Monoclonal* Antibodies Raised against Carp Myosin

Misako Nakaya and Shugo Watabe

Laboratory of Marine Biochemistry, Faculty of Agriculture, The University of Tokyo, Yayoi, Bunkyo, Tokyo 113, Japan

(Received August 30, 1993)

Several cell lines producing monoclonal antibodies against carp fast muscle myosin were obtained by fusion of immunized BALB/c mouse spleen cells with syngeneic NS-1 myeloma cells and subsequent cloning by the limiting dilution method. Monoclonal antibodies, which were obtained when myosin was injected to mice as an antigen, recognized myosin subfragment-1 (S1). Further assignments for antigenic sites in the S1 molecule revealed that antibodies from 35A11 and 41C2 cell lines were specific to 50 and 25 kDa fragments of S1 tryptic digests, respectively. No cell line producing monoclonal antibody specific to the 20 kDa fragment of S1 tryptic digests was established. On the other hand, cell lines for monoclonal antibodies against myosin rod were established only when the rod was administered to mice as an antigen. The 37G3 cell line produced the antibody specific to myosin rod and reacted with its light meromyosin part. However, all monoclonal antibodies thus raised failed to discriminate between two types of myosin from carp acclimated to 10 and 30°C, irrespective of myosin S1 or rod.

Key words: myosin, myosin subfragment-1, myosin rod, light meromyosin, carp, antibody, monoclonal antibody

Sarcomeric myosins from higher vertebrates are complex multimeric proteins of contractile systems, having two functionally different halves, a rodlike tail and a unit of two globular heads.¹⁾ The rod has a coiled-coil structure of two parallel α -helices wound about each other, and is responsible for the assembly of the myosin molecule to form the functional thick filaments. Two long, identical myosin heavy chains separate from each other and fold into two globular heads, or myosin subfragment-1 (S1) units, which contain a site for ATP hydrolysis and a site for interaction with actin, each with two noncovalently bound light chains likely to be involved in the regulation of ATP hydrolysis and the actin-myosin interaction.²⁾ The heads of the myosin molecules protrude from the thick filament surface and form cross-bridges with actin-containing thin filaments.

Fish skeletal myosins^{3–5)} are composed of two heavy chain subunits with a molecular mass of about 200 kDa and four light chain subunits of about 20 kDa as those from higher vertebrates.¹⁾ Fish myosins can also be divided into two major parts, the head and tail, by α -chymotrypsin in low-ionic-strength buffer.^{6,7)} In comparison with the large quantities of information available for myosin subfragments of mammalian myosins, however, little is known about fish S1 and rod, although fish myosins have high species-specificity in structural and enzymatic properties.^{3,8–12)}

There are several strategies aiming at elucidation of the relationship between the structure and function of the myosin molecule. One of the potential methods is to establish antibodies specific for a certain functional part of the protein molecule.¹³⁾ For example, the antibody raised against the tryptic phosphopeptide of bovine aortic smooth muscle myosin heavy chain of 204 kDa effectively distinguished it from the isoform of 200 kDa.¹⁴⁾ Therefore, it seems promising to adopt such immunological methods with specific antibodies for elucidating species-specificity and structural and functional properties of fish myosins.

The purpose of this study was to prepare monoclonal antibodies specific to carp myosin S1 and rod. Although we could establish several cell lines for antibodies which recognized different part of the carp myosin molecule, none of them was specific to 10°C- or 30°C-acclimated myosin.

Materials and Methods

Materials

Several specimens of carp *Cyprinus carpio* (600–900 g) were acclimated to either 10 or 30°C according to the procedures reported before¹⁵⁾ and their dorsal ordinary muscle was used for preparation of proteins. The reagents were purchased from the following sources: complete and incomplete Freund's adjuvants from Yeatron; RPMI 1640 (430–1800) and fetal bovine serum (FCS) from GIBCO; myeloma cells (P3-NSI-1-Ag4-1=NS-1) from Dainippon Pharmaceutical; 2,6,10,14-tetramethylpentadecane (pristane) from Aldrich; aminopterin, bovine serum albumin (BSA) RIA grade, goat anti-mouse immunoglobulin G (IgG) and IgM conjugated to alkaline phosphatase or horseradish peroxidase, high molecular weight standard mixtures SDS-6H, hypoxanthine, phenylmethylsulfonyl fluoride (PMSF), polyethylene glycol 3350, *N*-tosyl-L-lysine chloromethyl ketone-treated α -chymotrypsin, *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin, and thymidine from Sigma; 3,3'-diaminobenzidine tetrahydrochloride (DAB) from Dojindo; dithiothreitol (DTT) from Boehringer Mannheim GmbH. All other reagents were of analytical grade.

