Noodleman, L., Norman, J. G., Osborne, J. H., Aizman, A., & Case, D. A. (1985) *J. Am. Chem. Soc. 107*, 3418–3426.
Orme-Johnson, W. H., & Beinert, H. (1969) *Biochem. Biophys. Res. Commun. 36*, 337–344.
Perutz, M. F., & Raidt, H. (1975) *Nature 255*, 256–259.
Prince, R. C., & Adams, M. W. W. (1987) *J. Biol. Chem. 262*, 5125–5128.
Rabinowitz, J. (1972) *Methods Enzymol. 24*, 431–446.
Rall, S. C., Bolinger, R. E., & Cole, R. D. (1969) *Biochemistry 8*, 2486–2496.
Seki, Y., Seki, S., & Ishimoto, M. (1989) *J. Gen. Appl. Microbiol. 35*, 167–172.
Sharp, K., & Honig, B. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 301–332.
Smith, E. T., & Feinberg, B. A. (1990) *J. Biol. Chem. 265*, 14371–14376.
Smith, E. T., Feinberg, B. A., Richards, J. H., & Tomich, J. M. (1991a) *J. Am. Chem. Soc. 113*, 688–689.
Smith, E. T., Bennett, D. W., & Feinberg, B. A. (1991b) *Anal. Chim. Acta 251*, 27–33.
Tanaka, M., Nakashima, T., Benson, A. M., Mower, H. F., & Yansunobu, K. T. (1966) *Biochemistry 5*, 1666–1681.
Tanaka, M., Mitsuru, H., Matsueda, G., Yasunobu, K. T., Himes, R. H., Akagi, J. M., Barnes, E. M., & Devanathan, T. (1971) *J. Biol. Chem. 246*, 3953–3960.
Tanaka, M., Mitsuru, H., Matsueda, G., Yasunobu, K. T., Jones, J. B., & Stadtman, T. C. (1974) *Biochemistry 13*, 5284–5289.
Tsunoda, J. N., Yasunobu, K. T., & Whiteley, H. R. (1968) *J. Biol. Chem. 243*, 6262–6272.
Veber, D. F., Milkowski, J. D., Varga, S., Denkewalter, R. G., & Hirschman, R. (1972) *J. Am. Chem. Soc. 94*, 5416.
Warshel, A. (1987) *Nature 330*, 15–17.
Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagoma, G., Profeta, S., Jr., & Weiner, P. (1984) *J. Am. Chem. Soc. 106*, 765–784.

A Synthetic Peptide of the N-Terminus of Actin Interacts with Myosin†

Jennifer E. Van Eyk and Robert S. Hodges*

Department of Biochemistry and MRC Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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ABSTRACT: Research reported from numerous laboratories suggested that the N-terminal region of actin contained one of the binding sites between actin and myosin. A synthetic peptide corresponding to residues 1–28 of skeletal actin was prepared by solid-phase peptide methodology. The formation of a complex between this peptide and myosin subfragment 1 (S1) was demonstrated by high-performance size-exclusion chromatography (pH 6.8). The actin peptide precipitated S1 at higher pH (7.4–8.2) but remained soluble when bound to heavy meromyosin (HMM) or S1 in the presence of F-actin. The actin peptide 1–28 bound to S1 and HMM and activated the ATPase activity in a manner similar to that of F-actin. These results demonstrate that the N-terminal region of actin, residues 1–28, contains a biologically important binding site for myosin.

The use of synthetic peptides that mimic biologically important regions of proteins has been extremely informative in investigating complex biological systems. For example, the studies by Talbot and Hodges (1979) initiated extensive synthetic peptide research on muscle proteins that has contributed to our understanding of the mechanism of muscle contraction (Suzuki et al., 1987; Van Eyk & Hodges, 1988; Shaw et al., 1990; Keane et al., 1990). Muscle contraction involves the regulation of the actin and myosin (acto–myosin) interaction by the regulatory protein complex, troponin–tropomyosin (Tn–TM). The troponin (Tn) subunits (TnC, TnI, TnT) interact with each other and work in concert with actin–TM to produce conformational changes which alter the acto–myosin interaction. When either Mg^{2+} or Ca^{2+} is bound to the two high-affinity Ca^{2+}-binding sites of TnC, muscle relaxation is promoted due to the induction of inhibition of the ATPase activity by TnI (Van Eyk et al., 1986). In contrast, Ca^{2+} binding to the two low-affinity Ca^{2+}-binding sites of TnC results in muscle contraction due to the release of TnI inhibition and potentiation of the ATPase activity.

Immunological data, cross-linking, and NMR experiments have strongly indicated that the N-terminal region of actin contains one of the binding sites between actin and myosin. Cross-linking experiments have shown that the acidic residues of the actin region (1–12) bound to the 20- and 50-kDa tryptic fragments of myosin subfragment 1, S1 (which contains one globular head of myosin) (Sutoh, 1982, 1983). NMR studies have confirmed that residues in the amino terminus of actin were perturbed during the acto–S1 interaction (Moir & Levine, 1986, Moir et al., 1987). Two independent laboratories produced antibodies specific to sequences within the N-terminal region of actin. Mejéan et al. (1986, 1987) used two antiprotein antibodies with different specificities in the N-terminal region of actin (1–28). The antibody affected by sequence changes at positions 2 and 3 (skeletal vs cardiac actin) only partially inhibited the acto–S1 interaction (determined by ELISA assays). Their second antibody with a suggested epitope favoring the C-terminal region of residues

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1–28 completely blocked the interaction of actin and S1. Miller et al. (1987) used an antipeptide antibody to residues 1–7 to confirm that this region was not directly involved in the acto–S1 interaction (determined by centrifugation studies). However, this antipeptide antibody inhibited the S1-induced polymerization of G-actin by blocking the initial binding of these proteins (DasGupta et al., 1990). The recent actin structure determined by X-ray crystallography of the actin–DNAase I complex showed that regions within the N-terminal residues 1–32 of actin, in particular, residues 1–7 and 20–28, are located on the surface of the actin molecule (Kabsch et al., 1990). In the actin structure and the proposed model of the F-actin filament, the N-terminal residues are exposed and not involved in either the DNAase–actin or the proposed actin–actin interactions (Holmes et al., 1990). As well, the actin residues 1–7 have high temperature factors which indicate that this region is surface exposed and highly mobile. In this paper, a peptide of actin residues 1–28 was synthesized by solid-phase methodology. The actin peptide 1–28 bound S1 and the larger tryptic fragment of myosin, HMM (which contains both "heads" of myosin), and activated the S1 and HMM ATPase activities. Therefore, the actin peptide 1–28 is another example of a small synthetic peptide capable of mimicking the biological activity of its native protein (approximately 14 times larger).

