Isolation of a Tripeptide from a Random Phage Peptide Library That Inhibits P^1,P^4-Diadenosine 5′-Tetraphosphate Binding to Its Receptor†

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ABSTRACT: Extracellular P^1,P^4-diadenosine 5′-tetraphosphate (Ap_4A) has been implicated as a modulator of cell stress. We have previously demonstrated specific receptors for Ap_4A at the surface of cardiac myocytes (Walker et al., 1993a). In addition, we have isolated a monoclonal antibody (mAb TL4) that recognized the Ap_4A receptor and inhibited binding of Ap_4A to its receptor (Walker & Hilderman, 1993). As part of our effort to characterize the Ap_4A receptor building domain, we screened a random phage peptide library with mAb TL4. After affinity purification of specifically bound phage, we isolated 38 individual phage clones. Twenty-eight of these clones bound mAb TL4 in ELISA and dot blot analyses. Twenty-two of the twenty-eight individual clones contained inserts with an RGS tripeptide sequence. Synthetic RGS peptide specifically inhibits the binding of mAb TL4 to its membrane receptor. Furthermore, the RGS peptide also inhibits [^{3}H]Ap_4A binding to its receptor. These data are consistent with the RGS peptide mimicking part of the mAb TL4 recognition site on the Ap_4A receptor. The RGS peptide may be used to help characterize the Ap_4A receptor binding domain and to help determine the physiological significance of the interaction between Ap_4A and its receptor.

Diadenylated nucleotides have been studied for only a relatively short period of time. Nevertheless, our current understanding of their metabolism and mechanism of action in prokaryotic and eukaryotic cells suggests that these dinucleotides may constitute a versatile system of cell regulation. P^1,P^4Diadenosine 5′-tetraphosphate (Ap_4A, CAS registry number is 102783-36-8) has been termed an alarmone [reviewed in Kitzler et al. (1992); Remy, 1992; Ogilve, 1992] since it is released into the blood following stress (Flodgaard et al., 1982; Rodriguez et al., 1988; Castro et al., 1990; Zamecnik et al., 1992; Pintor et al., 1992) and modulates diverse physiological functions.

Ap_4A is stored at concentrations greater than 10^{-4} M in both chromaffin cells and dense secretory granules of platelets (Flodgaard et al., 1982; Rodriguez et al., 1988; Zamecnik et al., 1992). Under physiological conditions, the circulating concentration of Ap_4A is too low for detection. In contrast, the circulating concentration of ATP is 0.1 μM (Vassort et al., 1993). By using purified plasma membranes, we have calculated a K_D value of 0.074 μM for Ap_4A and a K_D value of 0.8 μM for ATP binding to the Ap_4A receptor (Walker et al., 1993a). This indicates that there is not a significant amount of ATP occupying the Ap_4A receptor. Thus, localized release of Ap_4A and ATP resulting from platelet activation will favor Ap_4A binding and supports the notion that this molecule is involved in signal transduction.

The effect of Ap_4A binding to its cell surface receptor has been characterized in several cell types. It inhibits ADP-induced platelet aggregation (Zamecnik et al., 1992), influences smooth muscle tone of isolated rabbit arteries (Luthje & Ogilve, 1987), increases basal secretion of catecholamines from isolated chromaffin cells (Castro et al., 1990), and activates glycogen phosphorylase in isolated liver cells (Crik et al., 1993).

The pleiotropic nature of Ap_4A may be due to the activation of a second messenger system with distinctly different ramifications in different cell populations or activation of distinct second messenger systems in differing cell populations. There is indeed evidence that binding of extracellular Ap_4A initiates a redistribution of intracellular calcium (Castro et al., 1990, 1992; Colvin et al., 1991; Pintor et al., 1992; Sen et al., 1993), a ubiquitous second messenger. This internal calcium mobilization, induced by Ap_4A, modulates catecholamine secretion in chromaffin cells (Castro et al., 1992), inhibits cardiac sarcolemma adenyl cyclase (Colvin et al., 1991), and activates protein kinase C (Sen et al., 1993). These data are consistent with the notion that extracellular Ap_4A alters diverse physiological processes through the activation of second messenger systems.