Preparation of Proteins

Myosin and its S1 and rod parts were prepared as reported in our previous paper.¹⁶⁾ Tryptic digestion of S1 was performed at an enzyme-to-protein weight ratio of 1 : 100 at 10°C in 20 mM phosphate buffer (pH 7.0) containing 0.12 M NaCl, 1 mM EDTA, and 0.1 mM DTT to obtain three fragments with molecular masses of 25, 50, and 20 kDa, aligned in order from the N-terminus. The proteolytic reaction was terminated by the addition of soybean trypsin inhibitor at a weight ratio of 10 : 1 to trypsin. α -Chymotryptic digestion of rod was carried out at 10°C for 30 min at a weight ratio of 1 : 130 to rod in 20 mM Tris-maleate (pH 7.5) containing 0.5 M KCl and 0.1 mM DTT to produce light meromyosin fragments (LMM). The digestion was stopped by adding PMSF to a final concentration of 0.5 mM.

Antigen Preparation and Immunization of Mice

Myosin from 10°C-acclimated carp and myosin rod from 30°C-acclimated carp were dialyzed against phosphate-buffered saline (PBS) at 4°C. Myosin from 10°C-acclimated carp was selected since it contained a specific, tryptic peptide of 23 kDa which was not observed in the 30°C-acclimated S1 (see Fig. 2). On the other hand, myosin rod from 30°C-acclimated carp was used as an antigen because of its larger number of LMM isoforms than the 10°C-acclimated LMM.¹⁶⁾ 200 µg of each protein in 1 ml was emulsified with an equal volume of complete Freund's adjuvant and injected to 8-week-old BALB/c female mice intraperitoneally. After two weeks, the same amount of antigen emulsified with incomplete Freund's adjuvant was injected similarly. Mice were given the antigen 3 more times under the same conditions once every two weeks. On day 3 after a booster injection of the antigen, the spleen was isolated and its cells were fused to BALB/c mouse myeloma cells.

Cell Fusion and Cloning

Monoclonal antibodies were prepared by a method first described by Kohler and Milstein.¹⁷⁾ NS-1 myeloma cells of BALB/c mouse of 1×10^5 in a RPMI 1640 medium containing 10% FCS (NS-1 medium) were fused with immunized spleen cells of 5×10^5 in the same medium. To the fused cells was added a NS-1 selected medium containing 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine (HAT medium) at 37°C to make a cell concentration of 5×10^6 cells/ml.^{18,19)} Aliquots of 0.1 ml cell suspension were added per well to microtiter plates containing 96 wells (Falcon) and were cultured at 37°C in a 5% CO₂ and humidified atmosphere. A HAT medium was added to the microtiter plates on days 4 and 6 to grow cells, then a solution containing 100 µM hypoxanthine and 16 µM thymidine in an NS-1 medium (HT medium) was added on day 9.

Selected positive cultures were cloned by the limiting dilution method²⁰⁾ using thymocytes as feeder cells from BALB/c mice at a concentration of 1×10^7 cells/ml in a HT medium. Hybridoma cells with a high titer were mixed with thymocyte suspension in three serial 10-fold dilutions and added to 96-well microtiter plates at a hybridoma cell concentration of 1–10 cells per well. Microtiter plates containing hybridoma cells were incubated at 37°C with 5% CO₂. The same cloning procedures were repeated two more times to establish cell lines which produce monoclonal antibodies.

