

Recognition of cell-specific binding of phage display derived peptides using an acoustic wave sensor

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Abstract

ASSLNIA, a peptide selected for murine myofibers using phage display technology, was immobilized onto an acoustic wave sensor. The sensor responded to murine and feline muscle homogenates indicating crosspieces interactions. Kidney, liver, and brain preparations produced insignificant responses. © 2002 Elsevier Science B.V. All rights reserved.

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The efficiency of drugs and therapeutic genes depends strongly on tissue-specific delivery. Identification of cell-specific binding ligands may be required for success in this approach. Development of ligands for many differentiated tissues such as mature muscle is limited by a lack of information on their cell-specific surface receptors. A solution to this problem can be found in the selection of ligands using phage display libraries, which is an approach requiring no prior knowledge of the target cell receptor expression and function [1]. However, in vivo screening protocol [2,4] cannot be applied to humans, as the procedure requires euthanasia. Therefore, we have developed an in vitro assay to examine the cross-species properties of phage display derived peptide ligands. This method allows detection of ligand receptor interactions directly in tissue homogenates, opening the possibility to estimate the apparent affinity of peptides selected in animals to human tissues.

Peptide specific to murine skeletal muscle, ASSLNIA, was identified [4] by screening of a 7-mer

phage display peptide library (New England Biolabs, Inc., Beverly, MA, USA). The peptide containing a GGGSK spacer added to C-terminus was synthesized, modified by coupling of a biotin molecule, and HPLC purified to 98% by Peptide Technologies Corporation (Gaithersburg, MD, USA). Samples of mouse and cat skeletal muscle, mouse kidney, liver and brain were harvested and tissue aliquots were homogenized in phosphate buffered saline (PBS). After homogenization samples were microcentrifuged for 10 min at 3000 rpm, and the supernatants containing tissue vesicles were separated from the pellets. Tissue homogenization resulted in the formation of vesicles, whose membranes are identical to the cell membrane. Vesicle concentration and integrity were examined by dark-field microscopy [3]. To perform the blocking experiment, free ASSLNIA peptide (1 mg/ml) synthesized by Research Genetics, Inc. (Huntsville, AL, USA) was added to murine muscle tissue homogenate (50 mg/ml) and incubated at room temperature for 1 h with agitation.

Monolayers containing biotinylated phospholipid (N(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) were transferred onto the gold surface of an acoustic wave sensor using the Langmuir–Blodgett (LB) technique. Biotinylated peptide

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(ASSLNIAGGGSK-Biotin) was coupled with the phospholipid via streptavidin/biotin binding. The stable LB film [5] of biotinylated monolayers was treated for 2 h with streptavidin, diluted to a final concentration of 0.01 mg/ml in the subphase solution [5], rinsed with distilled water, and dried for 2 min in ambient air. Then, the film was treated with the biotinylated peptide (0.001 mg/ml) in subphase solution for 2 h, rinsed and dried again as above.

Measurements were carried out using a PM-700 Maxtek plating monitor as previously described [5]. One ml PBS was delivered to the dry sensor surface and voltage was recorded for 4–8 min. Then PBS was removed and tissue homogenates of different dilutions were added sequentially to the sensor. Each experiment was replicated 2–4 times. Temperature of all samples was 25 °C. The ratio of occupied (Y) and free ($1 - Y$) peptide molecules on the sensor surface were analyzed by the Hill Plot [6]

$$\log\left(\frac{Y}{(1 - Y)}\right) = \log K_b + n \log[C] \quad (1)$$

where K_b is the association binding constant, C is a protein concentration in tissue homogenate, and n is the number of molecules bound to a single peptide.

Fig. 1 shows the sensor response curves obtained by exposing the sensor to PBS containing different concentrations of the murine muscle homogenate. The initial time of the response ($\tau_1 = 8 \pm 1$ s) does not depend on the vesicle concentration. For each vesicle concentration the sensor signal approaches a steady-state value, corresponding to that concentration within 100 s ($\tau_2 = 70 \pm 20$ s). The response curves are distinguished by the fast reaction, the attainment of a steady state, and low non-specific binding [5].

