Anti-peptide monoclonal antibody imaging of a common binding domain involved in muscle regulation

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Abstract

Multiple-component regulatory protein systems function through a generalized mechanism where a single regulatory protein or ligand binds to a variety of receptors to modulate specific functions in a physiologically sensitive context. Muscle contraction is regulated by the interaction of actin with troponin I (TnI) or myosin in a Ca^{2+} -sensitive manner. Actin utilizes a single binding domain (residues 1–28) to bind to residues 104–115 of TnI (Van Eyk JE, Sönnichsen FD, Sykes BD, Hodges RS, 1991, In: Rüegg JC, ed, *Peptides as probes in muscle research*, Heidelberg, Germany: Springer-Verlag, pp 15–31) and to myosin subfragment 1 (S1, an enzymatic fragment of myosin containing both the actin and ATP binding sites) (Van Eyk JE, Hodges RS, 1991, *Biochemistry* 30:11676–11682) in a Ca^{2+} -sensitive manner. We have utilized an anti-TnI peptide (104–115) monoclonal antibody, Mab B4, that binds specifically to TnI, to image the common binding domain of actin and thus mimic the activity of actin including activation of the S1 ATPase activity and TnI-mediated regulation of the S1 ATPase. Mab B4 has also been utilized to identify a receptor binding domain on myosin (residues 633–644) that is recognized by actin. Interestingly, Mab B4 binds to the native protein receptors TnI and S1 with relative affinities of 100- and 25,000-fold higher than the binding affinity to the 12-residue peptide immunogen. Thus, anti-peptide monoclonal antibodies prepared against a receptor binding domain can mimic the ligand binding domain and be utilized as a powerful tool for the detailed analysis of complex multiple-component regulatory systems.

Keywords: actin; anti-peptide monoclonal antibodies, common binding site; multiple-component regulatory protein complex; myosin subfragment 1 (S1); troponin I

Multiple-component regulatory protein systems function through a generalized mechanism where a single regulatory protein or ligand binds to a variety of receptors to modulate specific functions in a physiologically sensitive context. The common binding site on the ligand must meet the binding requirements of the different receptors. The recognition between the ligand and the

various receptors lies in the complementarity between the tertiary structure of the ligand–receptor binding sites. This implies that the binding domains located on the various receptors for the ligand must be similar to each other in amino acid sequence or three-dimensional structure. In addition, there may be flexibility in the structure of either the ligand or the receptor binding sites to allow for induced fit between the ligand and the receptors. In nature, there are various examples of multiple-component protein complexes that are involved in the regulation of different cell processes. For example, there are a large number of transcriptional factors that interact with one another and DNA polymerase to control transcription (for review see Mitchell & Tjian, 1989) and proteins that are involved in signal transduction cascades, including the G-proteins, which interact with more than one protein (for review see Linder & Gilman, 1992; Stryer, 1993). However, in many of these cases, it is not known if the various regulatory proteins (receptors) that bind a single ligand are binding to a common binding site or to different binding domains on the ligand. Thus, it is of interest to

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Abbreviations: BSA, bovine serum albumin; CNBr, cyanogen bromide; F-actin, filamentous actin; HMM, heavy meromyosin contains two S1(A1)(A2) domains; Mab, monoclonal antibody; Mab B4, anti-TnI peptide 104–115 monoclonal antibody B4; S1, enzymatic fragment of myosin containing the S1 heavy chain and two alkaline-extracted light chains, A1 and A2; TnI, troponin I; troponin consists of a complex of TnI, troponin C, and troponin T; TSDS-PAGE, tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis CDR, complementarity-determining region of the variable region of an antibody light and heavy chain.

develop a general technique that can determine if multiple receptors interact with a common binding site on a ligand. This methodology is critical when it is difficult to study the interaction between the ligand and its receptors, for example, if the ligand is difficult to isolate or purify, and thus limited quantities are available for study, or if there is low binding affinity between the different receptors and the ligand or if there is poor solubility of the ligand.

Our approach utilizes anti-peptide monoclonal antibodies (Mabs) to image the conformation of a common binding domain of a ligand that binds to several receptors. Anti-peptide monoclonal antibodies are produced against a synthetic peptide that constitutes the receptor binding site. Thus, the receptor binding site for the ligand must have been previously identified using conventional techniques or predicted based on amino acid sequence homology of other known ligand binding proteins. An antibody that can mimic the conformation of the ligand binding site is a powerful tool to confirm if different receptors interact at the same site on a ligand. Furthermore, the antibody can identify and be utilized to purify previously unknown receptors that interact with the ligand. This is especially important if the different receptors do not contain any significant degree of amino acid sequence homology to each other. In this case, the anti-peptide antibody identifies structural homology independent of sequence homology. This methodology is effective only if the monoclonal antibody images the biologically active conformation of the ligand binding site. Therefore, it is critical that the antibody should not only mimic the ligand's binding properties but also its biological properties.