Preparation of Ascites Fluid

Monoclonal antibodies were obtained in ascites fluid from hybridoma cells.²¹⁾ Syngeneic mice (BALB/c) received two intraperitoneal injections of 0.5 ml pristane with a seven-day interval. Two to three weeks later, selected hybridoma cells were intraperitoneally injected to the pristane-primed mice at a concentration of 10^7 cells/mouse. One or two weeks later, 1 ml of physiological saline was injected into the abdominal cavity of the mouse through a #18 gauge needle and the ascites fluid developed was recovered together with the injected physiological saline. The ascites fluid collected was centrifuged at 1,500 rpm for 10 min and the supernatant was stored frozen at -80°C until use.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed essentially according to Engvall and Perlmann²²⁾ for immunochemical reactions. Microtiter plates containing 96 wells (Nunc, No. 439454) were coated with 100 µl/well of myosin, rod, or S1 at 50 µg/ml in PBS. The plates were washed exhaustively with PBS containing 0.05% Tween-20 (PBS-Tween) and the wells were supplemented with 1% BSA in the same buffer. After extensive washing with PBS-Tween, 100 µl of tissue culture supernatant, ascites fluid, or diluted mouse polyclonal anti-myosin antiserum was added to the plate. The plates were exhaustively washed with PBS-Tween and goat anti-mouse IgG conjugated to alkaline phosphatase (1 : 1,000 dilution in PBS-Tween) was added to the wells. The plates were washed with PBS-Tween and supplemented with a substrate solution containing 0.1% *p*-nitrophenylphosphate and 0.5 mM MgCl₂ in 923 mM diethanolamine-HCl buffer (pH 9.8). The reaction product formed in each well at room temperature was measured spectrophotometrically at 405 nm with a TOSO MPR-A4 microplate reader.

In competitive ELISA, a 1,000-fold diluted ascites fluid containing antibody was incubated in test tubes with various concentrations of antigens, and was applied as the primary antibody complex. The reaction mixture was applied to microtiter plates previously coated with an antigen (50 µg/ml) and the following procedures were carried out as in the case of the non-competitive ELISA.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli²³⁾ using 7.5–20% polyacrylamide gradient slab gels containing 0.1% SDS to check purity and estimate protein substructures. Molecular weights of proteins were determined using standard proteins.

Immunoblotting

Immunoblotting was performed essentially as described by Towbin *et al.*²⁴⁾ as follows: proteins in electrophoretic gels were transferred to a Millipore PVDF membrane in a blotting buffer (pH 8.3) containing 25 mM Tris, 195 mM glycine, 0.1% SDS, and 20% methanol at 190 mA/10 cm for 3 h using a Biocraft (Tokyo) model BE300 horizontal semidry electroblotter. Following transfer, the membrane was soaked in 50 mM Tris-HCl (pH 8.0) and 0.15 M NaCl (Tris-saline buffer, TBS) containing 1% BSA. The membrane was then soaked in TBS containing 0.1% BSA and the washed membrane incubated with an antibody-containing cell culture medium or ascites fluid diluted with TBS containing 0.1% BSA. The excess antibody was washed out with TBS, then the membrane was treated with TBS containing goat anti-mouse IgG (or IgM)-horseradish peroxidase conjugate (1 : 500 dilution) and 0.1% BSA. The excess antibody conjugate was washed with TBS and the membrane was pooled at room temperature in a peroxidase substrate solution containing 0.5 mg/ml DAB and 0.05% H₂O₂ in TBS for color development.

Protein Concentration

Protein concentrations were determined according to Gornall *et al.*²⁵⁾

Results and Discussion

Myosin prepared from the white ordinary muscle of carp acclimated to 10°C was injected intraperitoneally into BALB/c mice. On day 7 after the 4th injection, the mice were bled from the tail vein to obtain serum. Table 1 shows titers of each serum obtained by ELISA using 10°C-acclimated myosin as an antigen. Six mice all exhibited a strong reaction against myosin and were considered to be useful for subsequent cell fusion and cloning procedures to establish cell lines for monoclonal antibodies.

The mouse numbered 2 with the highest reactivity was subjected to the isolation of the spleen. Spleen cells were fused with syngeneic NS-1 myeloma cells. The fused cells were then poured into microtiter plates with 96 wells and incubated at 37°C in CO₂ incubator (5% CO₂ : 95% air). On days 4 and 6 after cell fusion, 50 µl of HAT medium was added to each of 96 wells, then 50 µl of HT medium was added on day 9. About one-third of the wells showed colonies of hybridoma cells. The wells which gave a positive reaction in the ELISA using S1 as an antigen were the most abundant, whereas wells reacting with myosin rod were much fewer.