Materials and Methods

Peptide Synthesis and Purification. The actin peptide 1–28 (Ac-D-E-D-E-T-T-A-L-V-A-D-N-G-S-G-L-V-K-A-G-F-A-G-D-D-A-P-R-amide) was synthesized using the standard procedures for solid-phase peptide synthesis (Erickson & Merrifield, 1976) on an Applied Biosystems 430A peptide synthesizer (Foster City, CA). The copoly(styrene–1% divinylbenzene) benzhydrylamine hydrochloride resin (Institute Armand Frappier Laval, Canada) was maximally substituted to 0.8 mmol of Boc-amino acid/g of resin. All α-amino groups were protected with the Boc group. The following side-chain protecting groups were used: Arg(Tosyl), Lys(2-ClZ), Asp(OBzl), Glu(OBzl), and Thr(Bzl). The N-terminal amino group of residue 1 was acetylated (as found in native actin) with a mixture of acetic anhydride/toluene/pyridine (1:3:3 v/v). The programs used for acetylation of the peptide and deprotection, neutralization, and coupling of each amino acid were previously described by Hodges et al. (1988a). A radioactive label was incorporated at position 15 by using Boc-Gly-*1*-^{14}C. Two syntheses of actin 1–28 were done which produced specific activity of 290 or 1550 cpm/nmol. The Cys at position 10 in the native actin sequence was substituted with an alanine in the synthetic peptide. This change eliminated any problems related to the oxidation of the cysteine. The synthetic peptide was cleaved from the resin as previously described (Hodges et al., 1988a).

The crude peptides were purified on a HPLC system comprised of a Spectra Physics SP8700 solvent delivery system and Kratos SF7697 detector and an analytical reversed-phase column (4.6 mm i.d. × 220 mm) (Aquapore RP-300, 300-Å pore size and 7.5-μm particle size, Pierce Chemicals, CA). A linear AB gradient (0.1% B/min) at 1 mL/min, where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile, was used. The sample loads varied between 20 and 50 mg per run (Mant et al., 1987a; Parker et al., 1987; Hodges et al., 1988b, 1991). One-minute fractions were collected. In order to identify the fractions containing the desired peptide, analytical runs of the various fractions were carried out using the same reversed-phase column with a linear AB gradient rate of 1% B/min. The fractions containing the pure peptide were pooled and lyophilized.

Preparation of Muscle Proteins. G-Actin was prepared from rabbit skeletal muscle acetone powder as described previously (Spudich & Watts, 1971). Myosin subfragment 1 (S1) was prepared by the method of Weeds and Taylor (1975), as modified by Talbot and Hodges (1981). The S1 purification by DEAE-cellulose chromatography provided two fractions, S1(A1) and S1(A2), which were pooled for these experiments. The K^+-EDTA ATPase activity of the S1 (A1, A2) preparations was between 550 and 650 nmol of PO_4^{2-}/(min·mg of S1). HMM was prepared using the procedure by Weeds and Pope (1977). HMM was supplied by Dr. Kay, University of Alberta, Edmonton, Canada. The purity of the proteins were checked by SDS–urea–polyacrylamide gel electrophoresis (Chong et al., 1983). The concentration of the peptides and proteins was determined by amino acid analysis, except S1 and HMM, which were determined by absorbance (Yagi et al., 1967; Young et al., 1964).

Size-Exclusion Chromatography. Mixtures of S1 and the actin peptide 1–28 were incubated at room temperature for 10 min. The peptide–protein mixtures were run on a high-performance size-exclusion column, Altex TSK G2000 SW (7.5 mm i.d. × 30 cm) (Beckman Inc., Berkeley, CA), in a buffer consisting of 10 mM Tris, 100 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EGTA, pH 6.8, at a flow rate of 0.5 mL/min. The salt was present in the buffer to eliminate nonspecific ionic interactions between the peptide or protein and the support and hence maintain ideal size-exclusion behavior (Mant et al., 1987b). One-minute fractions were collected and lyophilized for further analysis. The HPLC system used for the size-exclusion chromatography (SEC) was a Vista Series 5000 liquid chromatograph (from Varian Walnut Creek, CA), with a diode array HP 1040A detector and accessories (Hewlett Packard, Palo Alto, CA).

The quantity of peptide present in the various size-exclusion fractions was determined from radioactivity measurements on a Beckman scintillation counter. A hydrophobic interaction (HIC) column was used to separate and quantitate the S1 in each fraction (Bio-Gel Spherogel TSK-phenyl-5-PW, 7.5 mm i.d. × 750 mm, Bio-Rad, Richmond, CA). This column was operated in reversed-phase mode using a linear AB gradient (2% B/min) where solvent A consisted of 0.05% aqueous TFA and solvent B was composed of 0.05% TFA in acetonitrile at a flow rate of 1.0 mL/min (Ingraham et al., 1985). S1 is composed of a heavy chain and two light chains, A1 and A2. When run under these conditions, the heavy chain was resolved from the light chains. SDS–urea gel electrophoresis was also run on the fractions collected from this column to confirm the location of S1 heavy chain and the two light chains. The area of the peak containing the heavy chain S1 was quantitated to determine the relative amount of S1 present in each SEC fraction. The reversed-phase chromatography on the HIC column was carried out on an HP1090 fully automated liquid chromatograph (Hewlett Packard). Radioactivity measurements were also used to detect the actin peptide in each SEC fraction.