The multiple-component system that we have used as our model involves proteins that regulate skeletal muscle contraction. Regulation of muscle contraction is in part controlled by the binding of myosin (receptor A) or troponin I (receptor B) to actin (ligand) (Fig. 1). TnI is part of the troponin complex that consists of three proteins (troponin C, troponin T, and TnI). Troponin-tropomyosin forms the regulatory complex that provides Ca^{2+} -sensitive control of muscle regulation. Actin (41,859 Da for monomeric G-actin) exists as filamentous actin. Actin contains a single common binding domain located within the N-terminus of actin that interacts with both TnI (21,135 Da) and an enzymatic fragment of myosin, myosin subfragment 1 (S1, ~95,000 Da). S1 contains both the actin and ATP binding sites and has two associated alkali-extractable light chains, A1 and A2. It is an S1(A1)(A2) (~115,000 Da) complex that is isolated from the chymotrypsin digestion of myosin. Like actin, a synthetic peptide actin 1-28 (Van Eyk & Hodges, 1991) and a longer enzymatic actin fragment, residues 1-44 (Kögler et al., 1991), are able to activate the S1 ATPase activity. The location of the TnI binding site for the N-terminus of actin has been identified at or near the inhibitory region of TnI, residues 96-116 (Grabarek & Gergely, 1987; Levine et al., 1988; Van Eyk et al.,

1991). The location of the S1 binding site for the N-terminus of actin is near the junction of the 20- and 50-kDa tryptic fragments of S1, around residues 633-644 (Sutoh, 1982). Cheung and Reisler (1992) demonstrated that a synthetic peptide of S1, residues 632-642, was able to inhibit the acto-S1 ATPase activity, implying that the interaction between this region of the S1 and actin is important for the actin activation of the S1 ATPase activity. The interaction between F-actin and myosin activates the myosin ATPase and the myosin subfragment S1 ATPase activity (Spudich & Watts, 1971; Weeds & Taylor, 1975; Fig. 8A), providing the energy required for the movement of the myosin thick filament and the thin filament (actin-tropomyosin-troponin) with respect to one another, resulting in muscle contraction.

Our goal was to produce a Mab that was able to mimic the actin binding domain for TnI and myosin. Mabs were produced against a synthetic peptide of the TnI inhibitory region, residues 104-115. The synthetic TnI peptide 104-115 represents the minimum amino acid sequence required to inhibit the acto-S1 ATPase activity (Talbot & Hodges, 1981). Van Eyk and Hodges (1988) have carried out a detailed study of the interactions of this sequence with actin-tropomyosin and troponin C. Therefore, if the antibody is able to mimic the N-terminus of actin, then the anti-TnI peptide antibody should (1) bind to the TnI peptide 104-115, (2) bind to the native protein, TnI, (3) bind to S1, (4) activate the S1 ATPase activity, and (5) the TnI peptide should inhibit the antibody-activated S1 ATPase activity.

Results

The anti-TnI peptide B4 clone was selected for ascites production based on its ability to bind to the TnI peptide 104-115, TnI, and troponin. Competitive ELISA results show that the Mab B4 binds to TnI and the TnI peptide 104-115 because TnI and the peptide are able to compete with one another for binding to Mab B4 (Fig. 2). In fact, TnI and TnI peptide 104-115 compete for Mab B4 regardless of whether TnI was employed as the competitor or was the ligand bound to the ELISA well, indicating that surface immobilization of TnI does not reduce B4 binding to its epitope. The specificity of the Mab B4 for the TnI inhibitory region is illustrated by the difference in the ability of the TnI peptide 104-115 and a TnI peptide analog to compete with TnI when TnI is bound to the ELISA well. The amino acid sequence of the TnI peptide analog is the same as the TnI peptide 104-115, but with several semiconserved changes to its amino acid sequence (the four arginine residues were substituted with lysine and the valine residue by isoleucine, Fig. 3). Competition ELISA of TnI peptide 104-115 (Fig. 2), TnI peptide analog (Fig. 2), or TnI (data not shown) when TnI is immobilized in the ELISA well were performed in order to calculate apparent binding constants of Mab B4 for these antigens (Table 1). Interestingly, Mab B4 binds 100 times tighter to the TnI protein

Fig. 1. Schematic representation of the interaction between the ligand, actin, and its two receptors, TnI and myosin. These proteins are involved in the Ca^{2+} -dependent regulation of muscle contraction. The actin filament contains the regulatory complex consisting of troponin and tropomyosin, which provides the Ca^{2+} -dependent control.

Fig. 2. Anti-TnI peptide Mab B4 recognizes TnI and the peptide immunogen, TnI peptide 104-115. Solid symbols (●) show the competitive ELISA profile for rabbit skeletal TnI inhibition of the anti-TnI peptide monoclonal antibody, B4, binding to skeletal TnI peptide 104-115 conjugated to BSA. ELISA plates were coated with 0.014 μ M of TnI peptide-BSA conjugate (5 mol TnI peptide per mol BSA). Open symbols show the effect of the TnI peptide 104-115 (○), the TnI peptide analog (Δ), and S1 peptide 633-644 ($\square$) on the binding of Mab B4 to TnI plated in the ELISA well. ELISA plates were coated with 0.48 μ M of TnI. Mab B4 concentration was 0.025 μ M. The competitive ELISA protocol is described in the Materials and methods. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point.

than to the TnI peptide (K_S/K_N ratio), whereas the TnI peptide analog binds 800 times weaker ($K_S/K_N = 0.00125$ from Table 1) than the TnI peptide 104-115.