Hybridoma cells which showed a reasonable growth rate with high titer were selected and cloned by limiting dilutions. Several cell lines, 35A11, 35H11, 35D7, 35E6, 34G2, 41C2,

Table 1. Titers of serum from BALB/c mice immunized with myosin from carp acclimated to 10°C

Mouse No.	Absorbance at 405 nm
1	0.886 ± 0.092*
2	1.093 ± 0.014
3	0.935 ± 0.017
4	0.931 ± 0.072
5	0.971 ± 0.005
6	0.938 ± 0.048

* The average ± S.D. from four determinations. Color development in ELISA assay was performed for 7 min at room temperature using 100-fold diluted mouse serum after 4th injection with the 10°C-acclimated myosin as an antigen.

Fig. 1. Non-competitive ELISA pattern of the monoclonal antibody in ascites fluid from the 35A11 cell line with various types of antigens. The antibody was raised against myosin from carp acclimated to 10°C and examined for cross-reactivity with myosin (○, ●), rod (□, ■), and S1 (△, ▲) from carp acclimated to either 10°C (○, □, △) or 30°C (●, ■, ▲). Color development in ELISA was performed for 45 min at room temperature and monitored at 405 nm.

and 41D12, were obtained after cloning 3 times using S1 as an antigen. The 35A11 cell line was selected and injected into pristane-primed mice at a concentration of 1×10^7 cells/mouse. After one week or 10 days, the mice were tapped by inserting a #18 gauge needle into the abdominal cavity. The ascites fluid was collected into a tube, centrifuged to remove cells, sampled and stored at -80°C until use. The yield of ascite fluid was 5–10 ml/mouse.

Figure 1 shows the reactivity in non-competitive ELISA of the ascites fluid produced by the 35A11 cell line with several types of antigens from carp acclimated to 10 and 30°C. The antibody in the ascites fluid showed the highest reactivity with S1 antigens from 10- and 30°C-acclimated carp, whereas very weak reaction was observed with myosin and its rod from the two acclimation groups.

The monoclonal antibody in the ascites fluid produced by the 35A11 cell line was then examined for the localization of antigenic epitopes in the S1 molecule, together with culture media collected from 41C2 and 41D12 cell lines. The latter two cell lines also produced antibodies with reasonable titers for further analyses, although cloning by the limiting dilution method was not repeated. For this purpose, S1 was further subjected to limited proteolysis with trypsin followed by SDS-PAGE and immunoblotting. Tryptic digestion of S1 resulted in the production of three well-known fragments called 25, 50, and 20 kDa in the order from the *N*-terminus of the S1 molecule as reported before for S1s from carp²⁶⁾ and higher vertebrates.²⁷⁾ The antibody produced by the 35A11 cell line reacted only with the 25 kDa fragment (Fig. 2). A 75 kDa fragment as well as an intact S1 heavy chain also showed a positive reaction, probably because this fragment is composed of the 25 and 50 kDa fragments.²⁸⁾ On the other hand, the antibody from the

Fig. 2. SDS-PAGE patterns of tryptic digests of myosin subfragment-1 (S1-T) and α -chymotryptic digests of myosin rod (rod-C) (A) and their immunoblotting patterns using monoclonal antibody from the 35A11 cell line established with the 10°C-acclimated myosin (B).

Proteins (20 $\mu\text{g}/\text{lane}$) were prepared from carp acclimated to 10 and 30°C, which are indicated as numerals above the photographs. Abbreviations used are Ma, molecular weight markers of standard proteins; S1-HC, myosin subfragment-1 heavy chain; LMM, light meromyosin; A2, myosin alkali light chain 2; A1', tryptic peptide of myosin alkali light chain 1.

41C2 cell line reacted specifically with the 50 kDa fragment (Fig. 3). The culture medium collected from the 41D12 cell line showed immunoreactivity with all three tryptic fragments of S1. However, it was noted that the 41D12 cell line produced the antibody against the 23 kDa band specific to the 10°C-acclimated S1. The cloning for this antibody is now in progress.

Many types of monoclonal antibodies have been reported for different types of myosin heavy chain. These monoclonal antibodies together with polyclonal ones have been demonstrated to be very useful for structural and functional studies of myosin molecules.^{14,29–32)} However, the monoclonal antibodies for fish myosin heavy chains have not been available and structural studies on fish myosins using immunological methods have been restricted to their light chain subunits.^{33–37)} Myosin heavy chain subunit has a much bigger and more complex structure compared with light chain subunit, and thus is supposed to have more species-specific antigenic epitopes which can be used for determining specific structural and functional properties of fish myosins.

Although some wells of hybridoma cell culture plates were reactive with myosin rod after cell fusion with spleen cells immunized with myosin, it was impossible to establish cell lines which produce monoclonal antibodies specific to myosin rod. Therefore, myosin rod was prepared from the 30°C-acclimated carp and injected into BALB/c mice.

