HMGA2 transcription factor has also been shown to be important in fat cell proliferation and obesity [30].

The HMGA proteins bind to the minor groove of AT-rich sequences in DNA duplexes through three distinctive conserved DNA-binding domains called "AT-hooks" [6-9,13,20-23]. The central palindromic sequence, Pro-Arg-Gly-Arg-Pro (PRGRP) is referred to as an AT-hook motif. The core AT-hook motif, RGR, adopts a specific crescent conformation to obtain favorable contact interactions with the minor groove when binding to DNA [20-23]. The protein possesses an unfolded conformation in the free solution, but folds on DNA complex formation [32]. The *trans*- proline residues of PRGRP facilitate insertion of two arginine residues along the floor of the minor groove of AT sequence DNA. The central RGR core makes significant hydrogen-bonding interactions with AT base pairs on the floor of the minor groove, while the flanking lysine and arginine residues of the PRGRP binding domain contribute electrostatic interactions with phosphates on the DNA backbone and hydrophobic interactions of aliphatic methylenes of the side chains with adenine or thymine bases [19].

Since HMGA2 selectively recognizes AT-rich binding sites in the minor-groove of DNA through AT hooks [9,20,21], it offers an excellent opportunity for rational design of AT minor groove binders to achieve anti-cancer or anti-obesity activity. Such compounds could compete with HMGA proteins for binding to the same AT-rich binding site in the DNA promoter region and thus inhibit the HMGA activity as a transcription factor. To evaluate inhibition of protein or protein-DNA complexes by small molecules with different properties and structures, biosensor-surface plasmon resonance surface competition methods offer attractive advantages. Biosensor-SPR competition methods have been reported for use in characterizing low-molecular-weight compounds that have low affinities with the immobilized targets, and for screening and ranking competitive inhibitors for proteins [32-36]. In a biosensor-SPR surface competition assay, one of the interacting components is immobilized on the surface while another interacting component is mixed with a high-molecular-weight species that completes to bind to the same site on the immobilized component. The observed signal from the binding of high-molecular-weight molecule is influenced by the competitive binding from low-molecular-weight competitor. Such a competitive assay allows evaluation of the kinetics of the competitive interaction and rapid ranking of inhibitors.

The goal of the studies reported here is to design a biosensor-SPR screening assay, using the competition binding method, for inhibition of the HMGA2-DNA interaction by small molecules. A DNA model system with an HMGA2 site containing two AT target sequences, the minimum required for strong binding of HMGA2, was selected and immobilized on a sensor chip flow cell. The DNA target sequences were selected by SELEX (systematic evolution of ligands by exponential enrichment) for strong and specific HMGA2 interactions [37]. As a system to establish and test the screening assay for inhibition of HMGA2 binding by minor groove binders, a well-characterized polyamide minor groove binding compound, netropsin, was used. The crescent core AT hook motif in the HMGA2-DNA complex mimics the shape

of minor groove binding agents such as netropsin [19], and this makes minor groove agents promising inhibitors for the HMGA2-DNA interaction. An important feature of the designed assay is that the small molecule has two binding sites in the protein-DNA interaction sequence. This is clearly an advantage in terms of inhibition of transcription factor binding to DNA since a compounding at either or both AT sites. We present the results for the screening assay as well as an equation for analysis of the results when the inhibitor has two binding sites in the biopolymer complex recognition surface. To our knowledge, this is the first report of a surface competition method to evaluate nucleic acid-protein complex inhibition.

Materials and methods

Materials

The mouse HMGA2 was expressed, purified, and characterized as previously described [37, 38]. A molar extinction coefficient of $5,810 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm was used for HMGA2 concentration determination. 5'-Biotin labeled hairpin DNA oligomers from Integrated DNA Technology, Inc. (Coralville, IA) with HPLC reversed phase purification were utilized for SPR studies. Netropsin was purchased from Sigma Co. and used without further purification. HEPES 20 buffer containing 0.01 M HEPES, 0.003M EDTA, 0.2 M NaCl and surfactant P-20 at 0.05% (v/v) with an adjusted pH of 7.4 was used.