Centrifugation Studies. Mixtures of S1 (6.8 nmol) with increasing quantities of peptide were incubated at room temperature for 10 min and then centrifuged for 15 min at 15000 rpm at 4 °C (Figure 2). There was no difference in the amount of S1 precipitated if 0-, 10-, or 30-min incubations were done. The assay was done in the Mg^{2+}-ATPase buffer described below for the S1 and acto–S1 ATPase assays except ATP was not present. Aliquots of the supernatant were taken for radioactive measurements to quantify the peptide remaining in the supernatant and for amino acid analysis to

determine quantity of S1 in the supernatant. From these values the quantities of S1 and peptide in the pellet were determined.

To determine whether the actin peptide could bind to the acto–S1 complex, centrifugation studies were performed as described above except actin (5.4 nmol) was present in the S1 (5.1 nmol) solution (Table I). In the absence of peptide and S1, 87–90% of the actin was pelleted while in the absence of only peptide, 95–100% of S1 was found in the pellet in addition to actin. Centrifugation was carried out for 25 min at 15000 rpm at 4 °C. The quantity of radiolabeled actin peptide found in the supernatant following centrifugation was determined by radioactive measurements. From these values the amount of peptide present in the pellet (comprised of actin or actin–S1) was determined. Amino acid analysis and SDS urea gel electrophoreses of the supernatant were done to ensure the quantity of actin and S1 remained constant as increasing quantities of actin peptide were added.

ATPase Assays. The ATPase assays were performed using an automatic pH-stat apparatus consisting of a Brinkman Metrohm 614 Impulsomat, 655 Dosimat, 625 Dosigraph, and 635 pH meter with a 1-mL syringe (Van Eyk & Hodges, 1988). Assay samples, 2 mL in volume, were placed in glass vials and stirred continuously at 25 °C. The titrant was either 5, 2.5, or 1.25 mM KOH, the latter being used in the experiments monitoring S1 ATPase activity. The S1 and acto–S1 ATPase assays were measured, using standard protocols, in a Mg^{2+}-ATPase buffer consisting of 2 mM Tris, 0.1 mM EGTA, 5 mM $MgCl_2$, 30 mM KCl, and 2.5 mM ATP, pH 7.6. The HMM ATPase assays were measured, using standard protocols, in a buffer consisting of 2 mM Tris, 30 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, and 2.5 mM ATP, pH 8.0.

For experiments where both the ATPase rates and the amount of peptide–S1 precipitated were simultaneously determined (Figure 2), duplicate vials were prepared. The ATPase activity of the supernatant or remixed sample was determined after centrifugation. For all other ATPase assays, acto–S1 and HMM were performed by employing the continuous titration method (Van Eyk et al., 1986).

Results and Discussion

Actin Peptide 1–28 Interaction with S1. Complex formation between S1 and actin peptide 1–28 (in the absence of ATP) was determined by high-performance size-exclusion chromatography (SEC) as shown in Figure 1. A pH value of 6.8 was chosen for SEC because the peptide–S1 complex was soluble at this pH and showed no formation of a precipitate as observed at the higher pH values used in the bioassays (see below). Due to the large difference in molecular weight between S1 (A1, A2) (150000) and actin peptide (2890), these components are easily resolved by SEC. The formation of a peptide–S1 complex was indicated by the presence of peptide in the S1-containing fractions (Figure 1, panels B and C). It is interesting that the S1 and peptide–S1 complex were partially resolved and eluted in the opposite order to that expected on the basis of size contributions alone. Fraction 11 contained S1 only, while fractions 12 and 13 contained both peptide and S1 (compare panels B and C). This result indicates that a conformational change in S1 has occurred upon peptide binding such that the peptide–S1 complex is more compact and, hence, is eluted from the SEC column as if it had a smaller apparent molecular weight. RPC on a hydrophobic interaction column is ideal to monitor the fractions obtained from size-exclusion chromatography. The low ligand density on the hydrophobic interaction column allows an excellent

