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Characteristics of Monoclonal Antibody Binding with the *C* Domain of Human Angiotensin Converting Enzyme

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Abstract—Binding of a panel of eight monoclonal antibodies (mAbs) with the *C* domain of angiotensin converting enzyme (ACE) to human testicular ACE (tACE) (corresponding to the *C* domain of the somatic enzyme) was studied, and the inhibition of the enzyme by the mAb 4A3 was found. The dissociation constants of complexes of two mAbs, 1B8 and 2H9, with tACE were 2.3 ± 0.4 and 2.5 ± 0.4 nM, respectively, for recombinant tACE and 4.7 ± 0.5 and 1.6 ± 0.3 nM for spermatozoid tACE. Competition parameters of mAb binding with tACE were obtained and analyzed. As a result, the eight mAbs were divided into three groups, whose binding epitopes did not overlap: (1) 1E10, 2B11, 2H9, 3F11, and 4E3; (2) 1B8 and 3F10; and (3) 1B3. A diagram demonstrating mAb competitive binding with tACE was proposed. Comparative analysis of mAb binding to human and chimpanzee ACE was carried out, which resulted in revealing of two amino acid residues, Lys677 and Pro730, responsible for binding of three antibodies, 1E10, 1B8, and 3F10. It was found by mutation of Asp616 located close to Lys677 for Leu that the mAb binding epitope 1E10 contains Asp616 and Lys677, whereas mAbs 1B8 and 3F10 contain Pro730.

Key words: angiotensin converting enzyme, C domain, monoclonal antibodies

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INTRODUCTION

Angiotensin converting enzyme (ACE, peptidyl dipeptidase, EC 3.4.15.1), a Zn^{2+} dependent metallopeptidase belonging to the class of zincins, is one of the major regulators of blood pressure and content of vasoactive peptides in organism.[2] This enzyme is also involved in neuropeptide metabolism and immune and reproductive functioning [1, 2].

Two ACE isoforms are synthesized in mammalian cells. The ACE somatic form is composed of two homologous domains (*N* and *C*) within a single polypeptide chain, each of which contains a catalytic active site. A shorter testicular ACE form (tACE) synthesized in testicles corresponds to the *C* domain (with exception of 36 aa from the *N* terminus). Now crystal structures of single domains have been published [3, 4], but the structure of a full size enzyme remains unknown.

Localization of antibody binding epitopes on the ACE surface would provide identification of new functional regions of the enzyme molecule. Determination of binding of sheep polyclonal antibodies to human somatic ACE using potential linear antigen determinants (synthetic linear hexapeptides) showed that about 70% of these peptides actually comprise antibody binding epitopes and are located on the surfaces of both *C* and *N* domains of ACE protein globule, sometimes giving clusters [5]. Using monoclonal antibodies, a region on the ACE *N* domain surface responsible for the ACE carbohydrate-controlled dimerization, which may be related to ACE proteolytic cleavage from the cell surface, was determined [6]. Determination of binding epitopes for two mAbs to the ACE *N* domain along with available kinetic data provided the demonstration of conformational changes in the enzyme molecule upon the ligand (substrate or ligand) inhibitor binding [7]. The knowledge of binding epitopes for some mAbs allowed the development of detection methods of amino acid residue mutations in the perimembrane enzyme region, which lead to a considerable increase in ACE shedding from the cell surface [8, 9]. In addition, mAbs to human ACE recognizing conformational epitopes on the surface of the ACE *N* domain and

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[2] Abbreviations: ACE, angiotensin converting enzyme; tACE, testicular ACE; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; Hip, hippuryl.