In Fig. 2, upper curve, the mean values of the steady state output sensor voltages were plotted as a function of the relative concentration of the murine muscle homogenates. The interaction of the homogenate with the peptide was specific because preincubation of the murine muscle homogenate with free peptide resulted in significantly reduced signal (lower curve). When the

Table 1
Specificity of the interaction between ASSLNIA peptide and murine muscle homogenates demonstrated in the blocking experiment with free peptide

| Tissue homogenate | K_d (mg/ml) ^a | M_{\max} (ng/cm ²) ^b |
|-------------------|----------------------------|---|
| Murine muscle | 0.28 ± 0.05 | 54.0 ± 4.0 |
| Blocked muscle | 0.32 ± 0.05 | 34.4 ± 3.0 |

^a The apparent dissociation constant, $K_d = 1/K_a$, was calculated from Hill plots.
^b The apparent maximal surface concentration of protein, M_{\max} , was estimated from an increase of the sensor voltage assuming that the bound layer has a density ~ 1 g/cm³.

Table 2
Interspecies specificity of ASSLNIA peptide to murine and feline muscle

| Tissue homogenate | K_d (mg/ml) ^a | M_{\max} (ng/cm ²) ^a | Hill coefficient ^b |
|-------------------|----------------------------|---|-------------------------------|
| Murine muscle | $0.32 \pm \pm 0.05$ | 50.5 ± 3.0 | 0.92 ± 0.04 |
| Feline muscle | 0.37 ± 0.05 | 32.8 ± 2.0 | 0.93 ± 0.04 |

^a K_d and M_{\max} were calculated as explained in the legend to Table 1.
^b Hill coefficient was calculated by Eq. (1).

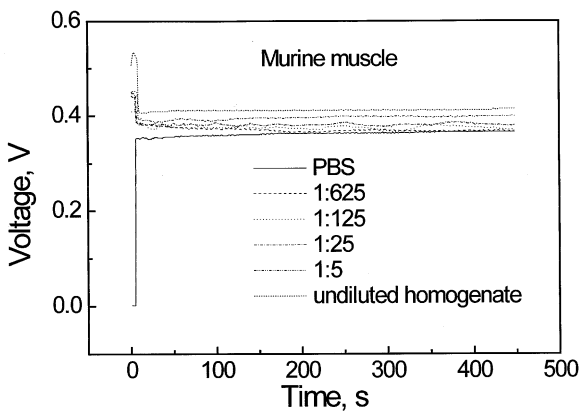


Fig. 1. Response curves obtained by exposing the sensor to murine muscle homogenate (3.8 mg/ml of protein) diluted in PBS at ratios of 1:5, 1:25, 1:125, and 1:625. Approximately 480 data points taken once a second during 8 min represent each line.

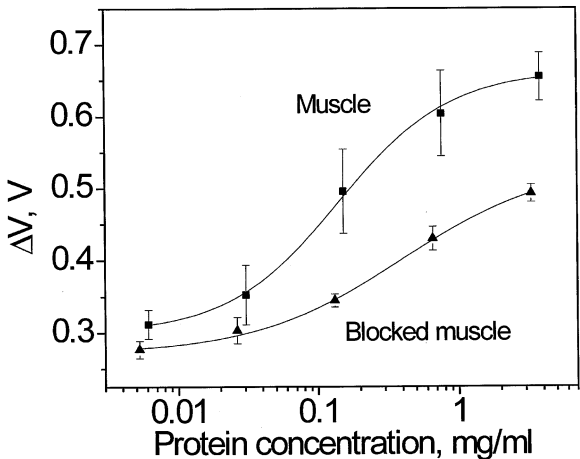


Fig. 2. Dose response plots. The curves represent the mean values of steady-state output sensor voltages as a function of protein concentrations in free and blocked murine muscle homogenates. Experimental values were obtained by averaging about 200 data points of each steady-state level of response curves exemplified by Fig. 1; bars are S.D. Each experiment was replicated 3–4 times. Smooth curves are the sigmoid fits to the experimental data ($\chi^2 = 0.199$, $R^2 = 0.999$, muscle; $\chi^2 = 0.053$, $R^2 = 0.999$, blocked muscle). ΔV were calculated by subtraction of the mean value of the voltage response to PBS from mean values of responses to homogenates.

