Journal of Biochemistry and Molecular Biology, Vol. 34, No. 5, September 2001, pp. 452-456

JOURNAL OF
Biochemistry
and
Molecular Biology

Determination of Monoclonal Antibodies Capable of Recognizing the Native Protein Using Surface Plasmon Resonance

Deok Ryong Kim*

Department of Biochemistry, College of Medicine Gyeongsang National University, Chinju 660-701, Korea

Received 25 June 2001, Accepted 20 July 2001

Surface plasmon resonance has been used for a biospecific interaction analysis between two macromolecules in real time. Determination of an antibody that is capable of specifically interacting with the native form of antigen is very useful for many biological and medical applications. Twenty monoclonal antibodies against the α subunit of *E. coli* DNA polymerase III were screened for specifically recognizing the native form of protein using surface plasmon resonance. Only four monoclonal antibodies among them specifically recognized the native α protein, although all of the antibodies were able to specifically interact with the denatured α subunit. These antibodies failed to interfere with the interaction between the τ and α subunits that were required for dimerization of the two polymerases at the DNA replication fork. This real-time analysis using surface plasmon resonance provides an easy method to screen antibodies that are capable of binding to the native form of the antigen molecule and determine the biological interaction between the two molecules.

Keywords: Surface plasmon resonance, Monoclonal antibody, Protein-protein interaction

Introduction

The study of biomolecular interaction is a crucial step in understanding biological functions. Signal transduction from one molecule to another during the biological event is achieved through molecular communication by direct contact between the two molecules. Many methods have been developed to detect these protein-protein interactions. For instance, an optical phenomenon of surface plasmon resonance (SPR) is used for the biospecific interaction analysis (BIA) in real time (Fägerstam *et al.*, 1992). The SPR response is correlated to changes in the refractive index at the sensor chip surface. The change in the refractive index depends on the amount of mass bound on the chip surface when the analyte binds to the immobilized ligand. The sensor chip consists of a glass slide with a thin layer of gold for good SPR response and chemical inertness. The gold film is in turn covered with a covalently bound carboxymethylated dextran matrix to facilitate immobilization of the biomolecules.

This BIA analysis has been applied to monitor many biological interactions. For example, the interaction between the α subunit of the *E. coli* DNA polymerase III holoenzyme and other subunits, such τ or β, was determined by this analysis (Kim and McHenry, 1996a, 1996b). The DNA polymerase III holoenzyme, a multi-subunit protein that is mainly responsible for DNA synthesis in *E. coli* consists of ten different subunits (McHenry, 1988). Each subunit coordinatively interacts with the other subunits during DNA replication (Kim *et al.*, 1995). The α subunit that possesses polymerase activity interacts with several DNA polymerase III subunits, or other proteins involved in DNA replication. In particular, the carboxyl terminus of the α subunit is responsible for binding to the τ subunit to dimerize the two DNA polymerases at the replication fork (Kim and McHenry, 1996a). Also, this technology was used to determine specificity between the antigen and antibody (Nechansky *et al.*, 1997; Ditzel *et al.*, 2000; Emanuel *et al.*, 2000; Gomes *et al.*, 2000; Li *et al.*, 2000; Pavlinkova *et al.*, 2000). Determination of the epitope map for a specific antibody, and the degree of its specificity, provides ample important information about the development of antibody-based drugs. The real-time SPR analysis can easily and rapidly determine the antibodys epitope and its kinetic parameters. In addition, many receptors on the cell surface receive signals from the outside and transmit them into the cell through a receptor-ligand interaction. Such interactions during cell signal transduction were also discovered by studies using surface plasmon resonance (Henry *et al.*, 1997; Mackenzie *et al.*, 1999; Naidenko *et al.*, 1999; Pullen *et al.*, 1999; Cormier *et al.*, 2000; Roseman and Baenziger, 2000; Urban *et al.*, 2000). Finally, this binding analysis was intensively used for

*To whom correspondence should be addressed.
Tel: 82-55-751-8734; Fax: 82-55-759-8005
E-mail: drkim@nongae.gsnu.ac.kr

understanding various regulatory mechanisms in many biological reactions (Yang and Mumby, 1995; Chrunyk *et al.*, 2000; Gomes and Burgers, 2000; Kang *et al.*, 2000; Nyholm *et al.*, 2000; Park and Sung, 2001). As mentioned previously, this SPR analysis is a very useful tool to determine the biospecific interaction between two molecules.