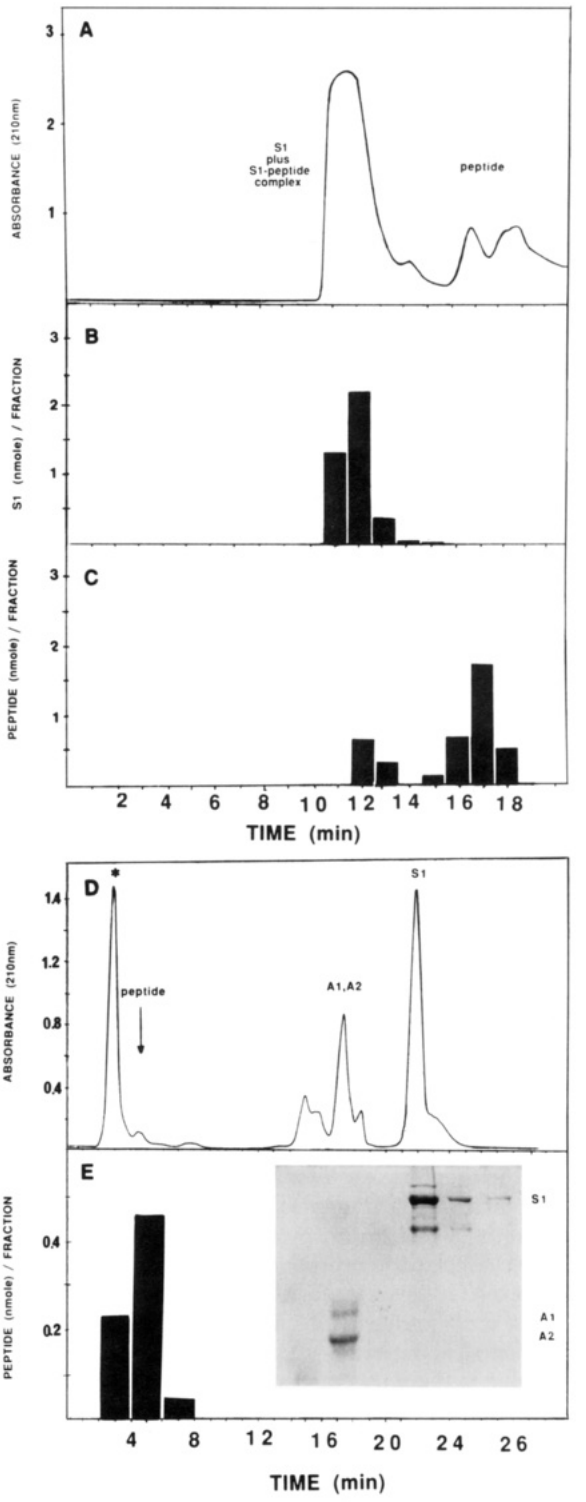

FIGURE 1: Actin peptide and S1 interaction determined by size-exclusion chromatography. Panel A shows the elution profile of a mixture of S1 and actin peptide at a 1:1 mole ratio (4.2 nmol of each component). The chromatographic conditions are described under Materials and Methods. Fractions were collected and analyzed for the presence of S1 and peptide. S1 was identified and quantitated in the SEC fractions by reversed-phase chromatography on a hydrophobic interaction column as described under Materials and Methods (panel B). A representative separation of peptide, S1 heavy chain, and the A1 and A2 light chains is shown in panel D. The * in panel D is the salt present from the SEC fraction (3 min), while the radiolabeled peptide is eluted at 5 min as detected in panel E. SDS–urea gel electrophoresis results of each 2-min fraction from RPC in the 14–28-min section of the chromatogram (panel D) are shown in panel E (insert). The quantity of peptide present in each SEC or RPC fraction was determined by radioactivity measurements (panels C and E).

separation between the actin peptide and S1 (A1, A2) (Figure 1, panel D). The S1 heavy chain and two light chains (A1, A2) could not be eluted from a standard reversed-phase column which has a ligand density approximately 10 times greater than that of the HIC column. The actin peptide does not bind to the HIC column under these conditions and is eluted just after the breakthrough salt peak (panel D).

S1 ATPase assays were examined to determine whether the actin peptide 1–28 interaction had any effect on the biological activity of S1. Unfortunately, mixtures of S1 and peptide formed a precipitate between pH 7.4 and 8.2, which is the pH range where S1 has optimal enzymatic activity. Therefore, centrifugation studies were done under these conditions to evaluate this interaction by comparing the ratio of peptide and S1 in the precipitate (in the absence and presence of ATP). In the absence and presence of ATP, the precipitate was composed of equivalent quantities of peptide and S1 at all ratios of peptide to S1 done in the assay (Figure 2, panel A). This result suggests that, on formation of a 1:1 complex, the peptide–S1 complex is less soluble, allowing aggregation and subsequent precipitation to occur. As expected, as the ratio of actin peptide to S1 increased, there was a linear increase in the amount of S1 precipitated (Figure 2, panel C). However, at a 1:1 ratio of S1 to peptide, in the absence of ATP, approximately 25% of the total S1 present in the assay was precipitated while in the presence of ATP, 15% of the total amount of S1 had precipitated (data not shown). Even though low levels of precipitation of S1–peptide complex occurred, the effect of the actin peptide on the S1 ATPase was examined (Figure 2, panel B). Under these conditions, the actin peptide 1–28 potentiated S1 ATPase activity from 0.032 s^{-1} in the absence of peptide to 0.058 s^{-1} in the presence of peptide.

To determine whether the S1–peptide complex in the precipitate and/or supernatant was biologically active, the ATPase activity of the suspension prior to centrifugation was compared to the activity of the S1–peptide complex in either the supernatant or pellet (after resuspension in its own supernatant or ATPase buffer). Individual tubes of S1 (in the absence of ATP) containing increasing quantities of actin peptide were prepared in triplicate. The three identical sets of tubes were centrifuged to pellet the aggregated S1–peptide complex. The quantity of peptide and S1 in the supernatant and pellet was determined from analysis of one set of tubes (Figure 2, panel C). The values obtained for S1 in the supernatant and pellet at each peptide concentration were used to calculate the ATPase activity in the supernatant and pellet after resuspension in ATPase buffer. Another set of tubes, after centrifugation, were vortexed to resuspend the pellet back into its own supernatant (control). The activity of this resuspended pellet was identical to that of the assay mixture containing the precipitate, which confirms that centrifugation of the S1–peptide complex and resuspension of the pellet have no effect on the ATPase activity of the mixture (Figure 2, panel B, open circles and open squares, respectively).

If the S1–peptide complex located in the pellet was not active, then one would expect the ATPase activity (s^{-1}) of the supernatant to be equivalent to or higher than the activity of the mixture containing suspended precipitate, since in the latter case the S1 aggregated in the complex is still present and would not contribute to the observed ATPase activity. To answer this question, following centrifugation of another set of tubes, the supernatant was removed and ATPase activity determined by addition of ATP (Figure 2, panel B, closed circles). As well, the pellet was resuspended in ATPase buffer and the activity determined (closed squares). The ATPase activity of