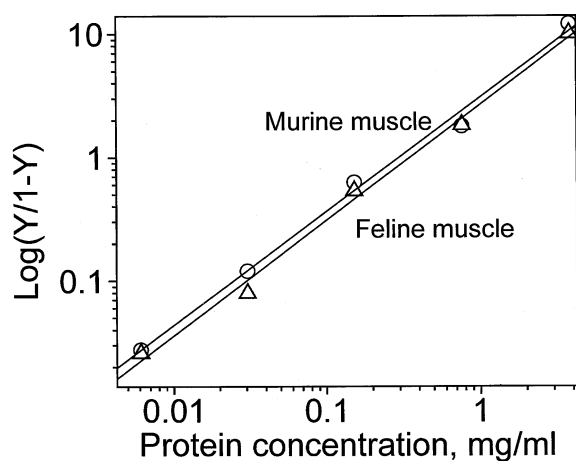


Fig. 3. Cross-species specificity of the peptide selected for murine muscle. Hill plots. Points represent experimental data. Lines represent the least square fit of Eq. (1) with coefficients shown in Table 2 (for both lines $R = 0.997$, $SD = 0.087$, $P < 0.0001$).

peptide (ASSLNIAGGGSK-Biotin) was replaced in the sensor structure with another peptide (CGHHPVY-ACGGGSK-Biotin) the sensor did not show any significant response to the murine muscle homogenate (data not shown). Binding parameters calculated from the Hill plot Eq. (1) are shown in Table 1. The data indicate that the apparent dissociation constants are not significantly different while the apparent maximal surface concentration decreased significantly ($P < 0.001$) when binding sites were blocked by free peptide. While the amplitudes of the responses to the feline muscle homogenates were lower compared to those of the murine muscles (Table 2, Fig. 3), the same K_d indicates that the peptide has cross-species affinity. As concluded from the value of the Hill coefficient, one binding site is needed to bind a single molecule to the sensor surface [6].

Fig. 4 shows selectivity of the sensor. The upper line represents the dose response of the peptide sensor to the murine muscle homogenate. Two lower lines show the dose responses of the peptide sensor to the murine

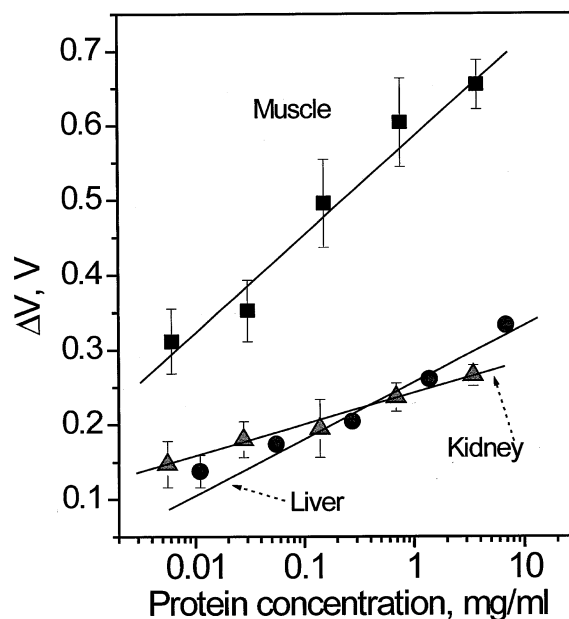


Fig. 4. Selectivity of the muscle-specific peptide. Dose response plots were recorded with the peptide sensor exposed to murine muscle, kidney, and liver homogenates. Points represent experimental data of 2–3 independent experiments. Lines represent the least square fit of Eq. (2) with coefficients shown in the footnote of Table 3 ($R = 0.988$, $P < 0.01$, muscle; $R = 0.997$, $P < 0.001$, kidney; $R = 0.977$, $P < 0.01$, liver).

liver and kidney homogenate. For any given concentration the sensor response for the muscle homogenate is greater than for liver or kidney. The voltage response (ΔV) as a function of protein concentration (C) is represented by the following empirical equation:

$$\Delta V = A + S \log C \quad (2)$$

where C is the protein concentration in a tissue homogenate, A is the constant, and S is the slope of the dose response dependence, defined as the sensitivity of the sensor [7].