Figure 4A shows that Mab B4 binds to S1 and that S1 can inhibit Mab B4 binding to TnI. In fact, Mab B4 binds with a 12.5-fold higher affinity to S1 than to TnI (1.25×10^7 M/ 1.0×10^6 M, Table 1). A western blot (Fig. 5) shows that Mab B4 binds to S1 and not to its two associated light chains, A1 and A2. One would expect that S1 would have an amino acid sequence homologous to the skeletal TnI peptide 104-115. A homology search that is capable of identifying extremely remote sequence homologies (NW_ALIGN; Wishart et al., 1994) was used to conduct an exhaustive pairwise alignment of the TnI peptide sequence with known skeletal myosin S1 heavy chain sequences (not including the A1 and A2 sequences) and was unable to identify any region of S1 exhibiting sequence homology. The matrix was modified to weigh totally toward positive charges, so that arginine and lysine are recognized as a match and no other amino acid was considered. This modified homology search program was able to identify one region of skeletal S1, residues 633-644 (for sequence see Fig. 3). Figure 4B shows that S1 and the synthetic S1 peptide (633-644) are able to compete with each other for binding to Mab B4. S1 and the S1 peptide 633-644 competed for B4 regardless if S1 was the competitor or bound to the ELISA well, indicating that surface immobilization of S1 did not reduce binding of B4 for its epitope. The apparent binding affinities of Mab B4 for S1(A1)(A2) and the

Protein	Amino Acid Sequence ^a	Net Charge
1) Skeletal Myosin		
rabbit 633-644 ^b	Ac-G-G-G- K-K -G-G-K- K-K -G-S-amide	+5
chicken 631-642	-G-G-G- K-K -G-G-K- K-K -G-S-	+5
chicken embryonic 631-643	-A-G-G-K- K-K -G-G-K- K-K -G-S-	+6
human fetal 631-643	-D-S-G-K- K-K -V-A-K- K-K -G-S-	+6
S1 peptide analog	Ac-G-G-G- K-K-G-G-K- G -K-G-S-amide	+4
2) Skeletal Troponin I		
rabbit fast 104-115	Ac-G-K-F- K-R -P-P-L- R-R -V-R-amide	+6
chicken fast 104-115	-G-K-F- K-R -P-P-L- R-R -V-R-	+6
rabbit slow 103-114	-G-K-F- K-R -P-P-L- R-R -V-R-	+6
TnI peptide analog	Ac-K-F-K- K -P-P-L- K K I K -amide	+6

Fig. 3. Amino acid sequences of the proposed binding sites of Mab B4 on skeletal myosin and skeletal TnI. ^aThe amino acid residues in myosin subfragment 1 and troponin I that are either conserved or semi-conserved and can be aligned with one another are boxed. The amino acid residues in the peptide analogs that differ from the native peptide sequence have been circled. Ac (acetyl) and amide represent the N- and C-terminal groups on the synthetic peptides used in this study. ^bThe amino acid position is from Tong and Eligna (1990).

Table 1. The apparent association constants of the monoclonal antibody B4 for various antigens compared to the antigen, TnI peptide 104–115 when troponin I or S1(A1)(A2) is immobilized in the ELISA well

Antigen	Relative K_a (M^{-1}) ^a		K_S/K_N ratio ^b	
	TnI ^c	S1 ^d	TnI ^c	S1 ^d
Myosin S1	—	1.25×10^7	—	25,000
S1 peptide 633–644	—	7.5×10^3	—	15
S1 peptide analog	—	5.0×10^2	—	1
TnI peptide 104–115	—	5.0×10^2	—	1
TnI peptide analog	—	5.0×10^2	—	1
TnI	1.0×10^6	—	100	—
TnI peptide 104–115	1.0×10^4	—	1	—
TnI peptide analog	1.25×10^1	—	0.00125	—
S1 peptide 633–644	3.3×10^2	—	0.033	—

^a K_a is the apparent association constant of Mab B4 for the corresponding antigens, which are calculated by the formula $K_a = 1/I_{50}$. The I_{50} value is the concentration of competitor in solution required to produce 50% inhibition of Mab B4 binding to an antigen on the ELISA well.

^b K_N is the apparent association constant of TnI peptide 104–115 binding to Mab B4 when either TnI or S1(A1)(A2) is plated in the ELISA well. K_S is the apparent association constants of TnI, S1, S1 peptide 633–644, or the TnI peptide analog binding to Mab B4 when either TnI or S1(A1)(A2) is bound to the ELISA well. A larger value indicates an increased binding affinity of Mab B4 for the antigen.

^c The concentration of TnI plated in the ELISA well was 2.0 μM when TnI was the competitor and 0.48 μM when the synthetic peptides were the competitors.

^d The concentration of S1 utilized to coat the ELISA well was 0.086 μM .

S1 peptide 633–644 is the same in the absence or presence of 0.2 mM ATP γS in the ELISA assay (data not shown), indicating that the binding of Mab B4 to S1(A1)(A2) is not effected by nucleotide binding to S1. The specificity of B4 for S1 is illustrated by the difference in the ability of the TnI peptide 104–115, TnI peptide analog, and an S1 peptide analog to compete B4 from binding to S1 bound to the ELISA well compared to the S1 peptide 633–644 (Fig. 4C). The S1 peptide analog has the same amino acid sequence as the native S1 peptide, except that the lysine at position 641 is substituted with a glycine (amino acid sequence in Fig. 3). Mab B4 binds 1,600- and 25,000-fold tighter to the S1 protein than to S1 peptide 633–644 and TnI peptide 104–115, respectively (Table 1).