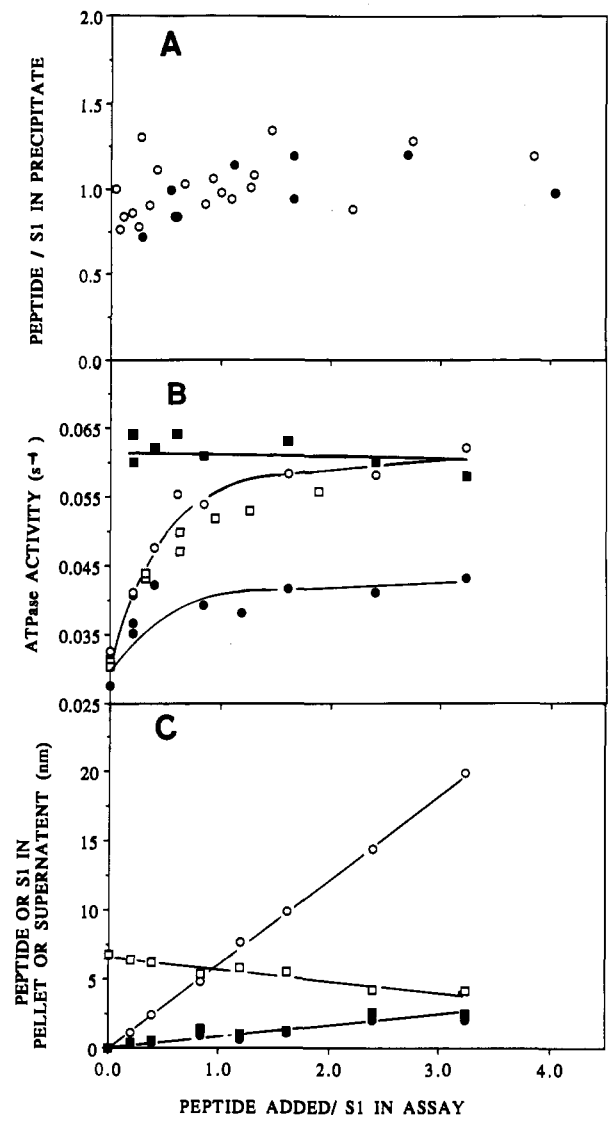

FIGURE 2: Interaction between actin peptide 1–28 and S1 in the absence and presence of ATP. Studies were carried out on mixtures of 6.8 nmol of S1 (A1, A2) in the absence of ATP (panels A and C) and in the presence of ATP (panels A and B) plus increasing quantities of actin peptide. Assay conditions are described under Materials and Methods. Composition of the pellet is shown in panel A. Following centrifugation, aliquots of supernatant were analyzed for S1 and radioactive peptide. From this data the ratio of peptide to S1 in the absence (○) and presence (●) of ATP in the precipitate was determined. The effect of the actin peptide on S1 ATPase activity is shown in panel B. Increasing quantities of peptide were added to a single vial of S1 such that the S1–peptide precipitate was suspended in the assay solution following the continuous titration method (□). As well, three identical sets of tubes containing S1 and different quantities of peptide were centrifuged to pellet the aggregated complex. To one set of tubes, the supernatant was removed and the ATPase activity determined by addition of ATP (●). As well, the pellet was resuspended in ATPase buffer and vortexed and the ATPase activity determined (■). The ATPase activities of the supernatant and pellet alone were calculated on the basis of the quantity of S1 in the supernatant and pellet respectively (panel C). These values were determined from analysis of the second set of tubes following the method used in panel A. Panel C shows the corresponding quantities of peptide (circles) and S1 (squares) located in the supernatant (open symbols) or pellet (closed symbols) at each specific quantity of peptide. The third set of tubes were vortexed to resuspend the pellet back into its own supernatant (control, ○). The ATPase activity for this resuspended sample was determined on the basis of the S1 concentration originally added to and still present in the assay mixture.

the supernatant was less than that of the resuspended precipitate, indicating that both the S1–peptide complex in the supernatant and that in the pellet are active. Since there was

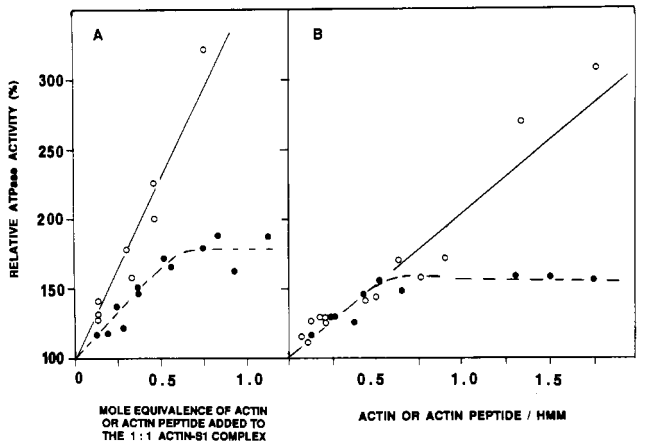

FIGURE 3: Effect of F-actin and actin peptide 1–28 on the acto–S1 (panel A) and HMM (panel B) ATPase activities. The acto–S1 ATPase activity in the absence of actin peptide or additional F-actin was taken to be 100%. The concentration of actin and S1 were both 3 μM. The HMM ATPase activity in the absence of peptide or F-actin was taken to be 100%. The concentration of HMM was 2 μM. The assay conditions are described under Materials and Methods. Symbols: F-actin (○) and actin peptide (●).

considerably more S1 present in the supernatant compared to the pellet (Figure 2, panel C), these results suggest that the 1:1 complex is the active species and that the aggregated 1:1 complex is more active than the 1:1 complex in solution.

Since the S1–actin peptide complex precipitated in the S1 ATPase assay, we concluded that this assay is inappropriate for detailing quantitatively the interaction of the peptide and S1. However, it did demonstrate that the peptide binds to S1 and is able to activate ATPase activity. It is preferable to find assay conditions where no precipitation occurs (see below).