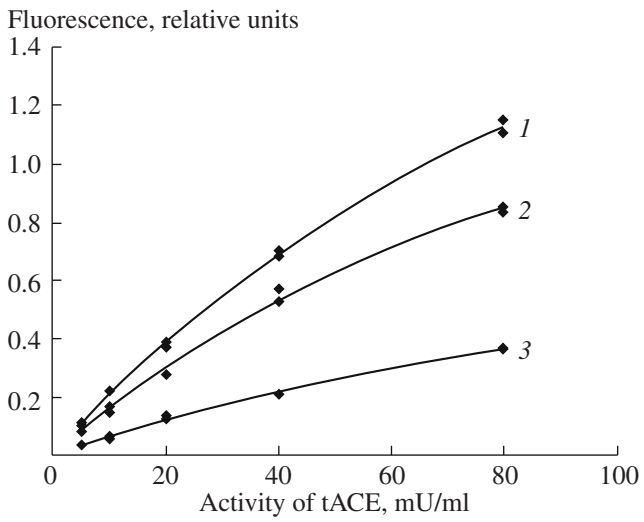

Fig.1. Dependence of tACE complex formation with mAb 1E10 on the concentration of the enzyme at mAb concentrations (*1*) 10, (*2*) 3, and (*3*) 1 μg/ml. Antibodies 1A10 were bound to the polyclonal antibodies to mouse immunoglobulins preliminarily sorbed in microplate wells and the enzyme at varied concentrations was added into the wells. The mAb–tACE complex formation was monitored by measuring the ACE enzymatic activity.

sequential epitopes on the denatured *C* domain are successfully used for the quantitative determination of both ACE forms [10] and enzyme inhibitors [11] in solution using ELISA, as well as of ACE on the cell surface using flow cytometry [12].

Whereas the epitope mapping of the ACE *N* domain has been rather complete [7, 11, 13, 14], binding epitopes for only two mAbs, 1B3 and 5C8, recognizing *C* domain, have been found so far [9], the mAb 5C8 binding only to the denatured enzyme.

We describe in this work a comparative study of binding of a panel of eight mAbs to tACE and the identification of some amino acid residues of the enzyme critical for binding.

RESULTS AND DISCUSSION

A comparison of binding of various mAbs to tACE was achieved by immunoprecipitation. For optimization of conditions of all further experiments, we used spermatozoid tACE and mAb 1E10.

For immunoprecipitation, polyclonal antibodies to mouse immunoglobulins, then mAbs, and finally tACE were successively adsorbed in microplate wells. In this case, we chose the concentration of polyclonal antibodies of 10 μg/ml, which was enough for saturation of the plate wells [14]; mAb and tACE concentrations were varied. The amount of the resulting mAb–tACE complex in microplate wells was determined by the enzymatic activity of bound ACE. As a substrate, Hip-His-Leu was used; its hydrolysis rate was determined fluorimetrically according to the product (His-Leu) accumulation. The reaction time was taken, so that the hydrolysis percentage did not exceed 10% and fluorescence values exceeded the background five times or more.

The curves showing the dependence of tACE complex formation with the immobilized mAb 1E10 on the enzyme amount at various mAb concentrations are given in Fig. 1. One can see that the curves corresponding to the selected mAb concentrations have a quasi linear region up to the enzyme concentration of 40 mU/ml, which corresponds to 0.3 μg/ml. In further experiments, both tACE preparations were used within the range of 5–20 mU/ml (0.04–0.15 μg/ml).

The mAb concentration of 3 μg/ml was chosen for further studies, because, in this case, the fluorescence signal was rather intensive and considerably (by 10–20 times) exceeded that of the background. This concentration also helped to avoid additional consumption of antibodies (10 μg/ml versus 3 μg/ml, practically, threefold difference). Since it turned out that mAb 1E10 is relatively weakly bound to tACE (see below), the conditions chosen for these antibodies were also suitable for other mAbs with the same or better binding to the enzyme.

Analysis of mAb Binding to tACE

Relative binding of mAb to ACE *C* domain was evaluated by determination of activities of tACEs (both recombinant and spermatozoid) bound to various plate-adsorbed mAbs. It turned out that fluorescence values obtained upon tACE binding to mAbs 1B3, 1E10, 3F11, and 4E3 were significantly lower than those bound to the 1B8, 2B11, 2H9, and 3F10 mAbs (Fig. 2). A relatively low fluorescence signal of ACE binding to the first mAb group could result from either lower binding constants to these mAbs or antibody-induced inhibition of tACE activity.