Immobilization of DNA

Surface plasmon resonance (SPR) experiments were carried out with a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). CM5 sensor chips with carboxymethylated dextran matrix and an amine coupling kit containing *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (EDC) and ethanolamine hydrochloride were obtained from Biacore. Although a number of other sensor chips are available, CM5 chips were used so that we could accurately monitor the small molecule-DNA interactions as well as those for the protein. CM5 allows immobilization of sufficient DNA to obtain excellent signal to noise for the both the small molecule and the protein binding to DNA (see Results). For experiments involving only high molecular weight material it would be possible to use sensorchips with lower charge and to immobilize less streptavidin and DNA. For capture of biotin-labeled DNA the chip was first activated by injecting a fresh mixture of 50 mM NHS and 200 mM EDCS at a flow rate of 5 $\mu\text{l}/\text{min}$. A 400 $\mu\text{g}/\text{ml}$ of streptavidin in 10 mM acetate buffer was then injected until approximately 3000 RU of streptavidin was covalently attached. Non-covalently bound esters were removed by injection of 1M ethanolamine hydrochloride, followed by a surface wash with buffer flow.

Biotin labeled DNA hairpins were then immobilized on three flow cells of the sensor chip via non-covalent biotin capture to the immobilized streptavidin [39]. After washing with 1M NaCl/50mMNaOH followed by buffer, DNA at a concentration of 25 nM was manually injected at a flow rate of 1 μl / min over a selected flow cell until the desired amount of DNA was immobilized. Three flow cells contained DNA and one flow cell was left blank as a reference.

SPR direct binding experiments

HMGA2 samples were prepared at a series of concentrations by dilution into degassed, filtered HEPES 20 buffer, which was also used as the running buffer. To study the binding interaction between HMGA2 protein and DNA, the series of protein samples was injected over the immobilized DNA surfaces followed by running buffer injection. A glycine solution (10mM, pH2.5) was used to regenerate the free DNA surface after each injection. All sensorgrams were processed by double subtraction, subtraction of the sensorgrams from the reference flow cell at same sample concentrations followed by subtraction of a sensorgram from the same flow cell at zero sample concentration to minimize the influence from specific flow cell factors

[40]. The RU values in the steady-state region of the sensorgrams at each concentration were averaged over a 60-second time zone and converted to r (moles of compound bound per mole of DNA hairpin) [39,41]. The binding affinity was determined by fitting r versus free compound concentration with a single site binding model ($K_2 = 0$) or a two-site binding model:

$$r = \left(K_1 * C_{\text{free}} + 2 * K_1 * K_2 * C_{\text{free}}^2 \right) / \left(1 + K_1 * C_{\text{free}} + K_1 * K_2 * C_{\text{free}}^2 \right) \quad (1)$$

where K_1 and K_2 are the macroscopic equilibrium binding constants; C_{free} is the free compound concentration at equilibrium and is the compound concentration in the flow solution [39].

Although it is useful to randomize the order of sample concentrations, in these experiments and the ones described below, we have injected the samples in order of increasing concentration. This was done due to significant absorption of the protein and to a lesser extent the small molecule in the entire flow system of the injection fluidics. The sensor chip surface could be regenerated quickly but cleaning the entire fluidic system between each injection was time consuming and cause some increase in chip surface deterioration. By injecting in increasing concentration order, the time for regeneration could be shortened considerably. Since we did the experiments in this manner, it was decided that it would be more appropriate to conduct complete replicate experiments for each different set of conditions rather than performing the usual procedure of replicate injections in a single experiment.

SPR competitive binding experiments

Competition experiments were conducted on a Biacore 2000 instrument with samples containing a fixed concentration of HMGA2 protein (0.1 μM) and a range of concentrations of the inhibitor in HEPES20 buffer. The samples were injected over the immobilized DNA surface at a flow rate of 50 $\mu\text{l/min}$ followed by HEPES20 buffer flow. A one-minute glycine solution (10mM, pH 2.5) injection was used for the surface regeneration. The binding responses (RU) at steady state were averaged and normalized by setting the RU with HMGA2 alone as 100% HMGA2 binding to DNA and the RU with saturation by the inhibitor as 0%. These values were then plotted versus inhibitor concentrations to evaluate IC_{50} for inhibition. IC_{50} values were determined by fitting the inhibition data with a model, which will be described below, for a competition system with a 1:1 binding stoichiometry for HMGA2 and a two-site binding for competitor:

$$\% \text{HMGA2 binding to DNA} = 100 / [1 + C(1 + K_{c2} * C) / [\text{IC}_{50}(1 + K_{c2} * \text{IC}_{50})]] \quad (1)$$

where K_{c2} is a macroscopic binding constant for inhibitor binding to DNA (Scheme 1), IC_{50} is the concentration of inhibitor which causes 50% inhibition of HMGA2 binding to DNA, and C is the concentration of inhibitor.

Derivation of the model equation for a competition system with one binding site for a macromolecule ligand and two binding sites for a competitor

In this competition model assay, the DNA duplex (D) contains two AT binding sites (Figure 1). A protein or ligand (L) that has a DNA binding domain with two AT recognition sequences (Fig. 1), such as the HMGA2 protein, binds to DNA at this domain with a 1:1 binding stoichiometry. A small AT-minor-groove-binding competitor (C) binds to the same site with a 2:1 binding stoichiometry as shown below, in which K_L is the equilibrium binding constant for binding of ligand to DNA, and K_{C1} and K_{C2} are macroscopic equilibrium binding constants for binding of small competitor to DNA. Equations have been presented for a 1:1 binding of macromolecule and competitor [42], but not for this more complex case.

For this competition model system, binding constants of DNA/ligand complex, where the ligand is typically a protein like HMGA2 and DNA/competitor complex formation are defined as follows:

$$K_L = [DL] / ([D][L]) \quad (2)$$

$$K_{C1} = [DC] / ([D][C]) \quad (3)$$

$$K_{C2} = [DC_2] / ([DC][C]) \quad (4)$$

where [D], [L], and [C] are free concentrations of DNA, ligand and competitor, respectively, [DL] is the concentration of DNA/ligand complex, and [DC] and [DC₂] are concentrations of DNA/competitor complexes. The ratio of bound ligand to total DNA can be written:

$$v_L = [L]_b/[D]_t = [DL] / ([D] + [DL] + [DC] + [DC_2]) \quad (5)$$

in which [L]_b is bound ligand concentration and [D]_t is total concentration of DNA. By substituting terms from equations (2), (3) and (4), Equation (5) can be rearranged:

$$v_L = [L]_b/[D]_t = K_L [L] / (1 + K_L [L] + K_{C1} [C] + K_{C1} K_{C2} [C]^2) \quad (6)$$

and

$$[L]_b = K_L [L] [D]_t / (1 + K_L [L] + K_{C1} [C] + K_{C1} K_{C2} [C]^2) \quad (7)$$

When the competitor concentration is zero, the bound ligand concentration reaches the maximum at any [D]_t and [L] is given by:

$$[L]_{b,c=0} = K_L [L] [D]_t / (1 + K_L [L]) \quad (8)$$

Therefore, the normalized fraction for ligand binding to DNA at any concentration of competitor is:

$$\begin{aligned} [L]_b/[L]_{b,c=0} &= \left[K_L [L] [D]_t / (1 + K_L [L] + K_{C1} [C] + K_{C1} K_{C2} [C]^2) \right] / \left[K_L [L] [D]_t / (1 + K_L [L]) \right] \\ &= (1 + K_L [L]) / (1 + K_L [L] + K_{C1} [C] + K_{C1} K_{C2} [C]^2) \end{aligned} \quad (9)$$

and

$$[L]_b/[L]_{b,c=0} = 1 / [1 + K_{C1} [C] (1 + K_{C2} [C]) / (1 + K_L [L])] \quad (10)$$

When the concentration of competitor is IC₅₀, the concentration for inhibition of 50% of ligand binding to DNA in the absence of competitor, equation (11) is obtained:

$$0.5 = 1 / [1 + K_{C1} IC_{50} (1 + K_{C2} IC_{50}) / (1 + K_L [L])] \quad (11)$$