Actin Peptide Interaction with Acto–S1 and HMM. Two alternative assays were used to measure the effects of actin peptide on myosin ATPase activity under conditions where no precipitation occurred. First, a larger myosin fragment, HMM, was employed instead of S1; and second, F-actin was added to the S1 ATPase assay prior to the titration with the actin peptide. The actin peptide did not precipitate HMM (in the absence of actin) or S1 in the presence of F-actin. Figure 3, panel B, compares the activation of the HMM ATPase activity by the actin peptide 1–28 and F-actin. At low concentrations of peptide (below 0.5:1 mole ratio of peptide to HMM), the peptide was as effective as F-actin and increased the ATPase activity by 62%. At higher concentrations (above a 0.5:1 mole ratio of HMM), F-actin continued to activate the ATPase rate while the peptide had reached its maximum activation. This suggests that F-actin filament formation may be required for the increased activation of HMM ATPase activity above the 0.5:1 ratio of peptide or F-actin to HMM. Since actin is filamentous, it may not act the same as monomeric actin does. There have been numerous studies suggesting that the binding of S1, and presumably HMM, to the F-actin filament induced long-range conformational changes along the filament (Ikkai et al., 1979; Yanagida et al., 1984; Rouayrenc et al., 1985). As well, in skinned muscle fibers there appears to be cooperativity within a regulatory unit, which consists of 7 actin and 1 TM–Tn, that causes an increase in force induction (Bremel & Weber, 1972; Brandt et al., 1984; Moss et al., 1985, 1986). These results suggest that the binding of myosin or a myosin fragment to actin is influenced by the remaining unbound actin in the filament, resulting in the alteration of the ATP hydrolysis rate.

The effect of the actin peptide and F-actin on the acto–S1 ATPase activity was similar to its effect on HMM (Figure 3, panel A). The starting conditions for the acto–S1 assay consisted of a 1:1 molar ratio of actin to S1 to which increasing concentrations of either actin or actin peptide (1–28) were added. The actin peptide was able to increase the acto–S1 ATPase activity in a manner similar to F-actin. At a 0.5:1 mole ratio of additional F-actin or actin peptide, the ATPase rate was activated by 130% and 65%, respectively. At a 1:1 mole ratio of additional F-actin or peptide, F-actin increased the ATPase activity by 260% while the actin peptide had reached its maximum activity.

Table I: Centrifugation Studies of Actin Peptide with Actin or Acto-S1

peptide added (nmol)	amount of peptide in pellet/actin in pellet		
	actin[a]	acto–S1[b]	
		–ATP	+ATP
0	0.00	0.00	0.00
1.0	0.04	0.03	0.07
2.1	0.09	0.13	0.05
2.9	0.12	0.25	0.14
3.8	0.20	0.17	0.25
5.9	0.16	0.17	0.20
11.1	0.29	0.17	0.17
22.5	0.38	0.34	0.23

[a] 5.1 nmol of actin was pelleted in the absence of S1 and peptide; hence there was 0.3 nmol of actin remaining in the supernatant. These assays act as a control to determine background levels of peptide pelleted. [b] 5.1 nmol of actin and S1 was pelleted in the absence of peptide; hence there was virtually no S1 remaining in the supernatant.

Table II: Apparent Binding Constant and Maximum Activation of Actin Peptide 1–28 for S1 and HMM

enzyme	K_{ATPase} ($\times 10^{-6}$ M)	V_{max} (% activation)
acto–S1	0.39 ● 0.05	173 ± 3
HMM	0.42 ± 0.09	162 ± 9

[a] Kinetic constants were obtained from Eadie–Hofstee plots of experimental data from Figure 3. 100% is the ATPase rate of HMM or acto–S1 (1:1 ratio) in the absence of actin peptide (V_0).

When actin was present in the S1 ATPase assay, there was no precipitation of peptide–S1 complex at low ratios of peptide to S1. There are two possibilities that would explain this decrease in peptide–S1 precipitate. First, the F-actin bound S1, decreasing the concentration of S1 in solution available to interact with the peptide. Therefore, at any given time there would be less S1–peptide complex in solution, which would reduce the opportunity for aggregation of the peptide–S1 complex to occur. Second, it is possible that the peptide and F-actin could bind to S1 simultaneously, resulting in a complex with increased solubility. To determine whether the actin peptide would bind simultaneously to the acto–S1 complex, centrifugation studies were performed. Minimal amounts of peptide were found in the acto–S1 pellet. In fact, the quantity of peptide present in the acto–S1 pellet was equivalent to the quantity of peptide found in the actin pellet in the absence of S1 (Table I). It is clear that peptide does not bind to the acto–S1 complex. Hence, when F-actin is present in the S1 ATPase assay (Figure 3, panel A), the actin peptide binds to S1 free in solution and not the S1 bound to actin. Thus, the activation of the ATPase activity observed upon addition of the peptide is most likely due to the binding of peptide to S1 that is not bound to F-actin.

ATPase data obtained from the actin peptide titrations of acto–S1 and HMM assays were analyzed by Michaelis kinetics to determine the concentration of peptide required to induce 50% of the maximum activation (K_{ATPase}) and the maximum activity (V_{max}). The actin peptide had the same K_{ATPase} of 0.4