The common features that underlie the pleiotropic effects of Ap_4A are the properties of the membrane receptor and possibly the mechanism through which it activates one or more second messenger systems. Therefore, an understanding of the molecular basis for the diverse roles Ap_4A plays in cellular physiology must begin with a detailed analysis of the membrane receptor. An understanding of the structure of the receptor not only will allow the preparation of perturbational reagents to directly assess the physiological importance of Ap_4A but may also reveal features common to other receptor systems. This information will allow us to direct our efforts toward the most productive avenues for analysis of the second messenger pathways activated by the binding of Ap_4A.

These considerations have led us to focus our efforts on the characterization of the membrane receptor for Ap_4A. Our

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laboratory has demonstrated the presence of specific, saturable membrane receptors for Ap_4A in brain, kidney, cardiac, spleen, and adipose tissues with the greatest density of receptors being found in cardiac tissue (Hilderman et al., 1991). Our data demonstrating that the Ap_4A receptor is present in diverse tissue types and localized on the cell surface (Walker et al., 1993a) support the notion the circulating Ap_4A is a modulator of cellular function. We have isolated a monoclonal antibody (mAb TL4) that blocks the binding of Ap_4A to its receptor (Walker & Hilderman, 1993). The inhibitory effect of mAb TL4 suggests that the epitope recognized by the antibody may coincide with the sequences recognized by Ap_4A. To identify these sequences, we have used mAb TL4 to screen a random phage peptide library (Scott & Smith, 1990). The library consists of phage-bearing random hexapeptides fused to the amino terminus of the phage coat protein PIII. Screening of the library was accomplished by using mAB TL4 to affinity-purify fusion phages that display a high affinity for the antibody. The amino acid sequences of hexapeptides displayed in the purified phages were determined by sequencing the corresponding coding region in the phage DNA (Scott & Smith, 1990). By using this procedure, we have isolated 28 positive clones and sequenced their DNA inserts. Twenty-two of these clones contain inserts having the RGS peptide. This synthetic tripeptide inhibited both mAb TL4 and [^{3}H]Ap_4A binding to the membrane receptor. The availability of simple synthetic ligands (e.g., peptide mimotopes) that mimic the natural mAb TL4 epitope may contribute to the elucidation of the molecular basis of Ap_4A binding to its receptor.

MATERIALS AND METHODS

Materials. Swiss mice were obtained from Charles River Laboratories. The animals were housed in an animal facility maintained with a photoperiod of 15L:9D and room temperatures of 22−25 °C. [^{3}H]Ap_4A was purchased from Amersham International. The RGS and SGR peptides at 98% purity were purchased from American Peptide Company, Inc. The PWY peptide at 98% purity was kindly supplied by J. Lilien (Wayne State University). Goat anti-rat IgG and goat anti-rat IgG conjugated with alkaline phosphatase were purchased from Bio-Rad. All nucleotides were purchased from Sigma Chemical Co. All other reagents were of analytical reagent grade or better. Isolation and purification of mAb TL4 have been described elsewhere (Walker & Hilderman, 1993).

Epitope Library. The hexapeptide epitope library used in this study was provided by George P. Smith (University of Missouri, Columbia) and was constructed by use of the phage fd-derived vector FUSE5 as described (Scott & Smith, 1990). This library consists of 2×10^8 original phage clones and was amplified about 500 times. The phage clones each contain a hexapeptide fused to the minor coat protein PIII. Theoretically, the library can represent 69% of the 6.4×10^7 possible hexapeptides (Scott & Smith, 1990).

Panning of the Phage Epitope Library. A library sample containing 3.8×10^9 infectious phage particles was subjected to three rounds of selection (panning) and amplification. Petri dishes were coated with goat anti-rat IgG overnight at 4 °C and then blocked for 60 min at room temperature with phosphate-buffered saline (pH 7.4), which contained (per liter) 5.46 g of Na_2HPO_4, 1.53 g of NaH_2PO_4, 8.47 g of NaCl, and 0.5% gelatin. The mAb TL4 was absorbed onto the goat anti-rat IgG-coated plates by incubating for 60 min at room temperature. The affinity isolation and amplification of phage from the hexapeptide epitope library were carried out as described (Scott & Smith, 1990).