By rearrangement:

$$1 + K_L [L] = K_{C1} IC_{50} (1 + K_{C2} IC_{50}) \quad (12)$$

and substituting for 1+K_L [L] in equation (10):

$$[L]_b/[L]_{b,c=0} = 1 / [1 + K_{C1} [C] (1 + K_{C2} [C]) / (K_{C1} IC_{50} (1 + K_{C2} IC_{50}))] \quad (13)$$

and

$$[L]_b/[L]_{b,c=0} = 1 / \{1 + [C] (1 + K_{C2} [C]) / [IC_{50} (1 + K_{C2} IC_{50})]\} \quad (14)$$

The IC₅₀ value, then, can be determined by nonlinear least squares fitting of normalized data with equation (14) for a competition system with 1:1 binding for ligand/protein and two-site binding for small competitor.

Results

Characterization of HMGA2-DNA interactions

As a first step in development of a biosensor SPR assay for exploring the inhibition of HMGA2 protein-DNA interactions by small molecules, it is essential to understand the direct interactions between HMGA2 protein and DNA. Electrophoretic mobility shifts, DNase I footprinting ITC and NMR studies show that the AT hooks of HMGA bind to AT rich sequences in the minor groove of DNA in a cooperative interaction [23,38,43]. Two DNA sequences, SELEX 1 and SELEX 2 (Fig. 1), which show the strongest binding with HMGA2 protein obtained from a SELEX screening method [31], have been used in the SPR experiments. Both DNA sequences have two sites containing five or six base-pair AT sequences (Fig. 1).

Several buffers with different compositions were compared to obtain optimized sensorgrams for protein-DNA binding. MES10 buffer (10mM MES, 100 mM NaCl, 1mM EDTA, pH 6.25, 0.005% (v/v) Biacore P-20 surfactant) has been widely used as running buffer for studying binding interaction of small molecules to DNA [39]. It was found, however, to be unsuitable for biosensor analysis of HMGA2 binding to DNA (see Fig. S1 in Supplementary materials). The distorted sensorgrams obtained in this buffer suggest that HMGA2 adsorbs on the flow cell surfaces and they are unsatisfactory even after double subtraction. Increasing NaCl concentration to 0.2M improves the sensorgram (Fig. S2 in Supplementary materials), revealing that nonspecific electrostatic interaction from the high positive charge of HMGA2 interaction with the negative surface is inhibited by an increase in salt concentration. The result is still not satisfactory, however, since many sensorgrams still have significant distortions. In order to minimize the nonspecific protein absorption HEPES 20 buffer with a higher P-20 surfactant concentration was evaluated and the sensorgrams in that buffer have more stable baselines. The higher concentrations of P20 and HEPES buffer have been used successfully in other studies of protein interactions [32,40,44].

Example sensorgrams for the HMGA2- SELEX 2 DNA interaction are shown in Fig. 2a and binding curves for HMGA2 with SELEX 1 and SELEX 2 DNAs are in Fig. 2b. A 2:1 binding model (Methods Section) provides an excellent fit to the results and shows that HMGA2 has one strong binding site with K_1 near 10^7 M^{-1} and a second site that is over 100 times weaker for both sequences (Table 1). The weak site suggests that some non-specific binding occurs, probably due to electrostatic interactions between the cationic HMGA and the anionic backbone of DNA, and this is confirmed in the netropsin experiments described below. The binding strength is somewhat weaker for HMGA2 with SELEX 2 than with SELEX 1, probably due to the loss of one AT base pair (Figure 1).

An SPR screening assay for small-molecule inhibitors of the DNA HMGA2 interaction

The HMGA2 (MW 10,800) molecular weight protein is much higher than for AT-minor-groove binding small molecules (typical MW 300 ~ 500). Netropsin (MW 432), a well characterized AT minor groove binder with antibiotic and antiviral activities [14,45-47], was used to develop an SPR inhibition assay for the transcription factors that target the DNA minor groove. In the screening assay a series of samples with a fixed concentration of HMGA2 and varied concentrations of netropsin were injected over the immobilized DNA surface in each flow cell. The observed RU response for competition binding at the AT sites is the sum of the mass from bound HMGA2 and bound netropsin on the sensor chip surface. If netropsin competes with HMGA2 at the same sites and inhibits the binding of HMGA2, the RU response signal will decrease since the molecular weight of netropsin is much smaller than that of HMGA2. If netropsin binds at a different binding site without inhibiting the binding of HMGA2, or forms a cooperative complex with HMGA2, the RU should increase.