To further establish the S1 binding site for Mab B4, a western blot of a CNBr digest of S1(A1)(A2) was carried out. Mab B4 bound to a band on the TSDS-PAGE gel with an approximate molecular weight of 5,000 Da. The CNBr fragment that contains the proposed S1 binding site is residues 617–661, which has molecular weights of 4,656 and 4,674 Da for the homoserine- and homoserine lactone-peptides, respectively. The other CNBr fragment of S1 with a similar molecular weight (homoserine molecular weight of the fragment) is residues 166–214 (5,305 Da), in addition to numerous fragments from the A1 and A2 chains. To confirm that the CNBr fragment that Mab B4 binds to is 618–644, a western blot was done in the presence of the synthetic S1 peptide 633–644 (Fig. 5). The synthetic peptide eliminated Mab B4 binding to S1 or the CNBr fragment, sug-

Fig. 4. Anti-TnI peptide Mab B4 recognizes a second protein receptor, myosin subfragment 1(S1) containing the two alkaline-extractable light chains, A1 and A2. **A:** Competition ELISA profile for S1 inhibition of Mab B4 binding to TnI. ELISA plates were coated with 0.48 μM of TnI. **B:** Closed symbols (■) show the competitive ELISA profile for S1 inhibition of Mab B4 binding to S1 peptide 633–644 conjugated to BSA (8 mol peptide per mol BSA). ELISA plates were coated with 0.015 μM S1 peptide 633–644–BSA conjugate. Open symbols (□) show the effect of the S1 peptide 633–644 on binding of Mab B4 to S1 immobilized in the ELISA well. ELISA plates were coated with 0.086 μM of S1. **C:** Competitive ELISA profiles of S1 (■), TnI peptide analog (○), S1 peptide 633–644 (□), TnI peptide 104–115 (○), TnI peptide analog (▲), and the S1 peptide analog (▲) inhibition of Mab B4 binding to S1 immobilized in the ELISA well. ELISA plates were coated with 0.086 μM of S1. Concentration of Mab B4 was 0.016 μM , 0.025 μM , and 0.025 μM in A, B, and C, respectively. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point.

Fig. 5. TSDS-PAGE and western blot of Mab B4 and native and digested S1. **A:** Coomassie blue-stained blot of S1 (A1)(A2) (lane A), CNBr digest of S1(A1)(A2) (lane B) and molecular weight marker (lane C). Associated molecular weights of the markers are written beside the bands. **B:** Immunoblots for Mab B4 in the presence (lanes A, B) or in the absence of synthetic S1 peptide 633–644 (lanes C, D). The CNBr digest of S1 (A1)(A2) (lanes A, C) and undigested S1(A1)(A2) (lanes B, D). Mab B4 binds to the CNBr fragment with the approximate molecular weight of 5,000 Da as well as to the S1 heavy chain with an approximate molecular weight of 95,000 Da as indicated by arrows. Molecular weight standards (lane E) have their associated molecular weights written beside the bands. Protocols for the TSDS-PAGE and western blots are described in the Materials and methods.

gesting that B4 binds to the CNBr fragment that contains residues 633–644 and that B4 has a single epitope on S1.

Figure 6 shows that both Mab B4 and F-actin compete for binding to TnI or the TnI peptide 104–115 (Fig. 6A) and for binding to S1 and the S1 peptide 633–644 (Fig. 6B). Specifically, the synthetic actin peptide 1–28 is capable of inhibiting Mab B4 binding to the BSA-conjugated S1 peptide 633–644 bound to the ELISA well (Fig. 7). The actin peptide 1–28 was not sufficiently soluble to achieve concentrations above 5 mM and at an actin peptide concentration of 2 mM, the peptide was able to inhibit 66% of Mab B4 binding to the S1 peptide. An actin peptide analog in which the aspartic acid residue at position 1 was substituted by alanine, (D1A) actin peptide 1–28, was unable to inhibit the Mab binding to S1 peptide when S1 peptide conjugate was bound to the ELISA well. This actin analog, (D1A) actin 1–28, is unable to activate the S1 or HMM ATPase activity nor can this peptide bind to a CNBr-activated Sepharose 4C affinity column derivatized with HMM (unpubl. data). These results illustrate

the importance of the N-terminal residue of actin in the actin–S1 interaction.

It is well established that actin activates the S1 ATPase activity and TnI is able to inhibit the actin activation of the S1 ATPase activity. Figure 8A shows that, at a 1:1 mol ratio of F-actin to S1 (2 μ M F-actin), F-actin causes a ~4.5-fold activation of the S1 ATPase activity. Maximum activation (~26 fold) of the S1 ATPase by F-actin occurs at a mol ratio of 8:1 actin to S1 under our assay conditions. Mab B4 produced a 3.2-fold activation of the S1 ATPase rate at a 1:1 mol ratio of Mab B4 to S1 (2 μ M B4) (Fig. 8A). A control mouse IgG antibody had no effect on the S1 ATPase activity. In addition, because the TnI peptide is able to bind to Mab B4 in competition with S1, it is able to prevent the antibody-mediated activation of the S1 ATPase activity (75% inhibition at 325 μ M of TnI peptide, Fig. 8B). The concentration of TnI peptide (200 μ M) required to produce 50% inhibition of the Mab B4 activation of the S1 ATPase activity (S1 concentration was 2.0 μ M) reflects the higher binding affin-

Fig. 6. Anti-TnI peptide Mab B4 blocks the ligand, actin, from binding to the two different receptors and their cognate peptides. **A:** Competitive ELISA profiles of F-actin and Mab B4 competing for binding to TnI (●) or BSA-TnI peptide (○) bound to the ELISA well. ELISA plates were coated with 0.48 or 0.014 μ M for TnI and BSA-TnI peptide, respectively. **B:** Competition between F-actin and Mab B4 for binding to S1 (■) or BSA-S1 peptide (□). ELISA plates were coated with 0.032 or 0.15 μ M for S1 and BSA-S1 peptide, respectively. Concentration of Mab B4 was 0.016 μ M. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point.