Here I report a method to screen monoclonal antibodies that are capable of recognizing the native antigen and to test antibodies for their ability to block the molecular interaction between two molecules using SPR analysis. Twenty monoclonal antibodies against the α subunit of DNA polymerase III were tested to monitor their abilities to recognize the native α subunit of *E. coli* DNA pol III. Four of the twenty antibodies specifically bound to the α subunit on BIAcore analysis.

Materials and Methods

Western blotting analysis Reconstituted *E. coli* DNA polymerase III holoenzyme (700 ng) was loaded on a 12.5% SDS-polyacrylamide gel and separated at 75 voltage overnight. The proteins were transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl (TBS) plus 3% non-fat milk, then incubated with the supernatant of a monoclonal anti-α antibody culture (2 ml, no dilution) at room temperature overnight. The membrane was washed three times with TBS that contained 0.3% non-fat milk and 0.03% Triton X-100 and incubated with anti-mouse IgG-conjugated with alkaline phosphatase (obtained from PIERCE) for 1 h at room temperature. After washing three times in TBS plus 0.3% non-fat milk and 0.03% Triton X-100, the membrane was developed in a solution (10 mM Diethanolamine, pH 9.5, 10 mM $MgCl_2$) containing BCIP (Bromo chloro indolyl phosphate)/NBT (Nitro blue tetrazolium) (purchased from GIBCO BRL).

SPR binding analysis This SPR binding analysis was carried out as described in Kim and McHenry (1996a). A Pharmacia Biosensor BIAcore™ instrument was used to determine the interaction between the antibody and antigen. All of the materials were purchased from Pharmacia unless stated otherwise. All of the buffers were filtered before use. Rabbit anti-mouse IgG1 (RAMG1) was coupled to the CM5 sensor chip by injecting 400 ng of RAMG1 in 10 mM sodium acetate, pH 4.5 at 5 μl/min after activating carboxymethyl group on the chip with NHS (N-hydroxysuccinimide)/EDC (N-ethyl-N'(dimethylaminopropyl) carbodiimide) coupling reaction. This reaction typically immobilized 3700 RU of RAMG1 on the chip. The interaction of the α subunit with monoclonal anti-α antibodies was measured by two subsequential injections; individual monoclonal anti-α antibodies were captured by RAMG1, followed by the injection of the α subunit. All of the binding analyses were carried out in a HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% (v/v) P-20 surfactant). The kinetic parameters were determined using the BIAevaluation 2.1 software.

Results and Discussion

An antibody can interact with the native form of antigen as well as the completely denatured antigen. For many applications, the antibodies that specifically interact with the native protein are absolutely required. Recently, many monoclonal antibodies were utilized for therapeutic purposes by using their functional properties to inhibit, or enhance a specific interaction that is necessary for the control of a particular disease. Therefore, they must first be tested for their ability to recognize the native form of antigen before use for this purpose. In this paper, I present a simple method to screen monoclonal antibodies that specifically interact with the native protein using antibodies against the α subunit of the *E. coli* DNA polymerase III holoenzyme.

Screening of monoclonal anti-α antibodies by Western blot Twenty monoclonal antibodies from hybridoma cell lines that are capable of producing anti-α antibodies were