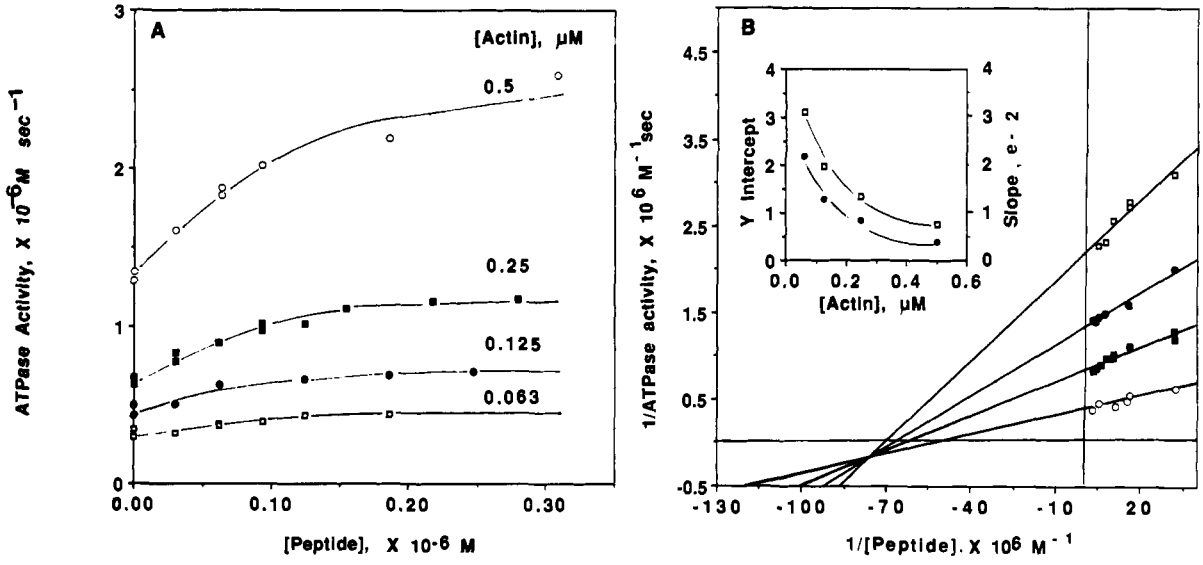

FIGURE 4: Competition results of F-actin and actin peptide in the S1 ATPase assay. The effect of the actin peptide on S1 ATPase activity in the presence of four concentrations of actin, 0.063 (□), 0.125 (●), 0.25 (■), and 0.5 (○) μM, is shown in panel A. The concentration of S1 was 1 μM. Panel B shows a Lineweaver-Burk plot of the data from panel A with the corresponding plot of the slope and *y* intercept terms versus concentration of actin or peptide (insert). The (●) and (□) symbols denote the slope and *y* intercept terms, respectively.

$\times 10^{-6}$ M and V_{max} of 162–173% (where 100% is equal to V_0), respectively, for both enzyme systems (Table II). Interestingly, under similar experimental conditions, the actin-activated HMM or S1 ATPase activity has an apparent binding constant of approximately $1 \times 10^{+5}$ M^{-1} (Stein et al., 1979, 1981). The lower apparent binding constant for F-actin may reflect the filamentous nature of actin and not the binding of monomeric actin or the actin peptide 1–28.

Competition between the Actin Peptide and F-Actin. To investigate whether the actin peptide was able to compete with F-actin for S1, a series of peptide titrations were carried out at various actin concentrations (Figure 4, panel A). Both the actin peptide and F-actin acted as activators of the S1 ATPase activity. At higher concentrations of F-actin, the actin peptide was more effective at stimulating the S1–ATPase hydrolysis rate, indicating that the presence of F-actin somehow acts to enhance the peptide's effect on S1 ATPase activity. A Lineweaver–Burk plot of 1/ATPase activity versus 1/[peptide] (Figure 4, panel B) shows that actin affects the V_{max} and K_{ATPase} of the peptide's activation of the S1 ATPase activity. In a similar manner, the peptide affects the V_{max} and K_{ATPase} of the actin activation. The slope and *y* intercept of the double-reciprocal plots of peptide activation at various actin concentrations (Figure 4, panel B) or actin activation at various peptide concentrations (data not shown), when plotted against the second activator, were hyperbolic (Figure 4, panel B insert). This indicates that both the actin and peptide act as complex nonessential activators (Segal, 1975).

Conclusion

The synthetic actin peptide 1–28 activated the S1 ATPase activity by approximately 81%. Under conditions used in the ATPase assays, actin peptide and S1 precipitated as a 1:1 complex (approximately 15–25% of the total S1 in the assay had precipitated at 1 equiv of S1). The actin peptide 1–28 did not precipitate HMM or acto–S1. The actin peptide caused the HMM and acto–S1 ATPase to be increased in a manner similar to that for F-actin at concentrations below 0.5 equiv of HMM or 1.0 equiv of S1. At higher concentrations, the peptide-induced activation leveled off, while F-actin continued to activate the ATPase rate.

These results indicate that the actin peptide can mimic, in part, the biological activity of F-actin. Hence, the actin peptide must contain a biologically important binding site between actin and myosin.

References

Brandt, P. W., Diamond, M. S., & Schachat, F. H. (1984) *J. Mol. Biol. 180*, 379–384.

Bremel, R. D., & Weber, A. (1972) *Nature, New Biol. 238*, 97–101.

Chong, P. C. S., Asselbergs, P. J., & Hodges, R. S. (1983) *FEBS Lett. 153*, 372–376.

DasGupta, G., White, J., Cheung, P., & Reisler, E. (1990) *Biochemistry 29*, 8503–8508.

Erickson, B. W., & Merrifield, R. B. (1976) in *The Proteins* (Neurath, H., & Hill, R. H., Eds.) Vol. II, pp 255–527, Academic Press, New York.

Hodges, R. S., Semchuk, P. D., Taneja, A. K., Kay, C. M., Parker, J. M. R., & Mant, C. T. (1988a) *Pept. Res. 1*, 19–30.

Hodges, R. S., Burke, T. W. L., & Mant, C. T. (1988b) *J. Chromatogr. 444*, 349–362.

Hodges, R. S., Burke, T. W. L., & Mant, C. T. (1991) *J. Chromatogr.* (in press).

Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature 347*, 44–49.

Ikkai, T., Wahl, P., & Auchet, J. (1979) *Eur. J. Biochem. 93*, 397–408.

Ingraham, R. H., Law, S. Y. M., Taneja, A. K., & Hodges, R. S. (1985) *J. Chromatogr. 327*, 77–92.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37–44.

Keane, A. M., Trayer, I. P., Levine, B. A., Zeugner, C., & Ruëgg, J. C. (1990) *Nature 344*, 265–268.

Mant, C. T., Burke, T. W. L., & Hodges, R. S. (1987a) *Chromatographia 24*, 565–572.

Mant, C. T., Parker, J. M. R., & Hodges, R. S. (1987b) *J. Chromatogr. 397*, 99–112.

Mejëan, C., Boyer, M., Labbë, J.-P., Derancourt, J., Benjamin, Y., & Roustan, R. (1986) *Biosci. Rep. 6*, 493–499.

