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Mapping of viral epitopes with conformationally specific monoclonal antibodies using biosensor technology

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ABSTRACT

An automated biosensor system (BIAcore) designed for measuring molecular interactions in real time and without labelling any of the reactants was used for mapping the epitopes of tobacco mosaic virus protein using conformationally specific monoclonal antibodies (MAbs). Some of the MAbs used as capturing antibody on the sensor chip allowed a conformational change to occur in the viral protein. As a result, MAbs specific for the quaternary structure of polymerized viral protein were able to bind to monomeric viral subunits. Compared with classical solid-phase enzyme immunosassay, the biosensor technology possesses several advantages for epitope mapping of viral proteins.

INTRODUCTION

The BIAcore instrument (Pharmacia, Uppsala, Sweden) is a biosensor-based system for real-time biospecific interaction analysis. Its detection principle is based on surface plasmon resonance, a phenomenon which can be used to measure the interaction of biomolecules close to a surface without labelling any of the reactants. One of the reactants (*e.g.*, an antibody) is immobilized on a dextran layer present on the sensor chip and the other (the antigen) is introduced in a solution that flows over the surface [1].

Tobacco mosaic virus (TMV) particles can be dissociated into subunits by acid degradation. Attempts to map TMV protein epitopes by enzyme-linked immunosorbent assay (ELISA) have been made using conformationally specific monoclonal antibodies (MAbs). These MAbs were specific for cryptotopes (epitopes present only on dissociated protein), metatopes (epitopes present on monomeric protein and virions) and neotopes (epitopes present only on intact virus particles) [2–4]. The region of the protein recognized by the four anti-cryptotope MAbs was established by the ability of these MAbs to bind to synthetic peptides [5]. Three MAbs were found to react only with neotopes present in virus particles which possess an intact quaternary structure. Many MAbs were specific for metatopes and these were found to react only with one extremity of TMV particles and with protein aggregates known as discs [6]. These MAbs have been shown to recognize the surface of the protein subunit designated as the bottom, which contains the right radial and left radial α-helices comprising residues 74–89 and 114–134, respectively [7]. Attempts were made by ELISA to determine which region of the polypeptide chain was recognized by the anti-metatope MAbs using anti-cryptotope MAbs as capturing Ab. It was assumed that if the metatope was located on the same side of the subunit as the cryptotope, steric hindrance would prevent the simultaneous binding of the two types of MAb. Unfortunately, no definitive conclusion about the location of metatopes could be drawn from ELISA competition experiments [8]. A major difficulty in such studies is linked to the fact that TMV protein binds very strongly to plastic surfaces, even when microtitre plates are blocked with protein. As a result, high background readings are invariably observed, which makes the interpretation of data difficult.

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In this study, we tested the ability of pairs of MAbs directed to neotopes, cryptotopes and metatopes to react with TMV protein using the biosensor technology. No non-specific reaction was observed with this technology.

A necessary control in this type of experiment consists in using the same antibody as both first and second antibody. If the same antibody is able to bind twice, it indicates the presence of polymeric protein which makes epitope mapping by such an approach impossible. No dual binding of the same antibody to the protein was observed in the BIAcore system, indicating that only monomeric protein was immobilized on the sensor chip by the first antibody. For self-aggregating proteins such as viral subunits, it seems, therefore, that mapping of epitopes is more easily performed with the BIAcore than by classical solid-phase enzyme immunoassay. In two-site BIAcore assays with antimetatope MAbs, it was found that in some pairwise combinations, two such antibodies could bind to the monomeric protein, suggesting that there are at least two different metatope sites per protein subunit.

EXPERIMENTAL

Antigen

The coat protein of tobacco mosaic virus (TMV, common strain) which is acetylated at its N-terminus was obtained by acetic acid degradation of the virus. The non-acetylated tobacco mosaic virus protein obtained as an expressed gene product in *Escherichia coli* was a gift from Dr. P. J. G. Butler (Cambridge, UK).

Antibodies

Purified rabbit anti-mouse immunoglobulin G(IgG) Fc (RAMFc) was obtained from Pharmacia Diagnostics (Uppsala, Sweden). Anti-TMV, antineotope MAbs 253P, 107P and 79P, antimetatope MAbs 6P, 16P, 25P, 151P and 167P and anticryptotope MAbs 161P, 174P and 270P were those used in previous studies [3,5].