Fig. 7. Actin peptide 1-28 competing with the Mab B4 for binding to the S1 peptide 633-644. Closed circles, competition ELISA profile between Mab B4 and actin peptide 1-28 for BSA-S1 peptide 633-644 bound to the ELISA wells. Open circles, effect of D1A actin 1-28 peptide analog (which does not bind to S1(A1)(A2)) on Mab B4 binding to BSA-S1 peptide 633-644 bound to the ELISA wells. ELISA plates were coated with 0.077 μ M of BSA-S1 peptide 633-644. Concentration of Mab B4 was 0.016 μ M. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point.

ity of Mab B4 for S1 than for the TnI peptide, as shown by the competitive ELISA results listed in Table 1. The TnI peptide analog was not able to inhibit the Mab B4 activation (Fig. 8B), probably due to the low affinity of Mab B4 for the TnI peptide analog as noted in the ELISA assays (Table 1).

Discussion

Based on the concept of common binding domains, we have proposed that an antibody raised against the inhibitory region of TnI, 104-115, should mimic the binding site on actin for this TnI peptide. Therefore, the antibody should mimic the binding and biological properties of actin and, in particular, those associated with the N-terminal residues of actin. Importantly, Mab B4 antibody was able to bind to TnI and the TnI inhibitory peptide 104-115 (Fig. 2) as well as to S1, which is composed of S1 heavy chain and two associated light chains A1 and A2 (Figs. 4, 5). A western blot of S1 indicated that Mab B4 binds to the S1 heavy chain and not to the two associated light chains, A1 and A2 (Fig. 5). Because Mab B4 binds to TnI peptide 104-115, TnI, and S1, it would be expected that S1 would contain an amino acid sequence that is homologous to the TnI peptide 104-115. However, the amino acid sequence homology between the TnI peptide 104-115 and S1 is very weak with the single sequence residue 633-644 that was identified. Even though there is low sequence homology, a synthetic S1 peptide 633-644 was able to compete with S1 for binding to Mab B4 in the competitive ELISA assay (Fig. 4B). Furthermore, the synthetic S1 peptide 633-644 eliminated binding of Mab B4 to a CNBr fragment(s) with the approximate molecular weight of the predicted CNBr fragment 617-661, which contains this proposed S1 binding site (Fig. 5). These data indicate that Mab B4 binds to residues 633-644 of S1.

Fig. 8. Mab B4 mimics the biological function of F-actin. **A:** Activation of the S1 ATPase activity by increasing quantities of F-actin ($\blacktriangle$) or Mab B4 ($\bullet$). A control antibody, normal mouse IgG ($\circ$), had no effect on the S1 ATPase activity. **B:** Inhibitory effect on Mab B4 activation of S1 ATPase activity by TnI peptide 104-115 ($\bullet$) or TnI peptide analog ($\circ$). Concentration of S1 in the assay was 2 μ M in A and B. In B, 0.8 μ M was present to activate the S1 ATPase activity by 1.84-fold prior to addition of TnI peptide or TnI peptide analog. S1 ATPase assay is described in the Materials and methods. Where error bars are not shown, the standard deviation is small and lies under the symbol of the data point.

Mab B4 competes with F-actin for binding to both TnI and S1 and their cognate synthetic peptides (Fig. 6). These results demonstrate that Mab B4 is able to mimic the binding properties of actin. In fact, Mab B4 mimics the N-terminal region of actin because the synthetic actin peptide 1-28 inhibits Mab B4 binding to BSA-S1 peptide 633-644 (Fig. 7). This indicates that both Mab B4 and actin 1-28 are able to interact with the S1 peptide 633-644. This is the first evidence that the two peptides actin 1-28 and S1 peptide 633-644 can form a complex. In addition, Mab B4 is able to image a biologically correct conformation of the N-terminus of actin because B4 was able to induce a 3.6-fold activation of the S1 ATPase activity at a 1:1 mol ratio of antibody to S1 (Fig. 8A). However, Mab B4 is less active than F-actin in activating the S1 ATPase activity, but this difference may be due to F-actin being a polymeric filament. This is supported by the fact that the synthetic actin peptide, residues 1-28, also activates the S1 ATPase activity but not to the same extent as F-actin (Van Eyk & Hodges, 1991). The actin 1-28 interaction with S1 is pH sensitive (Van Eyk & Hodges, 1993): at pH 7.6, actin 1-28 induces approximately a 1.8-fold activation of S1

(Van Eyk & Hodges, 1991). A longer actin fragment, residues 1–44, induces a 3.3-fold activation of the S1 ATPase activity (Kögler et al., 1991). The actin peptides 1–28 and 1–44 and Mab B4 function as actin agonists. Therefore, like actin and presumably like actin peptides 1–28 and 1–44, Mab B4 binding to S1 must induce conformational changes in S1 required for activation of the S1 ATPase activity. In addition, the TnI peptide 104–115 was able to inhibit the antibody activation of the S1 ATPase activity. The TnI peptide analog did not inhibit the antibody activation of the S1 ATPase activity (Fig. 8B), which may reflect the weak interaction between B4 and the TnI peptide analog when S1 is immobilized in the ELISA well (Table 1). In fact, a 10-fold higher quantity of TnI peptide analog is required to induce 50% inhibition of the actin-S1 ATPase activity compared to the native TnI peptide 104–115 (data not shown).

Few antibodies have been reported that are able to induce a conformational change in an antigen upon binding. An anti-protein antibody specific for LFA-1, a cell adhesion protein receptor, is able to recognize a unique Ca^{2+} -dependent epitope on the receptor and, when the antibody binds to the receptor, there is an enhancement of cell adhesion suggesting possible intramolecular changes in the receptor upon antibody binding (van Kooyt et al., 1991), whereas another antibody, when bound to its receptor, exposes a different ligand binding site on the receptor (Kouns et al., 1990; Frelinger et al., 1991). However, Mab B4 is unique in that it can induce the conformational changes required for activation of an enzyme's activity and the enzyme was not the original immunogen used in preparation of Mab B4.