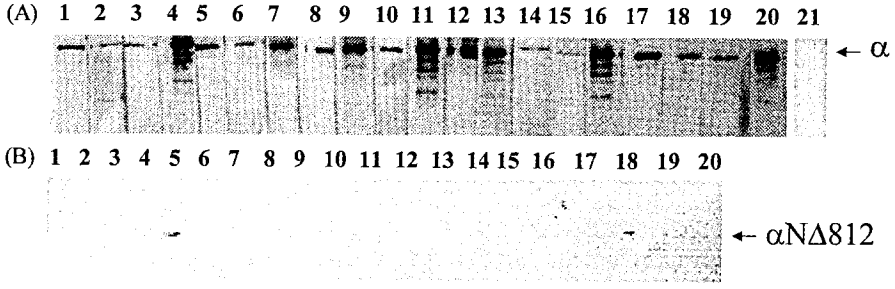

Fig. 1. Immunoblot using monoclonal anti-α antibodies. (A) A reconstituted holoenzyme (700 ng each lane) was separated by 12.5% SDS-PAGE and blotted as described in Materials and Methods. Each lane of the membrane was cut and incubated individually with the supernatant of each monoclonal antibody culture as follows: Lane 1, 178 A1; lane 2, 190 G8; lane 3, 210 E6; lane 4, 257 H2; lane 5, 279 H11; lane 6, 362 E4; lane 7, 370 H4; lane 8, 377 E8; lane 9, 520 C7; lane 10, 645 G6; lane 11, 889 H6; lane 12, 911 F12; lane 13, 1018 F12; lane 14, 1104 H12; lane 15, 1118 H1; lane 16, 1171 F10; lane 17, 1283 G11; lane 18, 1333 H12; lane 19, 1950 F3; lane 20, 1976 F8; lane 21, culture media control. (B) αNΔ812 (100 ng each lane) was separated by 10% SDS-PAGE (SE250 Mighty small gel from Hoefer). After the proteins were transferred onto a nitrocellulose membrane, the membrane was incubated individually with each monoclonal antibody culture in the PR 150 Mighty small Deca-Probe tray (Hoefer). Other steps of immunoblot were carried out as described in Materials and Methods. The number of the lane for each antibody is the same as above.

described (Kim, 1996). All of the monoclonal antibodies reacted positively with the denatured α subunit on Western blots, although some antibodies were highly reactive to the α subunit (Fig. 1A). The monoclonal anti-α antibodies, 257 H2, 889 H6, 1018 F12, 1171 F10, and 1976 F8 (lanes 4, 11, 13, 16 and 20), showed additional specificity to other smaller peptides rather than just the full-length α subunit. These smaller peptides might be some breakdown products of the α subunit, or other subunits of the DNA polymerase III holoenzyme. The hybridoma culture medium showed no reactivity to the DNA pol III subunits (Fig. 1A, lane 21).

Although the epitope for each monoclonal antibody was not mapped in detail, a few experiments using the deletional mutant protein of the α subunit provided information about the epitope map for some antibodies. Two antibodies of 1104 H12 and 911 F12 interacted very specifically with the αNΔ812 mutant protein that contained the C-terminal portion of the α subunit (Fig. 1B). According to the previous results, the C-terminal part of the α subunit participates in binding to the τ subunit (Kim and McHenry, 1996a). Thus, these two monoclonal antibodies might inhibit the interaction between α and τ that is important for the dimerization of the two polymerases at the DNA replication fork (data shown later).

Screening of antibodies capable of recognizing the native α subunit All of the antibodies do not react with the native form of protein. In order to identify the monoclonal anti-α antibodies that are capable of interacting with the native α subunit, I carried out a BIAcore analysis using indirect immobilization of the antibodies on the chip. First, RAMG1 (rabbit anti-mouse IgG1) was coupled to the chip (described in "Materials and Methods"), then each monoclonal anti-α antibody was captured by RAMG1. The interaction between the monoclonal anti-α antibody and α subunit was determined by the subsequential injection over the chip. For the screening of each antibody at the same chip, the chip was regenerated by injection of 10 mM HCl by destroying the interaction between RAMG1 and the anti-α-antibody. Most of the monoclonal anti-α antibodies did not react with the native α subunit although they were captured by RAMG1 at the compatible amount (about 200-400 RU range, shown in Table 1). Only four antibodies, 370 H4, 520 C7, 1104 H12, 911 F12, very specifically interacted with the α subunit (Table 1, Fig. 2). The monoclonal antibodies, 1104 H12 and 911 F12, were immobilized to RAMG1 at 484 and 249 resonance units respectively. The units of the bound α subunits to these two antibodies were 173 and 90. From this data, the calculated stoichiometric value of the interaction between the α subunit and these two antibodies is about 0.44, indicating that α does not bind to all of the binding sites of its antibody. The other two antibodies, 370 H4 and 520 C7, interacted with the α subunit at 1 : 1 stoichiometry. However, their equilibrium dissociation constant (K_D), determined from four sensorgrams (Fig. 2), was about ~10^{-8} M. When the culture medium was used as a negative control, the response unit of the bound α to

Table 1. Interactions of the α subunit of DNA pol III and monoclonal anti-α antibodies.