With the goal to evaluate the reason for a low fluorescence signal upon tACE binding to some antibodies, we analyzed 1B3, 1E10, 3F11, and 4E3 mAbs in solution for the inhibitory activity toward tACE. It turned out that a considerable excess of 4E3 antibodies (mAb/tACE 4E3 = 300/1) decreased the tACE activity nearly by 40%, whereas a similar excess of other antibodies did not affect the enzymatic activity. Thus, we can presume that monoclonal 4E3 antibodies display small inhibitory activity and all mAbs of this group are characterized by relatively low binding constants to tACE, antibodies 1B8, 2B11, 2H9, and 3F10 being more tightly bound to the enzyme.

We determined dissociation constants of the complexes of two effectively binding mAbs 1B8 and 2H9 with both tACE preparations using immunoprecipitation.

For mAbs 1B8 and 2H9, the obtained values were 4.7 ± 0.5 and 1.6 ± 0.3 nM for spermatozoid and 2.3 ± 0.4 and 2.5 ± 0.4 nM for recombinant tACE, respec-

tively. The difference in dissociation constants of the mAb complexes with two tACE preparations could be determined by both differences in glycosylation of the native and recombinant forms and a possibility of keeping the hydrophobic anchor in the native spermatozoid tACE.

Competition of Monoclonal Antibodies for Binding to tACE

We evaluated the binding of tACE–competing antibody complexes to the tested antibodies by ELISA to analyze the mAb capacity to compete with each other for tACE binding. If the tACE–competing antibody complex binds to the tested antibodies with the efficiency close to that of the free enzyme, binding epitopes for the two antibodies do not overlap. In the case if the tACE–competing antibody complex does not bind to the tested antibodies, one can claim that the binding epitopes for these antibodies are overlapped to a large degree. If the binding of the tACE–competing antibody complex to the tested antibodies is less effective than that of the free enzyme, one can propose that the binding epitopes for these antibodies are partially overlapped.

We believe that epitopes of two antibodies considerably overlap if the formation of tACE–competing antibody complex is inhibited by more than 75%; i.e., the overlapping degree of these epitopes exceeds 75%. The inhibition of tACE binding lower than 25% implied weak competition of two antibodies. If a degree of binding inhibition was lower 10%, we considered it within an experimental error and believed that mAb did not compete for binding to tACE.

We determined the capacity to compete with each other for binding to tACE for each mAb pair; both mAbs from each pair were used both as competing and tested. The obtained results are shown in Fig. 3*a*.

An analysis of the data obtained resulted in a diagram clearly demonstrating competitive and noncompetitive mAb–tACE binding (Fig. 3*b*). Based on the obtained results, we can state that there are three immunologically important regions on the ACE *C* domain surface, where mAb epitopes are located. One region contains five mAb binding epitopes (1E10, 2B11, 2H9, 3F11, and 4E3), the second contains two (1A8 and 3F10), and the third one, one mAb (1B3). At the same time, a considerable (mAb 1E10 and 4E3, 1B8 and 3F10) or partial (mAb 1E10 and 2B11, 3F11 and 2H9) competition occurs within the groups for binding to ACE *C* domain.

Comparative Binding of Monoclonal Antibodies to Human and Chimpanzee ACE

A comparison of mAb binding to somatic ACE from human and chimpanzee blood revealed three antibodies (1E10, 1B8, and 3F10) that could bind to human, but not to chimpanzee ACE (Fig. 4*a*). Other antibodies