BIAcore experiments

Immobilization of RAMFc. The BIAcore system, sensor chip CM5, surfactant P20, amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N′-(3-diethylaminopropyl)carbodiimide (EDC) and 1 *M* ethanolamine hydrochloride (pH 8.5) were obtained from Pharmacia Biosensor (Uppsala, Sweden).

Immobilization of the ligand to the sensor chip was performed as described previously [1,9,10]. The carboxylated dextran matrix was activated with EDC and NHS. Next the ligand, RAMFc at 100 μg/ml in 10 m*M* acetate buffer (pH 5) as described [1,9,10], was injected. To deactivate the unreacted groups, 35 μl of ethanolamine were injected. After conditioning with 15 μl of 100 m*M* hydrochloric acid, the sensor surface was ready for use.

The surface plasmon resonance detector in the instrument responds to changes in the refractive index close to the sensor surface. The refractive index increment for proteins is proportional to the mass present within the detected volume and varies little between protein species. The refractive index changes are monitored continuously over time and are registered as a sensorgram [1]. The *y*-axis of the sensorgram is denoted as the resonance signal indicated in resonance units (RU). A signal of 1000 RU corresponds to a surface concentration change of *ca.* 1 ng/mm^2.

Two-site binding assay cycle. A volume of 4 μl of the first MAb (MAb1) in 4-(2-hydroxy-ethyl)-1-piperazineethanesulphonic acid (HEPES) buffer saline (HBS) was injected at a flow-rate of 5 μl/min and bound to the immobilized RAMFc. The unoccupied sites of RAMFc were blocked with 4 μl of a non-specific MAb (in HBS) to avoid binding of the second MAb (MAb2) to unoccupied ligand sites. A 4-μl volume of TMVP at 100 μg/ml in HBS was injected followed by 4 μl of MAb2. The surface was regenerated with 15 μl of 100 m*M* hydrochloric acid. The non-specific background level of antigen binding was measured with an unrelated virus, cowpea mosaic virus.

Multi-site binding assay cycle. Multi-site binding experiments were performed by injecting two secondary MAb in sequence before regeneration. To ensure that each epitope corresponding to the secondary MAb was saturated, a volume of 10 μl was injected.

ELISA procedures

Control experiments were carried out by double antibody sandwich ELISA using the procedures 3 and 5 described in ref. 8.

RESULTS

Two-site binding assay

A RAMFc immobilization sensorgram is shown in Fig. 1. In this instance 3.7 kRU of RAMFc were immobilized. The results of a two-site binding assay cycle showing the reaction between TMV protein and anti-cryptotope MAb 161P and anti-metatope MAb 6P are presented in Fig. 2A. In a control experiment, the background binding observed with an unrelated virus (cowpea mosaic virus) was insignificant (Fig. 2B). Various combinations of pairs of MAbs specific for neotopes, metatopes and cryptotopes were also tested using the same assay. The results are summarized in Table I.

The five MAbs directed to metatopes and the two anti-cryptotope MAbs 174P and 161P, when immobilized on the RAMFc, were able to trap TMV protein. However, the three anti-neotope MAbs and the anti-cryptotope MAb 270P could not.

Anti-neotope MAbs used as first antibody. None of the three anti-neotope MAbs when used as first Ab were able to bind the TMV protein or the non-acetylated TMV protein.

Anti-metatope used as first antibody. TMV protein trapped by any anti-metatope MAb was recognized by all anti-neotope antibodies used as MAb2 (Table I). Among the anti-cryptotope antibodies tested as MAb2, only MAb 161P was able to recognize the protein trapped by a first anti-metatope MAb. When anti-metatope antibodies were used both a first and second MAb, only the two MAbs 151P and 16P were able to bind concurrently to the protein irrespective of which one was used as first antibody. In all other combinations of anti-metatope MAbs, no dual binding was observed (Table I). No difference was observed between normal TMV protein and the non-acetylated one (results not shown).

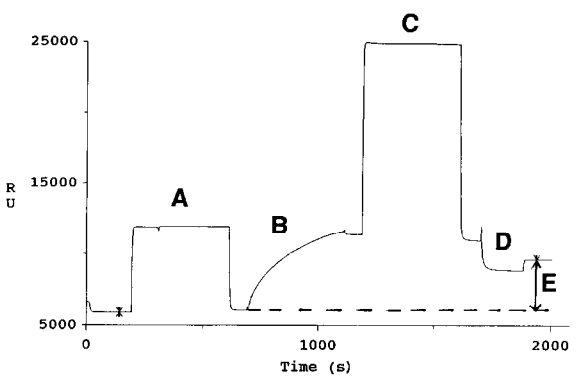

Fig. 1. Sensorgram showing immobilization of RAMFc on a sensor chip. A continuous HBS flow of 5 μl/min was allowed to pass over the sensor surface. The pulse of NHS/EDC (A) gives an increase in the SPR signal owing to the change in the bulk refractive index. B corresponds to the injection of 35 μl of RAMFc at 100 μg/ml in acetic acid (pH 5). Ethanolamine hydrochloride was then injected (C) to deactivate the surface and the RAMFc surface was conditioned (D) with 15 μl of 100 m*M* HCl. The amount of immobilized RAMFc (E) corresponds to 3.7 kRU.