Another unique feature of Mab B4 is that this antibody binds 25,000- and 100-fold tighter to S1 and TnI (Table 1) than to the peptide immunogen, TnI peptide 104–115 (Table 1). This is important for two reasons, the Mab B4 binds the receptor proteins (TnI and S1) tighter than the cognate peptides (TnI peptide 104–115 and S1 peptide 633–644) and B4 binds S1 protein 12.5 times tighter than TnI, which is the native receptor containing the peptide sequence used as the immunogen for preparation of Mab B4. Few anti-peptide or anti-protein antibodies have been reported that show even a small increase (less than 10-fold) in binding affinity to peptides containing specific changes in the amino acid sequence compared to the peptide immunogen (Wong et al., 1992) or with several heterologous proteins containing changes to the amino acid sequence compared to the native protein immunogen (Harper et al., 1987). As well, somatic mutations to a Fab raised against a small hapten have also been shown to have increased binding affinity to the hapten by up to 200-fold over the original immunogen (Parhami-Seren et al., 1990).

A sequence alignment of the proposed Mab B4 binding sites on TnI and S1 (Fig. 3) and of the 12 amino acids in the inhibitory region of TnI reveals one conserved and three semiconserved amino acid residues in the S1 sequence 633–644 that can be aligned. This indicates that Mab B4 is most likely identifying a structural homology on S1 rather than amino acid sequence homology. Mab B4 may bind to TnI and S1 with different affinities by binding to a different subset of residues within the CDRs of the antibody. On the other hand, the same residues may be critical for binding both receptors but are flexible and are able to adapt to a different conformation. Because the amino acid sequences of the receptor binding sites for the antibody are quite different from one another (Fig. 3) there presumably must be a degree of flexibility in the ligand and/or receptor to adapt

to these changes in the amino acid sequences. Whether this flexibility in the binding domain is a general phenomenon of antibodies or receptor proteins is not known. Recently, Friedman et al. (1994) have demonstrated the role of flexibility in peptide antigen-antibody interactions. Generally, antibodies are able to bind to related antigens but with varying affinities. The apparent association constants of Mab B4 for TnI peptide 104–115, TnI peptide analog, and S1 peptide analog when S1 is bound to the ELISA well are 15-fold less than for S1 peptide 633–644 (Table 1; Fig. 4C). Comparison of amino acid sequences shows the lysine at positions 636–637 and 641–642 of the S1 peptide 633–644 are conserved or semiconserved in the TnI peptide 104–115 and TnI peptide analog with arginine or lysine residues at equivalent positions, whereas the S1 peptide analog has the lysine at position 641 substituted with a glycine (Fig. 3). These results indicate that a cluster of four positively charged amino acid residues (either arginine or lysine) are critical for B4 binding to S1. On the other hand, when TnI is the receptor bound to the ELISA well, the apparent association constants of Mab B4 for TnI peptide analog and S1 peptide 633–644 are 800- and 30-fold less than the TnI peptide 104–115, respectively (Fig. 2; Table 1). This suggests that when Mab B4 binds to TnI, Mab B4 is not able to adapt to the arginine to lysine change in the amino acid sequence. These results suggest that the binding sites on TnI and S1 and/or the Mab B4 binding site possess a degree of flexibility to allow for some induced fit upon binding of the Mab B4 to TnI or S1. This also suggests that the conformation of Mab B4 is different when bound to TnI or S1.

The ability of two different receptors to bind to a common binding site on a ligand could be based on similarities in the tertiary structure rather than at the amino acid sequence. There is very little information available on the degree of sequence homology between two proteins that bind to the same ligand. For example, an anti-idiotypic antibody reactive against an antibody against reovirus was found to have sequence homology between two of the CDRs of the anti-idiotypic antibody and the reovirus (Bruck et al., 1986). In fact, a peptide of this amino acid sequence is able to compete with the anti-idiotypic antibody or the reovirus and prevent binding to the anti-reovirus antibody (Williams et al., 1988). As well, an IgM murine monoclonal antibody and fibrinogen, which compete for binding to platelet fibrinogen receptor, have a three-residue sequence that is homologous between the CDR of the antibody and the active binding site of fibrinogen (Taub et al., 1989). However, antibodies display considerable flexibility and can bind to antigens with mutations at the amino acid residues determined to be within the epitope (Strong et al., 1991a, 1991b; Tulip et al., 1992; Wong et al., 1992).

The results of this study with Mab B4 have shown that an anti-peptide monoclonal antibody is capable of imaging a binding domain in a conformation that is biologically relevant. An antibody that mimics the biologically active conformation of a ligand is a useful tool for determining whether a ligand can interact with various receptors. In addition, the antibody could lead to the identification of novel receptors that possess significantly different primary sequences at the ligand binding site and hence cannot be identified by sequence homology programs. Furthermore, the antibody can be used as a tool to purify the receptor and identify the amino acid sequence involved in the interaction with the ligand. This approach has been successfully employed in this study to identify the S1 binding site for the N-terminus