ELISA for Affinity-Purified Phage. After the affinity-purified phages were amplified as described (Scott & Smith, 1990), microtiter plates were coated overnight at 4 °C with the phage particles. The plates were than washed and blocked for 60 min at room temperature with Blotto (6.06 g of Tris and 8.77 g of NaCl per liter, 5% nonfat milk, and 0.1% Tween 20, pH 7.4). Aliquots (100 μL) of mAb TL4 (0.1 mg/mL) then were added to the wells and the plates were incubated overnight at 4 °C. Binding of the antibodies to the phage was identified by the use of goat anti-rat IgG conjugated with alkaline phosphatase (1:1000 dilution). Color was developed by adding 100 μL aliquots of developer containing 10 mg of 2-nitrophenyl phosphate in a 10 mL solution of 1% diethanolamine and 263 mM $MgCl_2$ (pH 9.5) and measuring the absorbance at 405 nm.

DNA Sequencing. Phages from the supernatants of positive clones were precipitated with poly(ethylene glycol), and their DNA was prepared by phenol extraction. DNA was sequenced by the chain termination method with the aid of described primers (Scott & Smith, 1990).

RGS Peptide Binding to mAb TL4. One hundred microliter aliquots of the RGS peptide (1 mg/mL) resuspended in 0.1 M Na_2CO_3 (pH 9.6) were incubated in microtiter wells overnight at 4 °C. Then the plates were washed three times with Tris-buffered saline (pH 7.4) (6.06 g of Tris and 8.77 g of NaCl per liter) containing 0.1% Tween 20, followed by blocking with Blotto for 30 min at room temperature. After washing, 100 μL of mAb TL4 (0.1 mg/mL) was added to the wells, and the plates were incubated for 2 h at room temperature. Binding of the antibodies was identified by the use of goat anti-rat IgG conjugated with alkaline phosphatase as described earlier under ELISA for Affinity-Purified Phage.

ELISA for RGS Peptide Inhibition of mAb TL4 Binding to Ap_4A Membrane Receptor. Mouse cardiac membranes were isolated as described (Walker et al., 1993a). These membranes were resuspended in Hepes-buffered saline with glucose and potassium [20 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM glucose, 3 mM KCl, and 1 mM $CaCl_2$ in addition to 1% Triton X-100], incubated for 60 min with stirring at 4 °C, and, then centrifuged at 100000*g* for 90 min. The pellet was resuspended in 67 mM Tris-HCl (pH 7.7), and 100 μM $MgCl_2$. One hundred microliter membrane aliquots (1.0 mg/mL) were incubated overnight at 4 °C in microtiter wells precoated with 100 μL of poly(L-Lys) (50 μg/mL) prior to washing three times with phosphate-buffered saline containing 0.1% Tween 20 and then blocked for 60 min at room temperature with phosphate-buffered saline containing 0.1% Tween 20 and 1% bovine serum albumin. One hundred microliter aliquots of varying concentrations of RGS peptide, resuspended in phosphate-buffered saline and preincubated overnight at 4 °C with 4 μL of mAb TL4 (2.5 mg/mL), were added to membrane-coated microtiter wells and incubated for 2 h at room temperature. Binding of the antibody was identified by the use of goat anti-rat IgG conjugated with alkaline phosphatase, as described earlier under ELISA for Affinity-Purified Phage.

Ap_4A Receptor Activation and Binding Assay. Receptor activation is required for Ap_4A binding to its receptor. Specific Ap_4A binding is enhanced 4−5-fold when the

Table 1: Panning of the Hexapeptide Library with Anti-Receptor mAb[a]

round of panning	goat anti-rat IgG (μg/mL)	anti-receptor mAb (μg/mL)	yield % of input phage
1	10	40	9.5×10^{-4}
2	10	20	9.2×10^{-2}
3	2	10	1.1×10^{-1}

[a] Panning was performed as described in the Materials and Methods. Phage concentrations were calculated from the titer of transducing units (TU).

Table 2: Internal Peptide Sequences with the Highest Homology from Hexapeptide Library Affinity-Purified Clones[a]

clones	mimotope	clones	mimotope
7	RGSGS	1	RGSSR
7	RGSSS	2	RGGSS
4	RGSAS	1	RSSSG
1	RGSAG	1	RSSSA
1	RGSSG	2	RSSSS
1	RGSGR		

[a] The phage epitope (single-letter amino acid code) was deduced from the DNA sequence.

membrane homogenates are incubated with binding buffer for 60 min at 29 °C prior to assay (Walker et al., 1993b). We refer to this incubation as receptor activation.