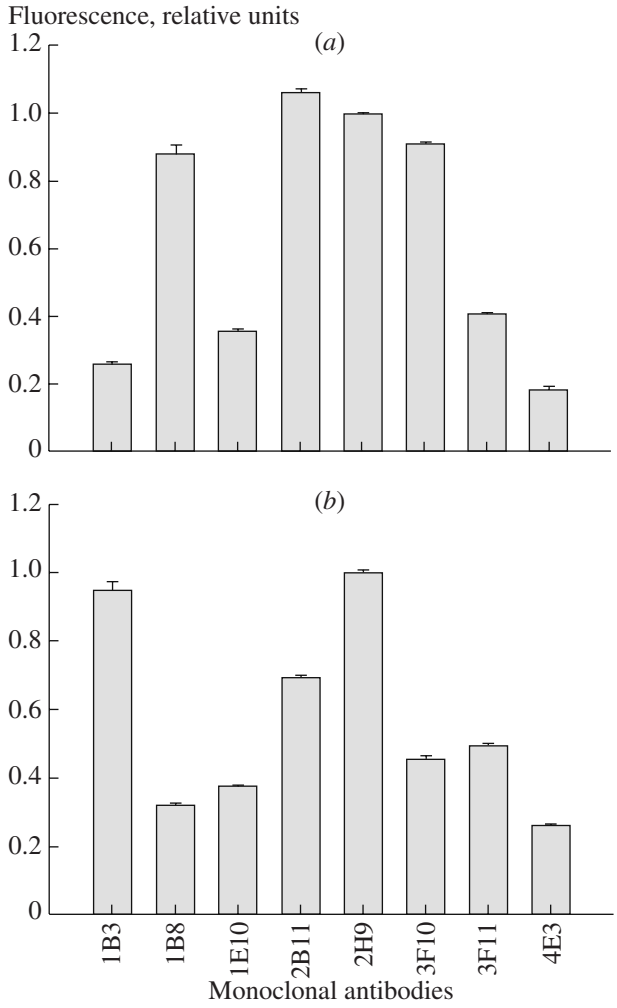

Fig. 2. Relative binding of (*a*) recombinant and (*b*) spermatozoid tACE to the mAb against the ACE *C* domain.

bound to these enzymes practically with the same efficiency. The analysis of amino acid sequences of human and chimpanzee ACE showed that their *C* domains differ by three amino acid residues, Lys677, Pro730, and Gly1269, with Gly1269 being located in the transmembrane region (Fig. 4*b*). Since we used in this work human and chimpanzee enzymes lacking a membrane anchor, the Gly1269 residue was not involved in the complex formation with the tested mAb. Thus, only two amino acid residues differing in human and chimpanzee ACE *C* domains (Lys677 and Pro730) are responsible for binding of three mAbs: 1B8, 3F10, and 1E10.

An analysis of the surface of human ACE *C* domain ([3], PDB: 1O8A) showed that Lys677 and Pro730 are located at the opposite sides of the enzyme globule. The binding epitopes for 1B8 and 3F10 antibodies are nearly completely overlapped, whereas mAb 1E10 does not compete with mAbs 1B8 and 3F10 for tACE binding. Therefore, we can conclude that Lys677 is responsible for binding of mAb 1E10, while Pro730 is