Anti-cryptotope MAb used as first antibody. Anti-cryptotope MAb 270P was not able to trap TMV protein or the non-acetylated protein. When TMV protein was trapped by anti-cryptotope MAb 174P, only the anti-cryptotope MAb 161P was able to bind as MAb 2. No reactivity was observed with any of the other MAbs (Table I). TMV protein trapped by anti-cryptotope MAb 161P was recognized by all anti-metatope and anti-neotope MAbs used as second antibody (Table I). Non-acetylated TMV protein trapped by anti-cryptotope MAb 161P was recognized by anti-metatope MAbs but not by anti-neotope MAbs used as second antibody (results not shown).

Multi-site binding assay

Multi-site binding assays carried out with combinations of anti-metatope MAbs 16P and 151P and anti-cryptotope MAb 161P were also performed in the BIAcore as described previously [9]. The results showed that TMV protein trapped by any one of them could bind a second MAb but not a third MAb.

DISCUSSION

In the BIAcore system no reactivity was observed when the same anti-cryptotope MAb was used as first and second Ab (Table I), indicating that no oligomers were trapped by the MAb. The absence of protein polymers mimicking the quaternary structure of virus particles was corroborated by the absence of antigen binding observed when anti-neotope MAbs were used as capturing MAb. Among the combinations of anti-cryptotope MAbs tested, only the pair 174P–161P gave a response when MAb 174P was used as trapping antibody. Thus the