Figure 3 presents a typical SPR binding sensorgram for netropsin competition with HMGA2 and SELEX 1 DNA. The large decrease in SPR signal and shape of the sensorgrams shows that netropsin directly competes with HMGA2 at the AT binding sites in both SELEX 1 DNA and SELEX 2 DNA. The top curve (red) and bottom curve (blue) in Fig. 3 are 0.1 μ M HMGA2 protein alone and 1 μ M netropsin alone, respectively. The middle curves (black) are 0.1 μ M HMGA2 protein in the presence of increasing concentrations of netropsin (top to bottom). A concentration of 0.1 μ M HMGA2 was used in the competition assay based upon the direct HMGA2 binding experiments (Fig. 2). At this concentration approximately 90% of the DNA binding sites are initially saturated by HMGA2. As the low-molecular-weight netropsin replaces higher-molecular-weight HMGA2 at the DNA binding sites, the bound mass on the surface is reduced and a decreased RU response relative to HMGA2 alone is easily observed. The sensorgram signal drops with the increase of netropsin concentration until complete inhibition of HMGA2 binding.

Figure 3 also illustrates an interesting and unusual “increase-decrease-plateau” phenomenon in the RU response in the association part of competition binding sensorgrams. The unusual shape can be understood in terms of the quite different kinetics and molecular weights of the protein and small molecule. In the case of HMGA2 alone the steady-state is reached rapidly while in the presence of netropsin the observed RU reaches a high response at the beginning of injection and then decreases. This sensorgram shape can be explained by rapid binding of the higher molecular weight protein with subsequent partial protein displacement by the slower binding small molecule. This results in an increase in RU on rapid HMGA2 binding, a decrease as HMGA2 is displaced by netropsin and, finally, a plateau at longer times as both compounds reach a steady state dynamic interaction with the immobilized DNA.

In addition, an unexpected RU response gap between the saturation of binding sites with netropsin in the presence of HMGA2 (bottom black curve) and in the absence of HMGA2 (blue curve) is observed in Fig. 3. The lowest response with the mixture sample does not drop to the level of netropsin alone, even at very high netropsin concentrations. The higher RU signal for saturation of netropsin in the presence of HMGA2 indicates that there is a type of bound HMGA2 that is not affected by netropsin. The direct binding experiments show that HMGA2 binds strongly at the AT sequence but also has a weaker nonspecific binding mode. Although netropsin displaces all HMGA2 in the AT binding site during competition, it seems that the nonspecific binding of HMGA2 is not significantly inhibited. The lack of inhibition of the HMGA2 nonspecific binding is a reasonable result given that netropsin has no significant nonspecific DNA interactions under the experimental conditions.

Inhibition data analysis

To provide a more quantitative comparison of compound inhibition potential, the small molecule concentration required for inhibition of 50% of HMGA2 binding to DNA, IC_{50} , should also be determined. In the SPR inhibition assay the observed RU signal in the SPR competitive binding experiment is the sum of the bound protein and bound netropsin. To evaluate the inhibitory effect on the binding interaction of HMGA2 with its target DNA, the total response in the steady-state region at each concentration was averaged and the signal from binding of netropsin at same concentration was subtracted. It should be noted that netropsin association is rate limiting for obtaining a steady-state or plateau response and longer collection times than are shown in Fig. 3 are required to obtain a steady state at low netropsin concentrations. The corrected SPR response values for HMGA2 binding were normalized by setting the top point (protein alone) as 100% HMGA2 protein binding to the specific binding site and the bottom point (maximum concentration of inhibitor, no specific binding from HMGA2), where the signal stops decreasing, as 0% HMGA2 specific binding to DNA. The normalized data were plotted versus the concentration of netropsin and the data were fitted

with equation (14) as described in the Methods Section. Figure 4a presents the best fit curves for inhibition of HMGA2-SELEX 1 DNA and HMGA2-SELEX 2 DNA interactions by netropsin. For comparison the data and fitting curves without correction from the netropsin binding experiment are shown in Fig. 4b and the results are summarized in Table 2. As expected, given the relatively low MW of netropsin, the IC_{50} values with and without correction from the netropsin binding experiments are identical within error. The inhibitory effect of netropsin on the binding of HMGA2 to both SELEX1 and SELEX 2 DNA is very similar.