of actin even though the sequence homology between the TnI inhibitory region and S1 was weak. In multiple-component protein systems, where the receptor and ligands are large and/or consist of multiple subunits, it is difficult to determine the 3-dimensional structure of the receptor-ligand complexes by X-ray crystallography or NMR. Under these circumstances, a minimalistic approach can be used in which small peptides representing the ligand binding site on the different receptors can be used to determine the structure of these receptor peptides bound to the ligand or the Fab fragment of the monoclonal antibody that mimics the ligand. In the case of muscle, the 3-dimensional structures of troponin C, actin (actin:DNAse complex), and S1 with the molecular weights of approximately 17,965, 41,859, and 115,000 Da, respectively, have been determined by crystallography. To date, there is no 3-dimensional structure of any muscle protein-protein or peptide-protein complexes. However, there are computer-generated 3-dimensional models of the actin-S1 (Rayment et al., 1993) and troponin C-TnI inhibitory peptide (Ngai et al., 1994) based on the 3-dimensional structures of the various individual components. Interestingly, the amino acid sequence that forms one of the actin-S1 sites of interaction (the same sequence that the Mab B4 binds) was not resolved in the S1 crystallographic structure. It may be possible to obtain the structure of the S1 peptide (residues 633-644) bound to Mab B4 and, because Mab B4 activates the S1 ATPase activity, the structure of this complex may be very similar to the active conformation of this region of S1. Finally, as demonstrated by the acto-TnI peptide Mab B4, it is possible to systematically produce antibodies that are able to mimic the biologically active conformation of a common binding domain.

Materials and methods

Purification and preparation of muscle proteins

All peptides were synthesized following standard procedures for solid-phase synthesis on co-poly (styrene 1% divinylbenzene) benzhydrylamine-hydrochloride resin on an Applied Biosystems 430A peptide synthesizer (Foster City, California) using conventional N^α -tert-butyloxycarbonyl chemistry and benzyl-type side-chain protection as described by Zhou et al. (1990). The peptides were purified by reversed-phase HPLC (Van Eyk & Hodges, 1991). The purity and authenticity of the peptides were examined by amino acid analysis and mass spectrometry. The peptides were dissolved in ELISA assay buffer (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). Crosslinking of peptides to keyhole limpet hemocyanin for immunization or BSA for ELISA experiments was accomplished using a benzophenone photoaffinity group, which was added following ornithine and two glycine (Gly) residues to the N-terminus of the peptide (Ngai et al., 1994). Ornithine was used to quantitate the ratio of peptide to carrier protein. Conjugates used in the ELISA assays had 8 mol S1 peptide 633-644 per mol BSA and 5 mol TnI peptide 104-115 per mol BSA.

Rabbit skeletal TnI was prepared and purified by reversed-phase HPLC according to Ingraham and Hodges (1988). TnI was dissolved in ELISA assay buffer including 6 M urea, 1 M potassium chloride, and then dialyzed against the ELISA buffer containing 1 M, 0.5 M, 0.25 M, 0.1 M, or 0.05 M potassium chloride and finally against the ELISA buffer. Myosin subfragment 1, S1(A1)(A2), was prepared by the method of Weeds and

Taylor (1975), as modified by Talbot and Hodges (1981). A1 and A2 are alkaline-extracted light chains associated with the S1 heavy chain. S1 was dialyzed against the ELISA assay buffer. Potassium-ethylenediaminetetraacetic acid ATPase activity of the S1 was 7.2 s^{-1} . CNBr digestion of S1(A1)(A2) was performed as follows: 15 mg of S1(A1)(A2) was dissolved in 1 mL of 70% formic acid. A 200:1 molar ratio of CNBr to S1 was added and the solution stirred at room temperature for 16 h. Deionized water (4 mL) was added and the sample lyophilized. Completeness of the digestion was monitored by reversed-phase HPLC. Rabbit skeletal actin was prepared according to Spudich and Watts (1971) and polymerized to F-actin by dialysis against the ELISA assay buffer plus 50 mM potassium chloride. Pipetting of F-actin was done such that the actin remained filamentous. The F-actin and some myosin S1 were the generous gift of Drs. L. Thomas and L.B. Smillie, Department of Biochemistry, University of Alberta. Concentration of the various peptides, TnI and F-actin concentrations were determined by amino acid analysis and S1(A1)(A2) concentration was determined by absorbance (Yagi et al., 1967).

Preparation and purification of Mab B4

Anti-TnI peptide monoclonal antibodies were prepared as described by Doig et al. (1990). The Mab B4 was chosen for ascites production because of its ability to bind to TnI peptide 104-115, TnI, and troponin in the absence of Ca^{2+} . In the absence of Ca^{2+} (muscle relaxation), the inhibitory region of TnI is proposed to interact with actin and not troponin C and should therefore be exposed in the troponin complex. At this stage, we could have screened the antibodies for binding to myosin. However, because myosin has different conformations in the absence and presence of ATP or ADP (Levitsky et al., 1992; Ostap et al., 1993), we elected to not include myosin in the initial screening of the clones due to the technical difficulties in controlling the ELISA assay buffers for the presence of nucleotide. Of the nine hybridomas that were selected for more extensive characterization, only two hybridomas produced monoclonal antibodies that were able to bind to S1 as well as to TnI peptide 104-115 and TnI. Mab B4 was selected for this study due to its immunoglobulin isotype (IgG_1) and that it did not have a different affinity for S1 in the absence or presence of ATP γS (data not shown). The Mab B4 clone was scaled into 5 mL Dulbecco-modified Eagle medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, hypoxanthine, aminopterin, and thymidine. The clone was frozen and subsequently subcloned twice in semisolid agarose (Kennett, 1980). Ascites tumors were produced by injecting 10^6 hybridoma cells into pristine primed BALB/c male mice (Kroprowski et al., 1977; Doig et al., 1990). Mab B4 was purified by HPLC on a protein G affinity column (21.6 mm $\times$ 100 mm, HiPAC protein G column, Chromatochem, Montana) at 6 mL/min. The ascite was initially filtered through a 0.22- μm Millipore filter, then loaded onto the column in ELISA assay buffer. The antibody was desorbed from the column using 2 M potassium thiocyanate, pH 7.0. The antibody was dialyzed against 10 mM sodium phosphate buffer, pH 7.4, and then concentrated using polyethylene glycol-8000. The Mab was identified as an IgG_1 by isotyping by using the Inno-Lia mouse immunoglobulin isotyping kit (Innogenetics N.V., Antwerp, Belgium). The concentration of Mab B4 was determined using the microtiter plate BCA protein assay (Pierce, Rockford, Illinois).