Antibody	Bound Abs (RU)	Bound α (RU)	Binding
190 G8	177	-10	-
178 A1	627	16	-
1104 H4	484	173	+
257 H2	387	5	-
646 G6	394	-5	-
210 E6	436	-11	-
1283 G11	485	-6	-
889 H6	356	-6	-
279 H11	260	-5	-
1171 F10	345	-8	-
1976 F8	369	-7	-
1018 F12	331	-7	-
362 E4	258	-1	-
370 H4	256	189	+
377 E8	262	10	-
520 C7	255	189	+
911 F12	249	90	+
1118 H1	265	9	-
1333 H12	240	6	-
1950 F3	283	4	-
Control[a]	13	3	-

[a]Control is the culture media

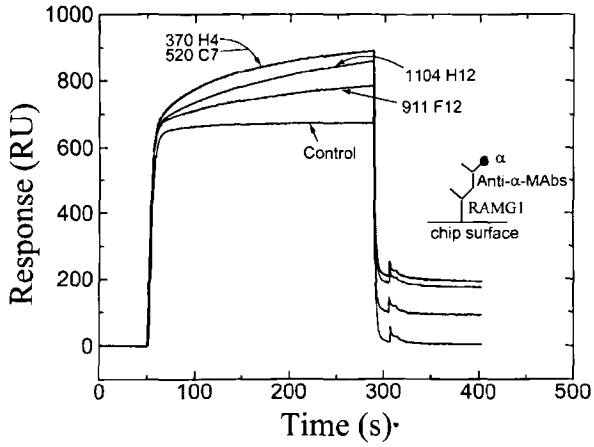

Fig. 2. Screening of antibodies recognizing the native α subunit on BIAcore. RAMG1 was immobilized to the sensor chip, and the monoclonal anti-α antibody was captured by RAMG1. The α subunit (20 μl of 20 ng/ml) was injected in order to examine their interaction with the individual antibody at a flow rate of 5 μl/min at 20°C. This figure shows only the interaction phase between the anti-α antibody and the α subunit. The other parts of the binding sensorgrams were deleted. Sensorgrams of 370 H4 and 520 C7 are indistinguishable. The control is the culture media. The inside picture represents the schematic drawing of the interactions. RU indicates the resonance unit.

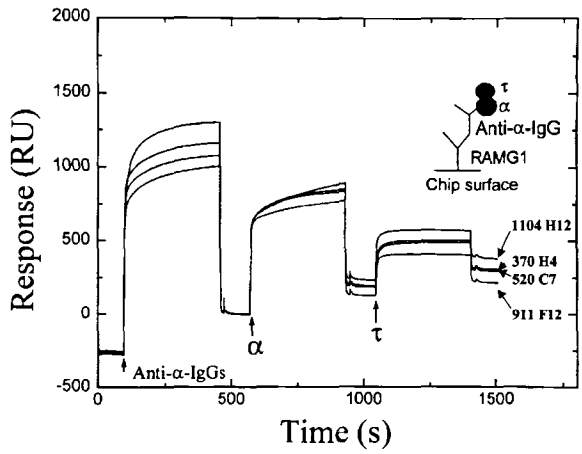

Fig. 3. Inhibition analysis of the α-τ interaction by monoclonal anti-α antibodies. RAMG1 was coupled to the sensor chip as described previously. Four antibodies, 1104 H12, 911 F12, 370 H4 and 520 C7, were immobilized by RAMG1 at the levels of 495, 275, 256 and 265 RUs, respectively. The α (30 μl of 20 ng/ml) and τ (30 μl of 210 nM) subunits were sequentially injected over individual monoclonal anti-α antibody chips at a flow rate of 5 μl/min at 20°C (indicated by arrows). The inside picture represents the schematic drawing of the interactions. RU indicates resonance units. Sensorgrams for each antibody clone at the first (anti-α-IgG injection) and second peaks (α injection) are placed in the same order (top (1104H12) to bottom (911F12)), as indicated at the third peaks (τ injection).

the antibody was similar to the unit values that were obtained from the antibodies that did not recognize the native α subunit (Table 1). Thus, most of the antibodies produced from the mouse hybridoma are unable to interact with the native form of the α subunit of the *E. coli* DNA polymerase III holoenzyme, although they are able to bind to the denatured α subunit.

Blocking of the α-τ interaction using monoclonal anti-α antibodies on BIAcore The previous data has shown that the C-terminal region of the α subunit was involved in binding to the τ subunit (Kim and McHenry, 1996a). Since the monoclonal antibodies, 1104 H12 and 911 F12, specifically recognized the native form of the C-terminal region of the α subunit, I tested whether these two antibodies can block the α-τ interaction using a SPR binding analysis. The anti-α antibody was captured by RAMG1 that was bound to the chip. The α and τ subunits were sequentially injected over the sensor chip to detect their interaction between the two molecules. The overall binding scheme of this analysis is depicted in the inside picture of Fig. 3. If the anti-α-antibody blocks the α-τ interaction, then the final binding profile for the α-τ interaction on the sensorgram will be undetected. The first binding profile of the sensorgram (Fig. 3) is for the interaction between RAMG1 and the monoclonal anti-α antibody. The second profile is for the binding of the anti-α antibody and the α subunit. The final binding phase is for the α-τ interaction.