Discussion

As described in the Introduction, some important regulatory proteins selectively bind to the minor groove, particularly at AT DNA sequences and are important in diseases from cancer to obesity. Inhibition of the interactions between such protein-DNA complexes is an attractive approach for selectively regulating expression of specific genes. Since the vast majority of regulatory proteins bind to DNA through the major groove and are relatively insensitive to minor-groove interactions, compounds that bind in the minor groove can be highly selective. Very little is known about small molecule inhibition of DNA-protein complexes, in general, and even less is known about inhibition of minor groove binding proteins. The AT hook HMGA proteins bind to the DNA minor groove and regulate many important biological functions. They are an attractive class for inhibitor design since much is known about minor groove binding small molecules. The biosensor-SPR based inhibition assay described here consists of a SELEX defined DNA sequence, the HMGA2 protein, and a small inhibitor that targets the DNA minor groove in AT sequences. The competition assay is based upon the SPR RU response, which is related to the bound mass on the sensor surface, and depends on the different molecular weights of HMGA2 and the small inhibitor.

Buffer composition and flow rate were found to be important factors for obtaining well-formed SPR sensorgrams. The HMGA2 protein can stick to surfaces and has a high positive charge at pH 7. High salt concentration, surfactant P-20, and high flow rate are very helpful in minimizing the protein absorption on flow surfaces and matrix of the flow cells. SPR binding studies show that HMGA2 binds to the target DNA with a strong affinity at specific binding sites that have 2-3 AT sequence tracts. The protein also has nonspecific interactions that are around 100 times weaker than the specific binding. The non-specific interactions are probably due to electrostatic interactions between negatively charged phosphate groups on the DNA backbone and multiple positively charged residues of HMGA2.

The SPR screening assay results confirm that HMGA2 and netropsin compete for binding to the AT binding sites of DNA. Netropsin blocks the strong binding site and efficiently inhibits the binding of HMGA2 to the biologically important sites on DNA. The HMGA2 protein has a significantly higher molecular weight than netropsin, and as a result, the SPR signals decrease with increased concentrations of netropsin until the protein is displaced from the strong binding site. The final RU signal is higher when the specific binding site is saturated with the compound in the presence of HMGA2 than in the absence of HMGA2. This reveals that netropsin only blocks the binding of the protein in the specific binding site and does not inhibit the non-specific binding. This is the first biosensor-SPR observation of highly specific competition in the DNA minor groove.

An “increase-decrease-plateau” is observed in the association phases of the competitive binding sensorgrams and the unusual shape is due to the different association kinetics of binding to DNA for netropsin and HMGA2 under these conditions. HMGA2 binds rapidly with a larger signal (the increase region) and is then displaced by netropsin (the decrease region). A plateau is reached at longer times when netropsin and HMGA2 are in a steady-state equilibrium with immobilized DNA. It can be seen that the displacement of the protein is rapid

at higher concentration where the bimolecular association rate of netropsin is increased. In addition, on the dissociation phase of the sensorgram, the kinetics becomes closer to the slow observed kinetics of netropsin, which also supports netropsin competition binding at the same site. The IC_{50} inhibition values can also be determined by fitting the normalized data with the model and equation (Methods Section, Eq. 14) that are appropriate for 2:1 (netropsin) and 1:1 (HMGA2) competition.