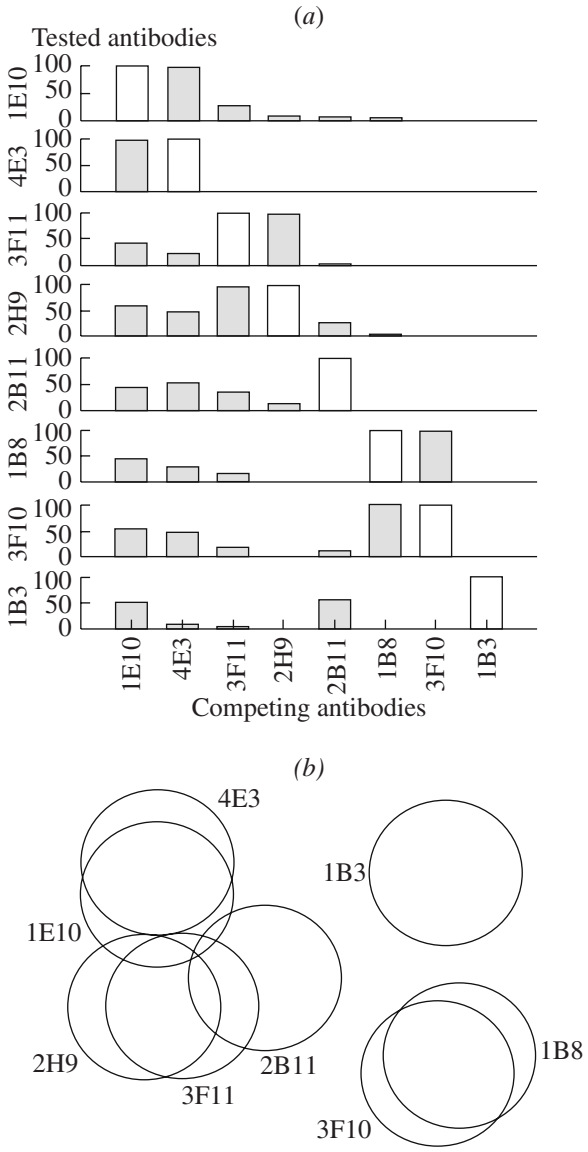

Fig. 3. Determination of mAb competing properties for binding to human tACE. (*a*) An inhibition degree (%) with competing mAbs of the recombinant tACE binding to the tested mAbs. A mixture of tACE and competing mAbs at a 100-fold excess was incubated before evaluation of binding of the resulting complex to microtitrator cells covered with the tested antibodies; (*b*) a diagram demonstrating a competitive and noncompetitive binding of the panel of monoclonal antibodies to tACE. Competitive binding is shown by overlapping circles; noncompetitive binding, with nonoverlapping circles.

a component of binding epitopes of mAbs 1B8 and 3F10 or vice versa. For determination of the amino acid residue composing any binding epitope, we studied the mAb interaction with the mutant ACE *C* domain (D616L), in which Asp616 located close to Lys677 on the ACE *C* domain surface was replaced by Leu. This substitution proved to dramatically affect the mAb 1E10 binding and practically completely inhibited it; it also partially weakened the mAb 2H9 binding to tACE (by 50% if compared with tACE lacking the D616L substitution) but did not affect binding of other mAbs to the *C* domain. Thus, we can presume that Asp616 comprises binding epitopes for mAb 1E10 and 2H9, although this mAb does not play an essential role for binding of the latter. Since the Asp616 residue on the ACE *C* domain surface is located close to Lys677, we can propose that the mAb 1A10 binding epitope contains Lys677 rather than Pro730, whereas binding epitopes for mAbs 1B8 and 3F10 contain Pro730.

EXPERIMENTAL

Antibodies. Preparation of monoclonal antibodies to the ACE *C* domain was described in [9]. Polyclonal antibodies to murine immunoglobulins were from Pierce.

Enzymes. Human recombinant and spermatozoid tACEs were used. The plasmid for recombinant tACE and tACE produced in Chinese hamster ovary cells were described in [15]. Culture medium containing a soluble form of recombinant tACE was used in this work. Human tACE were also obtained from spermatozoids of healthy donors; they were centrifuged at 2000 *g*, twice resuspended in physiological solution, and centrifuged again. The residue was dissolved in 0.05 M phosphate buffer, pH 7.5, containing 0.15 M KCl, 1 μM $ZnSO_4$ (buffer A), and 0.1% Triton X-100 and stirred overnight at +4°C. After centrifugation at 10000 *g*, dry ammonium sulfate was added to the supernatant in small portions up to 35% saturation, the mixture was stirred for 30 min, centrifuged at 10000 *g*, ammonium sulfate was added up to 80% saturation, and the mixture was stirred under the same conditions and centrifuged. The resulting residue was dissolved in buffer A and purified by affinity chromatography up to the electrophoretically homogenous state using the method described in [16].

In single experiments, human and chimpanzee sera as well as the mutant form D616L of *C* domain of human ACE were used (unpublished data).