Table 2. Blocking of α-τ binding by anti-α antibodies on BIAcore.

Antibody	Bound α (RU)	Bound τ (RU)	On-rate (k_{on})[b] ($M^{-1}s^{-1}$)	Stoichiometry[c]
1104 H12	238	144	7.14×10^5	1.09
911 F12	130	86	7.42×10^5	1.19
370 H4	187	187	7.01×10^5	1.03
520 C7	198	110	7.28×10^5	1.00
Control	9	12		

[a]As a negative control, the monoclonal anti-δ' antibody was used, and 432 RUs of the antibody was immobilized to the chip.
[b]On-rates were determined from Fig. 2 (τ binding phase to α) using BIAevaluation 2.1 software.
[c]Stoichiometry (τ/α) = τ bound (RU)/α bound (RU) XαMW/τ MW.

Results showed that both the monoclonal anti-α antibodies, 1104 H12 and 911 F12, had no effect on the α-τ interaction (Fig 3 and Table 2). The stoichiometry of all of the α-τ interactions that were calculated from the sensorgrams was about 1 : 1, which was quite similar to the number that was determined previously (Kim and McHenry, 1996a). The association rate constants (k_{on}) of the α-τ interaction in all cases were also about 7×10^5 ($M^{-1}s^{-1}$) (Table 2). Two other antibodies, 370 H4 and 520 C7, that are not specific to the C-terminal region of the α subunit showed similar results to those shown in 1104 H12 and 911 F12. These results suggest that none of these monoclonal anti-α antibodies disturb the interaction between the α and τ subunits, even though they are specifically binding to the carboxyl-terminus of the α subunit of the DNA polymerase III holoenzyme.

In this report, I have shown a simple method to screen monoclonal antibodies that are capable of recognizing the native structure of proteins using surface plasmon resonance. By using this method, four out of twenty monoclonal antibodies against the polymerase subunit of *E. coli* DNA polymerase III holoenzyme have been determined for their ability to interact with the intact protein. Also, I further tested its ability to block the molecular interaction that is required for co-operative function of the two subunits in DNA synthesis at the replicating fork. Recently, this antibody has been utilized in many applications, not only biological research, but also medicine development (Ditzel *et al.*, 2000; Emanuel *et al.*, 2000; Gomes *et al.*, 2000). Therefore, the determination of an antibody's epitope map, kinetic parameters, and binding ability to the native antigen is very important to improve its usage in various research fields.

Acknowledgments This study was supported in part by a Korean Research Foundation grant KRF-2000-041-D00286.

References

Chrunyk, B. A., Rosner, M. H., Cong, Y., McColl, A. S.,

Otterness, I. G. and Daumy, G. O. (2000) Inhibiting protein-protein interactions: a model for antagonist design. *Biochemistry* **39**, 7092-7099.

Cormier, E. G., Persuh, M., Thompson, D. A., Lin, S. W., Sakmar, T. P., Olson, W. C. and Dragic, T. (2000) Specific interaction of CCR5 amino-terminal domain peptides containing sulfotyrosines with HIV-1 envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. USA* **97**, 5762-5767.

Ditzel, H. J., Masaki, Y., Nielsen, H., Farnaes, L. and Burton, D. R. (2000) Cloning and expression of a novel human antibody-antigen pair associated with Feltys syndrome. *Proc. Natl. Acad. Sci. USA* **97**, 9234-9239.

Emanuel, P. A., Dang, J., Gebhardt, J. S., Aldrich, J., Garber, E. A., Kulga, H., Stopa, P., Valdes, J. J. and Dion-Schultz, A. (2000) Recombinant antibodies: a new reagent for biological agent detection. *Biosens. Bioeletron* **14**, 751-759.

Fägerstam, L. G., Frostell-Karlsson, A., Karlsson, R., Persson, B. and Ronnberg, I. (1992) Biospecific interaction analysis using surface plasmon resonance detection applied to kinetic, binding site and concentration analysis. *J. Chromatography* **597**, 397-410.

Gomes, P., Giralt, E. and Andreu, D. (2000) Direct single-step surface plasmon resonance analysis of interactions between small peptides and immobilized monoclonal antibodies. *J. Immunol. Methods* **235**, 101-111.

Gomes, X. V. and Burgers, P. M. (2000) Two modes of FEN1 binding to PCNA regulated by DNA. *EMBO J.* **19**, 3811-3821.