Fig. 3. SDS-PAGE (A) and immunoblotting (B) patterns of myosin subfragment-1 tryptic digests (S1-T) and rod α -chymotryptic digests (rod-C) from carp acclimated to 10 and 30°C.

NS-1 media were collected after cell cultures for 41C2 and 41D12 cell lines established with the 10°C-acclimated myosin and used for immunoreactivity. Proteins applied were 20 μ g/lane. Refer to the legend to Fig. 2 for abbreviations and further details.

Fig. 4. Non-competitive ELISA pattern of the monoclonal antibody in ascites fluid from the 37G3 cell line with various types of antigens.

The antibody was raised against myosin rod from carp acclimated to 30°C and examined for cross-reactivity with myosin (○, ●), rod (□, ■), and S1 (△, ▲) from carp acclimated to either 10 (○, □, △) or 30°C (●, ■, ▲). Color development in ELISA was performed for 30 min at room temperature and monitored at 405 nm.

Several wells with a positive reaction using myosin rod as an antigen were subjected to cloning by the limiting dilution method, resulting in the establishment of a 37G3 cell line. Figure 4 shows the reactivity in non-competitive ELISA of the ascites fluid from the 37G3 cell line with several kinds of antigens from either 10- or 30°C-acclimated carp. The antibody showed a high reactivity with myosin rod and

Fig. 5. Competitive ELISA pattern of the monoclonal antibody in ascites fluid from the 37G3 cell line with various types of antigens using microtiter plates coated with the 10- (A) and 30°C-acclimated rod (B).

The antibody was raised against myosin rod from carp acclimated to 30°C and reacted in test tubes before ELISA with myosin (●), rod (□, ■), and S1 (▲) from carp acclimated to either 10 (○) or 30°C (●, ■, ▲). Color development in ELISA was performed for 30 min at room temperature and monitored at 405 nm.

unexpectedly some reactivity with myosin S1. On the other hand, the competitive ELISA gave rise to striking differences in the reactivity between antigens (Fig. 5). The antibody raised against the myosin rod only reacted with the homologous antigen, rod, or intact myosin, and showed no reactivity with S1 preparations. No differences were, however, observed between protein preparations from the 10- and 30°C-acclimated carp.

The monoclonal antibody produced in the 37G3 cell line

Fig. 6. SDS-PAGE (A) and immunoblotting (B) patterns of myosin rod α -chymotryptic digests.

The ascites fluid used for immunoblotting was obtained from the 37G3 cell line established with the 30°C-acclimated rod. Numerals above photographs indicate periods of digestion time at 10°C of the 10- (L) and 30°C-acclimated rod (H). Roman numerals, I, II, and III represent light meromyosin (LMM) isoforms in the 10% polyacrylamide mini-slab gel (10 μ g/lane).

for myosin rod was used to examine its reactivity with LMM. LMM was prepared by limited proteolysis with α -chymotrypsin and subjected to SDS-PAGE and subsequent immunoblotting. Figure 6 shows multiple LMM isoform bands for 30°C-acclimated carp. Intact rod from the 30°C-acclimated carp reacted more strongly than that from the 10°C-acclimated fish, differing from the results in the ELISA (Fig. 5). This discrepancy remains to be elucidated. In the region of LMM isoforms, the bands of 73 kDa and 77 kDa from 30°C-acclimated carp showed a strong reactivity in immunoblots, while a 77 kDa band from the 10°C-acclimated carp was reactive.

We have recently observed that the myosin of carp fast skeletal muscle changes its properties by temperature acclimation.³⁸⁾ The acclimation by low water temperature was accompanied with the increase of myosin ATPase activity and susceptibility to thermal denaturation of myosin when Ca^{2+} -ATPase was used as a parameter. We found that the myosin rod of carp was also changed by temperature acclimation. Myosin from cold-acclimated carp showed lower solubility in a low-ionic-strength buffer than that from warm-acclimated fish.¹⁶⁾ In accordance with these findings, carp changes the structure of myosin rod by temperature acclimation. *In vitro* degradation patterns of myosin rod with α -chymotrypsin clearly differed between cold- and warm-acclimated carp. The monoclonal antibody by the 37G3 cell line showed stronger reaction to LMM isoforms from the 30°C-acclimated carp in immunoblotting. However, the localization of its epitope could not be determined. It would be of great interest to establish cell lines which could produce monoclonal antibodies specific to 10- and 30°C-acclimated rod, since these antibodies may be useful in disclosing structural differences between the two types of carp myosin rod.