Mejëan, C., Boyer, M., Labbë, J.-P., Marlier, L., Benjamin, Y., & Roustan, C. (1987) *Biochem. J. 244*, 571–577.

Miller, L., Kalnoski, M., Yunossi, Z., Bulinsky, J. C., & Reisler, E. (1987) *Biochemistry 26*, 6064–6070.
Moir, A. J. G., & Levine, B. A. (1986) *J. Inorg. Chem. 27*, 271–278.
Moir, A. J. G., Levine, B. A., Goodeart, A. J., & Trayer, J. P. (1987) *J. Muscle Res. Cell Motil. 8*, 68.
Moss, R. L., Giulian, G. G., & Greaser, M. L. (1985) *J. Gen. Physiol. 86*, 585–600.
Moss, R. L., Giulian, G. G., & Greaser, M. L. (1986) *J. Gen. Physiol. 87*, 761–774.
Mossakowska, M., & Strzelecka-Golaszewska, H. (1985) *Eur. J. Biochem. 53*, 373–381.
Parker, J. M. R., Mant, C. T., & Hodges, R. S. (1987) *Chromatographia 24*, 832–838.
Rouayrenc, J., Bertrand, R., Kasab, R., Walzhöny, D., Bähler, M., & Walliman, T. (1985) *Eur. J. Biochem. 146*, 391–401.
Segal, I. H. (1975) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Systems*, pp 227–240, Wiley-Interscience Publication, John Wiley and Sons, New York.
Shaw, G., Hodges, R. S., & Sykes, B. (1990) *Science 249*, 280–283.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Stein, L. A., Schwarz, R. P., Chock, P. B., & Eisenberg, E. (1979) *Biochemistry 18*, 3895–3909.
Stein, L. A., Chock, P. B., & Eisenberg, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1346–1350.
Sutoh, K. (1982) *Biochemistry 21*, 3654–3661.
Sutoh, K. (1983) *Biochemistry 22*, 1579–1585.
Suzuki, R., Nishi, N., Tokura, S., & Morita, F. (1987) *J. Biol. Chem. 262*, 11410–11412.
Talbot, J. A., & Hodges, R. S. (1979) *J. Biol. Chem. 254*, 3720–3723.
Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798–2802.
Van Eyk, J. E., & Hodges, R. S. (1988) *J. Biol. Chem. 263*, 1726–1732.
Van Eyk, J. E., Cachia, P. J., Ingraham, R. H., & Hodges, R. S. (1986) *J. Protein Chem. 5*, 335–354.
Weeds, A. G., & Taylor, R. S. (1975) *Nature 257*, 54–56.
Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–157.
Yagi, K., Yazawa, Y., & Yasui, T. (1967) *Biochem. Biophys. Res. Commun. 29*, 331–336.
Yanagida, T., Nakase, M., Nishiyama, K., & Oosawa, F. (1984) *Nature (London) 307*, 58–60.
Young, D. M., Himmelfarb, S., & Harrington, W. F. (1964) *J. Biol. Chem. 239*, 2822–2827.

Downregulation of GMP-140 (CD62 or PADGEM) Expression on Platelets by *N*,*N*-Dimethyl and *N*,*N*,*N*-Trimethyl Derivatives of Sphingosine†

Kazuko Handa, Yasuyuki Igarashi, Mohammad Nisar, and Sen-itiroh Hakomori*
The Biomembrane Institute, 201 Elliott Avenue West, Seattle, Washington 98119, and Department of Pathobiology, University of Washington, Seattle, Washington 98195

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ABSTRACT: GMP-140 (CD62 or PADGEM), a member of the selectin family, is a membrane glycoprotein in secretory granules of platelets and endothelial cells. When these cells are activated by agonists such as thrombin or AMP, GMP-140 is rapidly redistributed to the cell surface. The carbohydrate epitope defined by GMP-140 was identified as sialosyl-Le^x (as for ELAM-1), which may play an essential role in adhesion of leukocytes or tumor cells on endothelial cells, through aggregation with platelets. Redistribution of GMP-140 from α-granules of platelets to the cell surface, induced by thrombin and PMA, was strongly inhibited by preincubation of platelets with *N*,*N*-dimethylsphingosine (DMS) or *N*,*N*,*N*-trimethylsphingosine (TMS) at 10–20 μM concentration for a brief period (5 min). Inhibition of GMP-140 redistribution to the cell surface by DMS or TMS was also detected by a cell adhesion assay using HL60 cells, which highly express sialosyl-Le^x; i.e., HL60 cells adhered on platelets activated by thrombin or PMA but not on platelets which were briefly preincubated with DMS or TMS followed by activation. The inhibitory effect of DMS or TMS on GMP-140 redistribution is not due to cytotoxicity, since the TMS-treated platelets were fully capable of aggregating in the presence of ristocetin. Sphingosine (SPN) and protein kinase C inhibitors such as H-7 and calphostin C showed weaker inhibitory activity than DMS and TMS. Our results indicate that both DMS and TMS could be useful reagents to inhibit cell surface expression of crucial selectins which promote adhesion of Le^x- or sialosyl-Le^x-expressing cells with platelets and endothelial cells. They may therefore display effective inhibition of a variety of biological processes (e.g., inflammation and tumor metastasis) based on the expression and function of selectins.

Interaction of leukocytes with activated platelets and endothelial cells is an initial step in inflammatory processes and is mediated by various adhesion molecules including selectins. Selectins, which include LECCAM-1 (LAM-1 in man, MEL-14 in mice), ELAM-1, and GMP-140 (CD62/PADGEM), are all characterized by a similar structural motif consisting of a lectin domain at the N-terminal region, followed by an EGF sequence, a complement-regulatory domain, a transmembrane region, and a C-terminal domain (Stoolman, 1989; Osborn, 1990; Bevilacqua et al., 1989; Springer, 1990). A number of recent reports have focused on the identification of carbohydrate ligands recognized by members of the selectin family [e.g., Brandley et al. (1990), Lowe et al. (1990), Walz

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* Address correspondence to this author at the Biomembrane Institute.

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