Protein Determination. Protein concentrations were determined by dye binding (Bradford, 1976).

All experiments were performed a minimum of three times, with each data point in all experiments measured in triplicate.

RESULTS

Isolation and Identification of Peptide-Presenting Phage by mAb TL4. Repeated selection of the phage from a random phage peptide library resulted in an enrichment of phage capable of binding to mAb TL4. The yields from the second and third rounds of panning (Table 1) were significantly above the nonspecific background of 3×10^{-5}% yield (Parmley & Smith, 1988).

After three rounds of panning and phage amplification, 38 individual isolated bacterial colonies were grown, and their phages were assayed for antibody binding. ELISA and dot blot analyses revealed that 28 of these clones bound specifically to mAb TL4 (data not shown). DNA from each of the 28 clones was sequenced, and the deduced peptide sequences of these 28 clones are shown in Table 2. Twenty-two of these clones contain inserts with an RGS tripeptide sequence. Two additional clones had the tripeptide sequence RGG, and the four other clones had the tripeptide sequence RSS. All of the analyses described below using fusion phage were performed with phage containing the RGSSS insert (RGSSS phage), one of the two sequences found most often (Table 2).

mAb TL4 Recognition of Phage Containing the RGSSS Sequence. As a prelude to examining the effect of the isolated peptide sequence on binding of mAb TL4 or Ap_4A to the membrane receptor, we examined the interaction of the RGSSS-bearing phage with mAb TL4. Binding of mAb TL4 to the RGSSS phage was concentration dependent from 10^{10} to 5×10^{11} transducing phage particles (Figure 1A). In addition, mAb TL4 did not bind to an unrelated phage bearing the sequence IRWWHW, demonstrating the specificity of mAb TL4 binding. If the RGSSS-bearing phage is

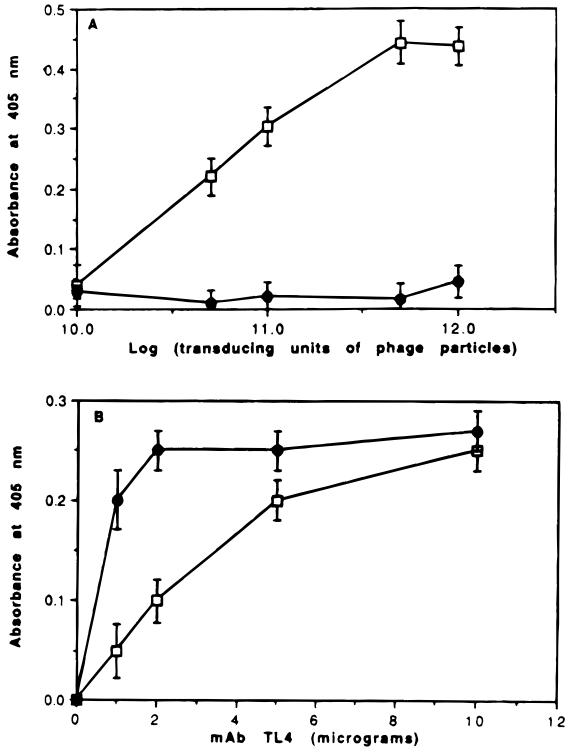

FIGURE 1: mAb TL4 recognition of phage containing the RGSSS sequence. (A) Varying concentrations of RGSSS phage (□) and IRWWHW phage (●) were resuspended in 0.1 M Na_2CO_3 (pH 9.6), incubated overnight at 4 °C prior to the addition of 10 μg of mAb TL4, and measured by ELISA as described in Materials and Methods. (B) One hundred micrograms of membrane homogenates resuspended in 0.1 M Na_2CO_3 (pH 9.6) was incubated overnight at 4 °C. Varying concentrations of mAb TL4 and 10^{11} transducing units of RGSSS phage particles were also incubated separately overnight at 4 °C. One hundred microliter aliquots of these antibody/virus mixtures (□) or antibody without phage (●) were then added to the membrane-coated microtiter plate wells and measured by ELISA as described in Materials and Methods. Error bars are shown as standard deviations.

mimicking a sequence in the receptor, a phage bearing this sequence should inhibit binding of mAb TL4 to the membrane receptor. This is indeed the case (Figure 1B). Furthermore, phage bearing the sequence IRWWHW did not inhibit binding of mAb TL4 to the membrane receptor (data not shown).