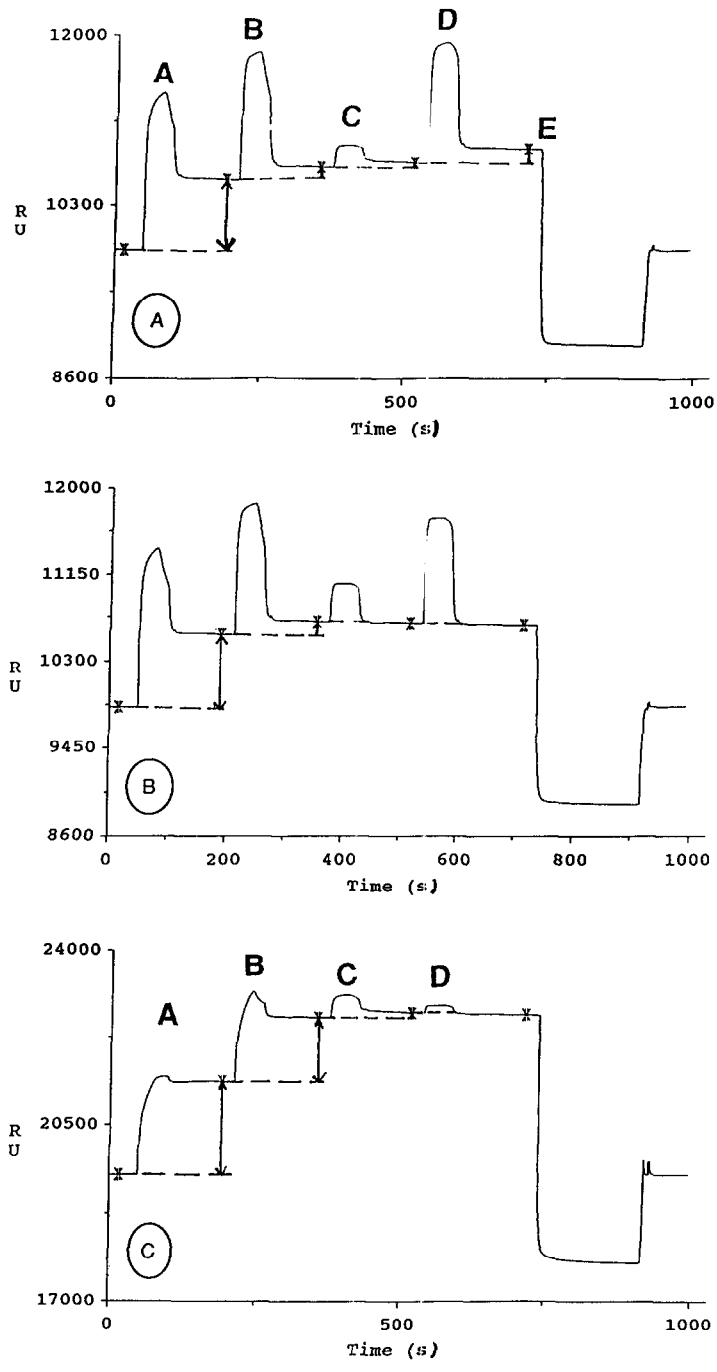

Fig. 2. (A) Sensorgram showing a double antibody sandwich assay. MAb1 (161P) was injected at A and bound to immobilized RAMFc (RU = 712). At B, a non-specific MAb (MAbsat) was injected to saturate the unoccupied sites of RAMFc (RU = 115). At C, TMV protein (4 μl at 100 μg/ml) was injected (RU = 42) and at D specific MAb2 (6P) was bound to the antigen (RU = 145). Ascitic fluids containing MAb1 161P and MAbsat were diluted 1:10 and MAb2 6P ascitic fluid 1:100. TMV protein and CpMV were used at 100 μg/ml. The double arrow indicates the amount of bound antibody or antigen. (B) Control assay corresponding to (A) in which TMV protein was replaced with CpMV. (C) Sensorgram showing a double antibody sandwich assay of 174P–TMV protein–253P, in which the second MAb does not bind to the antigen. MAb 174P was injected at A (RU = 1850). At B, a non-specific MAb was injected to saturate the RAMFc (RU = 1266). Note that more RAMFc had been injected and that sites had been left free compared with (A). At C, TMV protein (4 μl at 100 μg/ml) was injected (RU = 103). At D, MAb 253P (ascitic fluid diluted 1:100), which does not recognize TMV protein, was added.

TABLE I

RESULTS OF TWO-SITE BINDING ASSAYS BETWEEN MAbs AND TMV PROTEIN MEASURED IN BIAcore BIOSENSOR SYSTEM

MAb1: Immobilized antibody used to trap TMV protein. Only anti-cryptotope and anti-metatope antibodies were able to trap TMV protein. Negative results with anti-neotope antibodies reflect the fact that these antibodies did not capture TMV protein. MAb2: second antibody tested for its capacity to bind TMV protein captured by anti-metatope or anti-cryptotope antibodies. Plus signs: pairs of MAbs that bind concurrently to the TMV protein (typically 100–400 RU for the second MAb). Minus signs: pairs of MAbs that do not bind concurrently to the TMV protein (typically 0–40 RU for the second MAb).

MAb2	MAb1							
	Cryptotopes		Metatopes					Neotopes:
	174P	161P	6P	16P	25P	151P	167P	253P, 107P, 79P
Neotopes								
253P	−	+	+	+	+	+	+	−
107P	−	+	+	+	+	+	+	−
79P	−	+	+	+	+	+	+	−
Cryptotopes								
270P	−	−	−	−	−	−	−	−
174P	−	−	−	−	−	−	−	−
161P	+	−	+	+	+	+	+	−
Metatopes								
6P	−	+	−	−	−	−	−	−
16P	−	+	−	−	−	+	−	−
25P	−	+	−	−	−	−	−	−
151P	−	+	−	+	−	−	−	−
167P	−	+	−	−	−	−	−	−

epitope recognized by MAb 174P which is located on peptide 134–146 [5] is sufficiently distant from the epitope recognized by MAb 161P (known to bind to residues 90–95) to permit the simultaneous binding of the two MAbs on the same protein molecule. It is not clear why the protein when trapped by MAb 161P is not able to bind MAb 174P.

Of all combinations of anti-metatope antibody tested, only the two MAbs 151P–16P were able to bind as first and second antibody to the same protein molecule. In the case of intact virus particles, it was also observed that virus trapped by any one of these two antibodies could be recognized by the other [11]. Hence there are at least two spatially distinct metatopes present on TMV protein and these allow the simultaneous binding of the two MAbs on the monomeric protein and also on the virus particle.