Although evidence for structural differences between myosins from fish and higher vertebrates are poor except for myosin rod,³⁹⁾ fish myosins are very susceptible to proteolysis,¹⁰⁾ suggesting that structural differences must be really present between them. In this study, we obtained several cell lines which produce monoclonal antibodies recognizing various epitopes of the carp myosin molecule. The investigation in progress is to establish cell lines which can produce monoclonal antibodies specific to fish myosins

and 10- or 30°C-acclimated myosin.

Acknowledgments This study was partly funded by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan.

References

- 1) W. F. Harrington and M. E. Rodgers: Myosin. *Ann. Rev. Biochem.*, **53**, 35–73 (1984).
- 2) I. Rayment, W. R. Rypniewski, K. Schmidt-Base, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. M. Holden: Three-dimensional structure of myosin subfragment-1: A molecular motor. *Science*, **261**, 50–58 (1993).
- 3) S. Watabe and K. Hashimoto: Myosins from white and dark muscles of mackerel—Some physico-chemical and enzymatic properties. *J. Biochem.*, **82**, 1491–1499 (1980).
- 4) S. Watabe, Y. Ochiai, and K. Hashimoto: Identification of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and alkali light chains of piscine myosin. *Nippon Suisan Gakkaishi*, **48**, 827–832 (1982).
- 5) P. J. R. Barton and M. E. Buckingham: The myosin alkali light chain proteins and their genes. *Biochem. J.*, **231**, 249–261 (1985).
- 6) T. Ikariya, I. Kimura, and K. Arai: Preparation and characterization of myosin subfragment-1 from carp. *Nippon Suisan Gakkaishi*, **47**, 947–955 (1981).
- 7) Y. Ochiai, A. Handa, T. Kobayashi, S. Watabe, and K. Hashimoto: Isolation and enzymatic properties of myosin subfragment-1 isozymes from the ordinary muscle of tilapia *Oreochromis niloticus*. *Nippon Suisan Gakkaishi*, **55**, 2143–2149 (1989).
- 8) G. Hamoir, H. A. McKenzie, and M. B. Smith: The isolation and properties of fish myosin. *Biochim. Biophys. Acta*, **40**, 141–149 (1960).
- 9) I. A. Mackie and J. J. Connell: Preparation and properties of purified cod myosin. *Biochim. Biophys. Acta*, **93**, 544–552 (1964).
- 10) H. Buttkus: Preparation and properties of trout myosin. *J. Fish. Res. Bd. Canada*, **23**, 563–573 (1966).
- 11) I. Kimura, K. Arai, and S. Watanabe: Heavy meromyosin from skipjack tuna, *Euthynnus pelamis*—Preparation and enzymatic properties. *J. Biochem.*, **86**, 1629–1638 (1979).
- 12) S. Kanoh, S. Watabe, and K. Hashimoto: Isolation and some physicochemical properties of requiem shark myosin. *Nippon Suisan Gakkaishi*, **49**, 757–763 (1988).
- 13) J. N. Goding: Monoclonal Antibodies—Principles and Practice, Academic Press, London, 1983, pp. 134–187.
- 14) C. A. Kelley, J. R. Sellers, P. K. Goldsmith, and R. S. Adelstein: Smooth muscle myosin is composed of homodimeric heavy chains. *J. Biol. Chem.*, **267**, 2127–2130 (1992).
- 15) G.-C. Hwang, Y. Ochiai, S. Watabe, and K. Hashimoto: Changes of carp myosin subfragment-1 induced by temperature acclimation. *J. Comp. Physiol. B*, **161**, 141–146 (1991).
- 16) S. Watabe, G.-C. Hwang, M. Nakaya, X.-F. Guo, and Y. Okamoto:

- Fast skeletal myosin isoforms in themally acclimated carp. *J. Biochem.*, **111**, 113–122 (1992).
- 17) G. Kohler and C. Milstein: Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256**, 495–497 (1975).
 - 18) J. W. Littlefield: Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. *Science*, **145**, 709–710 (1964).
 - 19) J. F. Kearney, A. Radbruch, B. Liesegang, and K. Rajewsky: A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.*, **123**, 1548–1550 (1979).
 - 20) R. P. Siraganian, P. C. Fox, and E. H. Berenstein: Methods of enhancing the frequency of antigen-specific hybridomas, in "Methods in Enzymology" (ed. by J. L. Langone and H. V. Vunakis), Vol. 92, Academic Press, New York, 1983, pp. 17–26.
 - 21) P. Parham: Monoclonal antibodies against HLA products and their use in immunoaffinity purification, in "Methods in Enzymology" (ed. by J. L. Langone and H. V. Vunakis), Vol. 92, Academic Press, New York, 1983, pp. 110–138.
 - 22) E. Engvall and P. Perlmann: Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.*, **109**, 129–135 (1972).
 - 23) U. K. Laemmli: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
 - 24) H. Towbin, T. Staehelin, and J. Gordon: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354 (1979).
 - 25) A. G. Gornall, C. J. Bardawill, and M. M. David: Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.*, **177**, 751–765 (1949).
 - 26) M. Hamai and K. Konno: Structural stability of fish myosin subfragment-1. *Comp. Biochem. Physiol.*, **95B**, 225–259 (1990).
 - 27) E. Audemard, R. Bertrand, A. Bonet, P. Chaussepied, and D. Mornet: Pathway for the communication between the ATPase and actin sites in myosin. *J. Muscle Res. Cell Motil.*, **9**, 197–218 (1988).
 - 28) K. Yamamoto and T. Sekine: Interaction of myosin subfragment-1 with actin. I. Effect of actin binding on the susceptibility of subfragment-1 to trypsin. *J. Biochem.*, **86**, 1855–1862 (1979).
 - 29) V. R. Deutsch, S. Biadsi, A. Eldor, A. Muhlrad, and I. Kahane: Characterization of monoclonal antibodies to human platelet myosin that recognize highly conserved epitopes within the 50 kDa fragment of myosin subfragment-1. *Biochim. Biophys. Acta*, **1038**, 286–290 (1990).
 - 30) N. Murakami, P. Mehta, and M. Elzinga: Studies on the distribution of cellular myosin with antibodies to isoform-specific synthetic peptides. *FEBS Lett.*, **278**, 23–25 (1991).
 - 31) A. Horowitz and K. M. Trybus: Inhibition of smooth muscle myosin's activity and assembly by an anti-rod monoclonal antibody. *J. Biol. Chem.*, **267**, 26091–26096 (1992).
 - 32) H. Sugi, T. Kobayashi, T. Gross, K. Noguchi, T. Karr, and W. F. Harrington: Contraction characteristics and ATPase activity of skeletal muscle fibers in the presence of antibody to myosin subfragment 2. *Proc. Natl. Acad. Sci. USA*, **89**, 6134–6137 (1992).
 - 33) S. Watabe, T. N.-L. Dinh, Y. Ochiai, and K. Hashimoto: Immunological specificity of myosin light chains from mackerel ordinary and dark muscles. *J. Biochem.*, **94**, 1409–1419 (1983).
 - 34) T. N.-L. Dinh, S. Watabe, Y. Ochiai, and K. Hashimoto: Myosin light chains from the cardiac muscle of mackerel, *Pneumatophorus japonicus*. *Comp. Biochem. Physiol.*, **80B**, 203–207 (1985).
 - 35) F. Huriaux and B. Focant: Electrophoretic and immunological study of myosin light chains from freshwater teleost fishes. *Comp. Biochem. Physiol.*, **82B**, 737–743 (1985).
 - 36) Y. Ochiai, S. Watabe, and K. Hashimoto: Physicochemical and immunological properties of myosin light chains from the ordinary muscle of marine teleost fishes. *Comp. Biochem. Physiol.*, **90B**, 347–353 (1988).
 - 37) Y. Ochiai, T. Kobayashi, A. Handa, S. Watabe, and K. Hashimoto: Immunological cross-reactivity of myosin light chains from various species of fish. *Nippon Suisan Gakkaishi*, **55**, 2151–2156 (1989).
 - 38) G.-C. Hwang, S. Watabe, and K. Hashimoto: Changes in carp myosin ATPase induced by temperature acclimation. *J. Comp. Physiol. B*, **160**, 233–239 (1990).
 - 39) S. Kato and K. Konno: Filament-forming domain of carp dorsal myosin rod. *J. Biochem.*, **113**, 43–47 (1993).