Henry, A. J., Cook, J. P., McDonnell, J. M., MacKay, G. A., Shi, J., Suttor, B. J. and Gould, H. J. (1997) Participation of the N-terminal region of cepsilon3 in the binding of human IgE to its high-affinity receptor FcepsilonRI. *Biochemistry* **36**, 15568-15578.

Kang, H., Freund, C., Duke-Cohan, J. S., Musacchio, A., Wagner, G. and Rudd, C. E. (2000) SH3 domain recognition of a proline-independent tyrosine-based RYxxYxxY motif in immune cell adaptor SKAP55. *EMBO J.* **19**, 2889-2899.

Kim, Y. T, Lee, S. G. and Kim, H. J. (1995) Molecular and biochemical studies on the DNA replication of Bacteriophage T7; functional analysis of amino-terminal region of Gene 2.5 protein. *J. Biochem. Mol. Biol.* **28**, 484-489.

Kim, D. R. (1996) Deletion analysis to identify the limits of domains of the α subunit of *Escherichia coli* DNA polymerase III holoenzyme. Ph.D. thesis, pp. 157-165, University of Colorado, USA.

Kim, D. R. and McHenry, C. S. (1996a) Biotin tagging deletion analysis of domain limits involved in protein-macromolecular interactions: mapping the τ binding domain of the DNA polymerase III α subunit. *J. Biol. Chem.* **271**, 20690-20698.

Kim, D. R. and McHenry, C. S. (1996b) Identification of the β binding domain of the α subunit of *E. coli* DNA polymerase III holoenzyme. *J. Biol. Chem.* **271**, 20699-20704.

Li, S. L., Liang, S. J., Guo, N., Wu, A. M. and Fujita-Yamaguchi, Y. (2000) Single-chain antibodies against human insulin-like growth factor 1 receptor: expression, purification, and effect on tumor growth. *Cancer Immunol. Immunother.* **49**, 243-252.

MacKenzie, C. R., Hirama, T. and Buckley, J. T. (1999) Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance. *J. Biol. Chem.* **274**, 22604-22609.

McHenry, C. S. (1988) DNA polymerase III holoenzyme of *Escherichia coli*. *Annu. Rev. Biochem.* **57**, 519-552.

Naidenko, O. V., Maher, J. K., Ernst, W. A., Sakai, T., Modlin, R. L. and Kronenberg, M. (1999) Binding and antigen presentation of ceramide-containing glycolipids by soluble mouse and human CD1d molecules. *J. Exp. Med.* **190**, 1069-1080.

Nechansky, A., Pursch, E., Effenberger, F. and Kricek, F. (1997) Characterization of monoclonal antibodies directed against the alpha-subunit of the human IgE high-affinity receptor. *Hybridoma* **16**, 441-446.

Nyholm, T., Andang, M., Bandholtz, A., Maijgren, C., Persson, B., Hotchkiss, G., Fehniger, T. E., Larsson, S. and Ahrlund-Richter, L. (2000) Interaction between hammerhead ribozyme and RNA substrates measured by a surface plasmon resonance biosensor. *J. Biochem. Biophys. Methods* **44**, 41-57.

Park, G. H. and Sung, Y. C. (2001) Mapping of the interaction domain of DNA topoisomerase 2a and 2b with extracellular signal-regulated kinase 2. *J. Biochem. Mol. Biol.* **34**, 85-89.

Pavlinkova, G., Colcher, D., Booth, B. J., Goel, A. and Batra, S. K. (2000) Parmacokinetics and biodistribution of a light chain-shuffled CC49 single-chain Fv antibody construct. *Cancer Immunol. Immunother.* **49**, 267-275.

Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J. and Kehry, M. R. (1999) High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 mutimerization. *Biochemistry* **38**, 10168-10177.

Roseman, D. S. and Baenziger, J. U. (2000) Molecular basis of lutropin recognition by the mannose/GalNAc-4-SO4 receptor. *Proc. Natl. Acad. Sci. USA* **97**, 9949-9954.

Urban, S., Schwarz, C., Marx, U. C., Zentgraf, H., Schaller, H. and Multhaup, G. (2000) Receptor recognition by a hepatitis B virus reveals a novel mode of high affinity virus-receptor interaction. *EMBO J.* **19**, 1217-1227.

Yang, S. I. and Mumby, M. C. (1995) Analysis of the stoichiometry and the domain for interaction of Simion virus 40 small t antigen with protein phosphatase 2A. *J. Biochem. Mol. Biol.* **28**, 331-335.