Competitive ELISA protocol

Competitive ELISA assays were carried out as described by Doig et al. (1990). Wells on the ELISA plate were coated with 100 μ L of antigen in 10 mM sodium carbonate buffer, pH 7.4, and incubated for 12 h at 4 °C. A solution of Mab B4 and varying quantities of the competitor in ELISA buffer were incubated at 37 °C for 30 min. One hundred microliters of this mixture was added to the wells and incubated for 2 h at 37 °C. Next, 100 μ L of goat anti-mouse IgG conjugated to alkaline phosphatase (Jackson Immunoresearch Lab Inc., West Grove, Pennsylvania) was added to each well and incubated for 2 h at 37 °C. Finally, 200 μ L of 10% (v/v) diethanolamine solution was added and the absorbance at 405 nm was determined using a Titertek Multiskan Plus MKII microplate reader. Each ELISA data point was obtained in triplicate and all studies were independently replicated. The standard deviations of the data were calculated for all points.

TSDS-PAGE and Western blot analysis

TSDS-PAGE was performed with three layers or 4, 10, and 16.5% acrylamide gels as described by Schägger and Von Jagow (1987). Protein samples were incubated for 5 min at 100 °C in 4% SDS, 12% glycerol (w/v), 50 mM Tris, pH 6.8, 2% β -mercaptoethanol (v/v), 0.005% bromophenol blue (w/v) prior to electrophoresis. Using a mini-gel apparatus (Mini-protein II Dual Slab Cell, Bio-Rad, Richmond, California), the samples were electrophoresed for 1 h at a constant voltage of 30 V followed by 2 h at 120 V at room temperature.

Proteins on the TSDS-PAGE gels were transferred to polyvinylidene difluoride membrane (Bio-Rad, Richmond, California) using the protocol of Towbin et al. (1979) with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, California). The transfer was complete after 30 min at a constant current of 300 mA. Blots were either stained with Coomassie blue or used in a Western blot assay. In the Western blot assay, the membrane strips were incubated in a blocking buffer consisting of 50 mM Tris, pH 7.5, 150 mM sodium chloride, 0.05% (v/v) Nonidet-P40, 0.25% (w/v) gelatin, and 3% (w/v) BSA to eliminate nonspecific binding sites. For direct binding assays, the membrane strips were incubated with Mab B4 in the blocking buffer (antibody dilution 1:100) and shaken at 50 rpm for 1 h at room temperature. Then the strips were incubated with goat anti-mouse IgG (H+L) antibody-alkaline phosphatase conjugate (Jackson Immunoresearch Lab Inc., West Grove, Pennsylvania) in the blocking buffer (antibody dilution 1:5,000) and shaken at 50 rpm for 1 h at room temperature. After each antibody incubation, the membrane strips were washed three times with 50 mM Tris, pH 7.5, 150 mM sodium chloride, and 0.15% (v/v) Tween 20 for 10 min, followed by a final wash in 50 mM Tris, pH 7.5, 150 mM sodium chloride. Antibody binding was visualized by addition of alkaline phosphatase substrates (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate) in 100 mM Tris, pH 9.5, 100 mM sodium chloride, and 5 mM magnesium chloride. Color development was stopped by rinsing membrane strips in deionized water. The protocol for synthetic peptide competition assays is the same as for direct binding except, during the first antibody incubation, the membrane strips are incubated with Mab B4 (antibody dilution 1:100) and synthetic S1 peptide 633–644 (200 μ M).

S1 ATPase assay

The amount of Pi released with time was used to quantitate the myosin S1 ATPase activity. A colorimetric method based on Tobacman and Lees (1987) and modified by Chandra et al. (1994) was used except that the ATPase assay buffer consisted of 2 mM Tris, 6.5 mM potassium chloride, 3.5 mM magnesium chloride, 0.5 mM ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid, 1 mM dithiothreitol, pH 7.8. Actin was polymerized to filamentous actin by dialysis against ATPase assay buffer containing 50 mM potassium chloride. The Mab B4, control IgG, and S1 were dialyzed against the ATPase assay buffer. The TnI peptide 104–115 and TnI peptide analog were dissolved in ATPase buffer, pH 7.8. The reaction was started with the addition of 2.0 mM ATP and was allowed to proceed for 20–60 min depending on the experiment. During this time, the ATP hydrolysis rates were linear. The tubes were kept in a water bath at 25 °C and were covered with parafilm for the 60-min reaction time. The reaction was stopped and the Pi quantitated by the addition of 2 mL of a solution consisting of acetone:5 N sulfuric acid:10 mM ammonium molybdate (2:1:1, v/v/v), followed by 0.2 mL of 1 M citric acid. The absorbance of the individual tubes was determined at 355 nm. The concentration of S1 was 2.0 μ M and the assay volume was 0.2 mL. For the inhibition assay, 0.8 μ M Mab B4 was present, resulting in a 1.84-fold activation of the S1 ATPase activity prior to addition of the TnI peptide or TnI peptide analog. Each ATPase data point was done in duplicate or triplicate. Standard deviation of the data was calculated for each point.

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