ACE activity was determined by the initial hydrolysis rate of Hip-His-Leu (Sigma, United States) in 0.05 M Tris buffer, pH 8.3, containing 0.3 M KCl (buffer B) at 37°C. The accumulation of the reaction product His-Leu was fluorimetrically measured using *o*-phthalaldehyde [17]. Background fluorescence was measured under the same conditions, with buffer A used instead of the enzyme. Standard fluorescence was measured as the background one, with the reaction product His-Leu at known concentrations used instead of the substrate. The amount of the enzyme capable of hydrolysis of 1 μmol of the substrate for 1 min under the conditions was taken as an enzyme activity unit (U).

The enzymatic activity was measured in the absence and in the presence of antibodies after the preliminary incubation of tACE with mAb at various concentrations

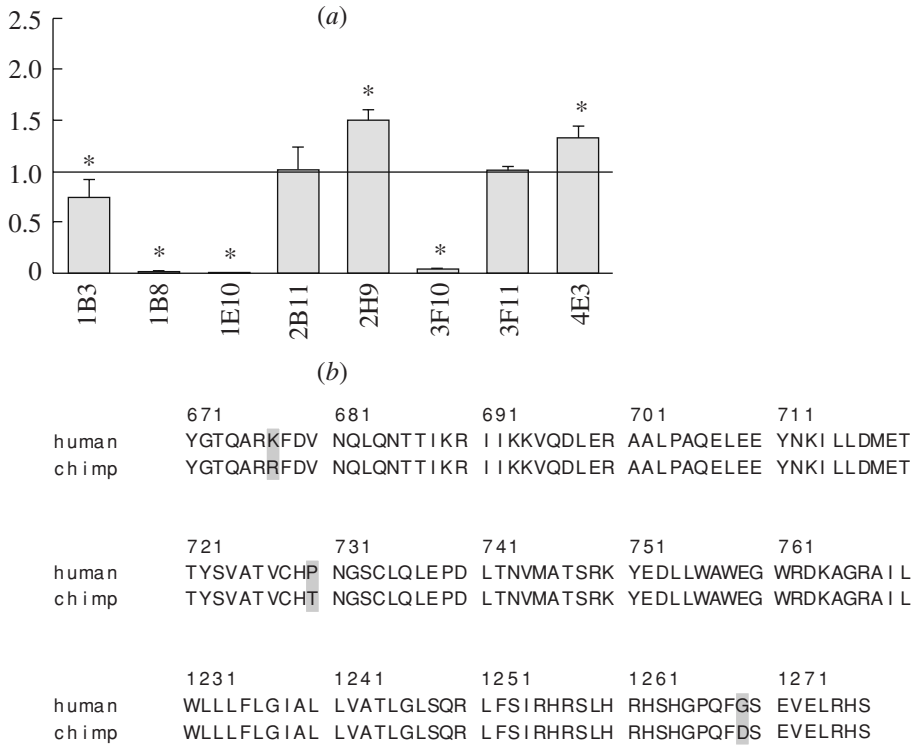

Fig. 4. Identification within ACE *C* domain of amino acid residues functionally important for binding to mAbs by comparative analysis of mAb binding to human and chimpanzee ACE. (*a*) Comparative binding of somatic human and chimpanzee ACE to various antibodies; (*b*) a comparison of primary amino acid sequences of human and chimpanzee ACE [http://cn.expasy.org/enzyme/3.4.15.1]. Amino acid residues different for human and chimpanzee ACE are shaded.

up to a 1000-fold excess for 2 h at 37°C for the determination of tACE inhibition affected by mAb.

Kinetic parameters of spermatozoid tACE–catalyzed hydrolysis of Hip-His-Leu substrate were determined in buffer B at 37°C by varying the substrate concentration within the range of 0.1–2 mM at the constant enzyme concentration of 0.5 nM. The content of tACE active molecules was preliminarily determined by lysinopril titration [18]; the kinetics of the enzymatic reaction obeyed to the Michaelis–Menthen equation. The activity was determined as described above; the hydrolysis time was chosen in the way, so that the hydrolysis degree of the substrate did not exceed 10%. The data were analyzed in $[S]/v$–$[S]$ coordinates [19], where $[S]$ is an initial substrate concentration, and v is the rate of enzymatic hydrolysis. Kinetic constants were k_{cat} 319 ± 22 s^{-1}, and K_m 1.9 ± 0.1 mM, which is in good agreement with the earlier data [20].