TMV protein trapped by any one of the five anti-metatope MAb could be bound by the anti-cryptotope MAb 161P. Further, when the protein was trapped by MAb 161P, it could be recognized by all anti-metatope MAbs (Table I). It seems, therefore, that residues 90–95 (recognized by MAb 161P) on the disordered loop of the viral protein [12] are sufficiently distant from the epitope recognized by anti-metatope antibodies to allow dual binding. This is not the case with the regions 76–88 and 134–146 recognized by MAbs 270P and 174P, respectively, which are probably too close to the epitope recognized by anti-metatope antibodies.

In view of the results obtained with anti-cryptotope MAb 161P and anti-metatope MAbs and also the pair 16P and 151P, we tried to bind simultaneously the three MAbs 161P, 16P and 151P to TMV protein. Only two of these antibodies were able to bind TMV protein concurrently while a third one could not. This may be due to the small size of the TMV subunit (relative molecular mass, M_r = 17 500), which permits only the simultaneous bind-

ing of two antibodies on the antigen in the dextran matrix. Another explanation may be that the three epitopes are so close to each other that steric hindrance prevents the simultaneous binding of the three Abs.

We also compared the reactivity in the BIAcore between normal TMV protein acetylated at its N-terminus and non-acetylated TMV protein. Only one difference in reactivity was observed. When protein trapped by anti-cryptotope MAb 161P was tested with a second anti-neotope MAb, only acetylated TMV protein was recognized by the second antibody whereas non-acetylated protein was not. Hence it seems that MAb 161P allows a conformational change mimicking the neotope conformation to occur in the acetylated protein, but not in the non-acetylated protein. It is also noteworthy that the anti-neotope MAbs were able to bind to TMV protein presented by any anti-metatope antibody, although they were unable, when used as capturing antibody, to trap the viral protein (Table I). Apparently, when anti-metatope antibodies were used as trapping antibody, a neotope conformation was also induced in both the acetylated and non-acetylated TMV protein. It is not clear if the induction of a neotope-like conformation in the monomeric viral subunit is brought about by the capturing antibody or by the second, anti-neotope MAb.

This study of TMV protein antigenicity demonstrates the advantage of using the BIAcore technology for epitope mapping compared with classical ELISA tests. In earlier studies by ELISA [8], high background readings prevented epitope mapping of TMV protein by the same MAbs used in this study. Subsequent control experiments carried out under the ELISA conditions used previously [8] showed that non-specific adsorption of TMV protein to the plastic surface of microtitre plates led to erroneous interpretations of the data in particular concerning the presence of aggregated protein in the sample being studied. The very low background reading observed in the BIAcore avoids these pitfalls completely. Further, the ability to visualize in the BIAcore each successive binding step during a two-site binding assay permits the unambiguous demonstration of allosteric changes occurring during antigen–antibody interaction.

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REFERENCES

1 A. Lofas and B. Johnsson, *J. Chem. Soc., Chem. Commun.*, 21 (1990) 1526–1528.
2 D. Altschuh, Z. Al Moudallal, J. P. Briand and M. H. V. Van Regenmortel, *Mol. Immunol.*, 22 (1985) 329–337.
3 I. Dore, D. Altschuh, Z. Al Moudallal and M. H. V. Van Regenmortel, *Mol. Immunol.*, 24 (1987) 1351–1358.
4 Z. Al. Moudallal, J. P. Briand and M. H. V. Van Regenmortel, *EMBO J.*, 1 (1982) 1005–1010.
5 Z. Al Moudallal, J. P. Briand and M. H. V. Van Regenmortel, *EMBO J.*, 4 (1985) 1231–1235.
6 I. Dore, E. Weiss, D. Altschuh and M. H. V. Van Regenmortel, *Virology*, 162 (1988) 279–289.
7 I. Dore, P. Ruhlmann, P. Oudet, M. Cahoon, D. L. D. Caspar and M. H. V. Van Regenmortel, *Virology*, 176 (1990) 25–29.
8 E. L. Dekker, C. Porta and M. H. V. Van Regenmortel, *Arch. Virol.*, 105 (1989) 269–286.
9 L. G. Fägerstam, A. Frostell, R. Karlsson, M. Kullman, A. Larsson, M. Malmqvist and H. Butt, *J. Mol. Recogn.*, 3 (1990) 208–214.
10 R. Karlsson, A. Michaelsson and L. Mattsson, *J. Immunol. Methods*, 145 (1991) 229–240.
11 M. C. Dubs, D. Altschuh and M. H. V. Van Regenmortel, *Immunol. Lett.*, 31 (1991) 59–64.
12 A. C. Bloomer, J. N. Champness, G. Bricogne, R. Staden and A. Klug, *Nature (London)*, 276 (1978) 362–368.