In conclusion, biosensor-SPR methods have been used to measure the direct binding of the HMGA2 transcription factor to immobilized DNA. The protein binds in a 1:1 complex to two closely spaced DNA sequences that have 5-6 adjacent AT base pairs. The binding is strong with K_D values of 20 - 30 nM depending on the exact DNA sequence. An inhibition assay was developed for small molecule competition with HMGA2 binding. An equation for quantitative analysis of the results for the case of different stoichiometry for the small molecule (2:1) and protein (1:1) binding to DNA has been derived and applied to this system. The results indicate that netropsin directly competes with HMGA2 for binding to the same AT binding sites on the DNA and thus inhibits the specific binding of HMGA2. This method is very effective for evaluating complex inhibition results when a small inhibitor has multiple binding sites in the DNA-protein complex recognition sites. Competition kinetics can also be monitored using the biosensor-SPR method and this is clearly an advantage over other inhibition assays. In terms of drug design and development, the method provides an approach for screening and quantitatively characterizing the inhibition of transcription factor binding to DNA. It should be an advantage in discovery of new compounds to regulate gene expression by modulating transcription factor binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HMGA2, high mobility group A2 protein; SELEX, systematic evolution of ligands by exponential enrichment; SPR, surface plasmon resonance; RU, resonance unit; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); NHS, N-hydroxysuccinimide; EDC, N-ethyl-N'-(dimethylaminopropyl) carbodiimide.

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Scheme 1.

Competition model for 1:1 binding by a protein or ligand (L) and a two-site binding for competitor (C) with DNA (D). K_L is the equilibrium binding constant for binding of ligand to DNA, and K_{C1} and K_{C2} are macroscopic equilibrium binding constants for binding of small competitor to DNA. Both DC and DC₂ complexes inhibit binding of L to DNA.

Netropsin

DNA:

SELEX 1: 5'-biotin-CC**ATATT**CGCG**ATTATT**GC
GG**TATAAG**CGCT**AATAAC**G

SELEX 2: 5'-biotin-CC**ATATT**CGCGG**TTATT**GC
GG**TATAAG**CGCC**AATAAC**G

HMGA2 protein:

MSARGEGAGQPSTSAQGQPAAPVPQKRGRGRPRKQQQEPTCEPSPKRPRGRP
KGSKNKSPSKAAQKKAETIGEKRPRGRPRKWPQQVVQKKPAQETEETSSQESAE
ED

Figure 1.

Netropsin structure, DNA sequences, and HMGA2 protein sequence.

Figure 2.

(a) SPR sensorgram of HMGA2 binding to SELEX 2 DNA in HEPES buffer at 25 C. The slight decrease in RUs at the near the end of the injection of protein in several sensorgrams is probably due to some slight differences in baseline drift between the sample and reference flow cells over long periods.

(b) Binding data for interactions of HMGA2 with SELEX 1 DNA (solid dots) and SELEX 2 DNA (open squares) and best fit curves for a 2:1 binding model (see Methods).

Figure 3.

The black curves are SPR competition sensorgrams for a mixture with a fixed concentration of HMGA2 (0.1 μ M) and varied concentrations of netropsin (0 to 1 μ M) binding to DNA in HEPES 20 buffer at 25 C at a flow rate of 50 μ l/min. The red curve is for HMGA2 binding without competitor and the blue curve is for netropsin binding (1 μ M) without HMGA2.

Figure 4.

Comparison of plots of normalized RU versus the concentrations of netropsin in SPR competition binding experiments with 0.1 μ M HMGA2 (see Methods): (a) Curves based on original RU response in the SPR inhibition assay (b) curves based on corrected RU response by subtracting the RU for netropsin binding in the absence of protein.

Table 1

Binding constants of HMGA2 protein with SELEX DNA sequences in HEPES 20 buffer at 25 °C

	Ka1 (M⁻¹)	Ka2 (M⁻¹)
SELEX 1	4.8×10^7	2.0×10^7
SELEX 2	2.5×10^7	2.9×10^7

Table 2IC₅₀ values for inhibition binding of HMGA2 to DNAs by netropsin in HEPES20 buffer at 25 °C

	IC ₅₀ ^a (nM)	IC ₅₀ ^b (nM)
SELEX 1	5.5	5.6
SELEX 2	5.9	5.8

All data were obtained using the SPR inhibition assay with a fixed concentration of HMGA2 (0.1 μM) and varied concentration of netropsin as shown in Figure 4

^aBased original RU response in SPR inhibition assay

^bBased upon corrected RU response by subtracting RU from netropsin