The binding of tACE to mAb. Polyclonal antibodies to mice immunoglobulins (50 μl, 10 μg/ml of each) were loaded into a 96-well polystyrol plate and incubated at 4°C overnight. The solution was removed and the wells were washed with buffer A containing 0.05% Tween 20. A solution of mAbs in buffer A (3 μg/ml, 50 μl) was added into each well and incubated at 37°C for 1 h. The wells were washed again with buffer A containing 0.05% Tween 20 and recombinant tACE solution (0.15 μg/ml, 50 μl) in buffer A was added. The mixture was incubated for 2 h at 37°C, and the wells were washed again. For developing antibody-bound tACE, a solution of 5 mM Hip-His-Leu (100 μl) in buffer B was added into each well and incubated at 37°C for a certain time period. The amount of the formed product was determined fluorimetrically using His-Leu solutions at the known concentration as a standard.

Competition of mAbs for tACE binding. Polyclonal antibodies to murine immunoglobulins were sorbed in microplate wells as described above and the mAbs tested for the antibody–antibody competition and tACE preliminarily incubated with a 100-fold excess of all competing mAbs were successively added into the wells. The tACE complex formation with the tested antibodies was compared in the presence and absence of competing mAbs.

Dissociation constant of tACE complexes with mAb 1B8 and 2H9. Polyclonal antibodies to murine immunoglobulins were sorbed in microplate wells, followed by successive addition of mAbs 1B8 or 2H9 and 0.5–5 nM (0.05–0.5 μg/ml) tACE, and the fluorescence of the product of enzymatic hydrolysis of the Hip-His-Leu substrate was determined at a fixed time. Initial concentrations of the active enzyme were calculated using the Michaelis–Menthen equation and the rate of

the enzymatic reaction determined under our conditions as $\Delta[P]/\Delta t$:

$$v = \frac{k_{cat}[E]_0[S]_0}{[S]_0 + K_m} \quad (1)$$

Thus, substituting kinetic parameters of the substrate hydrolysis in the equation, the substrate initial concentration, and experimental value of the reaction rate, we calculated initial tACE concentrations in the microplate wells for both enzyme preparations, provided that both recombinant and spermatozoid tACE hydrolyze Hip-His-Leu with the same kinetic parameters.

For calculation of dissociation constants of mAb–tACE complexes, we used $\Delta[P]/\Delta t$, measured fluorescence in the microplate wells for each tACE concentration; P_f, mAb fluorescence in the absence of the enzyme (background fluorescence, a lower plateau of the complete titration curve); and P_b, fluorescence at the enzyme saturating concentration (an upper plateau of the complete titration curve).

Concentrations of the tACE–mAb complex (C_b) were calculated using fluorescence values for each experimental point considering an equimolar ratio of tACE : mAb = 1 : 1 in the complex and assuming that the enzymatic activity is not changed upon the mAb complex formation.

The total concentration of mAb ($[mAb]_0$) precipitated on polyclonal antibodies to murine immunoglobulins was calculated similarly using the P_b fluorescence value and assuming that all mAbs are bound within the tACE–mAb complex at the enzyme saturating concentration. The concentration of free tACE (C_f) was determined by the difference between the total enzyme concentration (i.e., tACE concentration added to the well) and the tACE–mAb complex concentration (C_b).

Dissociation constants of tACE–mAb complexes were obtained by treatment of the obtained data in Scatchard coordinates C_b/C_f versus C_b according to the following equation:

$$\frac{C_b}{C_f} = -\frac{1}{K_a}C_b + \frac{1}{K_a}[mAb]_0 \quad (2)$$

The complex dissociation constant K_a was determined as a reciprocal of the tangent of slope in Scatchard coordinates.

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