The selectivity coefficient for any tissue homogenate to the muscle homogenate (K) can be estimated from the voltage responses at different concentrations using a

Table 3
Selectivity of the muscle-specific peptide

| Tissue, murine | Sensitivity, S (V/decade) | Selectivity coefficient, K (relative units) ^a | Activity ratio, R (relative units) ^b | Phage selectivity (relative units) ^c |
|----------------|--------------------------------|---|--|--|
| Muscle | 134 ± 20.0 | 1.0 | 1.0 | 1.0 |
| Kidney | 41.8 ± 7.0 | $(3.1 \pm 1.0) \times 10^{-1}$ | $(9.3 \pm 3.2) \times 10^{-5}$ | 1.1×10^{-1} |
| Brain | 62.5 ± 16 | $(4.6 \pm 1.4) \times 10^{-1}$ | $(3.4 \pm 1.1) \times 10^{-3}$ | 0.5×10^{-1} |
| Liver | 67.3 ± 6.0 | $(5.0 \pm 1.5) \times 10^{-1}$ | $(1.1 \pm 0.4) \times 10^{-3}$ | 0.7×10^{-1} |

^a Change of the output voltage is empirically described by the Eq. (2). The coefficient of selectivity for a certain tissue is calculated from Eq. (4).

^b The activity ratio R Eq. (3) was calculated using experimental equations: $\Delta V_{\text{muscle}} = 0.593 + 0.134 \log C$; $\Delta V_{\text{kidney}} = 0.240 + 0.0418 \log C$; $\Delta V_{\text{brain}} = 0.296 + 0.0625 \log C$; $\Delta V_{\text{liver}} = 0.260 + 0.0673 \log C$ at the protein concentration in tissue homogenate equal to 0.01 mg/ml.

^c Phage selectivity was calculated from the binding of ASSLNIA phage to muscles, brain, liver, and kidney compared to the binding of control wild-type phage in vivo experiments in mice [4].

method similar to the matched potential method [7]. The selectivity coefficient (K) is defined as the activity ratio (R) of primary to interfering species ($\Delta C_{\text{muscle}}/\Delta C_{\text{tissue}}$) that gives the same response change at the same condition. By using the definition of the selectivity coefficient and Eq. (2) the following is derived:

$$R = \frac{\Delta C_{\text{muscle}}}{\Delta C_{\text{tissue}}} \quad (3)$$

$$K = R = \frac{S_{\text{tissue}}}{S_{\text{muscle}}} \quad \text{when } \Delta C_{\text{muscle}} \Rightarrow 0 \quad (4)$$

where S_{muscle} and S_{tissue} are slopes of voltage responses to muscle and another tissue (kidney, liver, or brain) homogenates.

Table 3 illustrates the selectivity of the muscle-specific peptide. A marked response difference for the muscle homogenate is observed over all other tissues even when the concentrations of other tissues substantially exceed the concentration of the muscle homogenate. For example, the activity ratio for the muscle and kidney homogenates at 0.01 mg/ml is equal to 9.3×10^{-5} . This means that more than 100 mg/ml of the kidney homogenate is needed to induce the same response as induced by only 0.01 mg/ml of the muscle homogenate. Thus, the peptide sensor at these conditions selectively prefers muscle to kidney homogenates by factor of more than 10 000.

Novel delivery ligands can be selected in vivo in

animals from phage display peptide libraries. In this study, we have designed the biosensor to quantify specific interactions of phage display derived peptides with a cell surface component of a tissue homogenate. It opens the possibility to examine the cross-species properties of the peptides and may accelerate their use as site-specific delivery vehicles in clinical applications.

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