RGS Peptide Inhibits Binding of mAb TL4 to Its Membrane Receptor. It is possible that inhibition of mAb TL4 binding to its membrane receptor by the RGSSS phage is due to an interaction of the peptide in combination with sequences present in the phage PIII coat protein. To test this, we determined whether free RGS peptide interacted directly with mAb TL4 and whether the isolated peptide inhibited binding of mAb TL4 to its membrane receptor. Binding of mAb TL4 to increasing concentrations of immobilized RGS peptide shows a dose dependency between 25 and 75 μg of peptide (Figure 2A). In contrast, mAb TL4 did not bind to a tripeptide with the reverse orientation (SGR) nor to an unrelated peptide (PWY) (Figure 2A). Furthermore, binding of increasing concentrations of mAb TL4 to a constant amount of immobilized RGS peptide shows a dose dependency between 5 and least 20 μg of antibody (Figure 2B). Neither an unrelated mAb (JW17) nor bovine serum albumin significantly bound to the RGS peptide (Figure 2B).

Having established that the isolated peptide binds specifically to mAb TL4, we examined the ability of RGS peptide

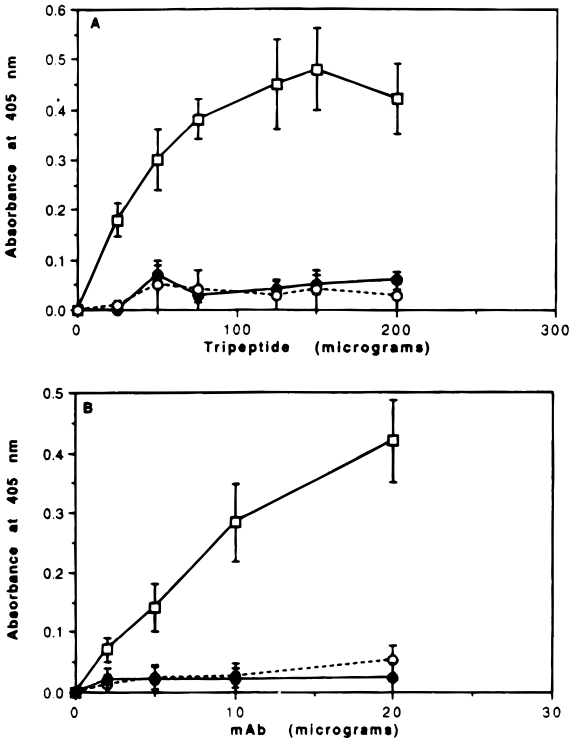

FIGURE 2: mAb TL4 recognition of the random peptide library-derived RGS peptide. (A) Varying concentrations of RGS (□), SGR (○), and PWY (●) were resuspended in 0.1 M Na_2CO_3 (pH 9.6), incubated overnight at 4 °C prior to the addition of 10 μg of mAb TL4, and measured by ELISA as described in Materials and Methods. (B) One hundred micrograms of RGS resuspended in 0.1 M Na_2CO_3 (pH 9.6) was incubated overnight at 4 °C prior to the addition of varying concentrations of mAb TL4 (□), mAb JW17 (○), or bovine serum albumin (●) and measured by ELISA as described in Materials and Methods. Error bars are shown as standard deviations.

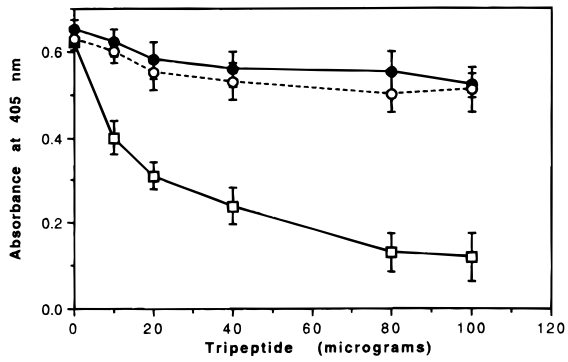

FIGURE 3: RGS peptide inhibition of mAb TL4 binding to the Ap_4A membrane receptor. One hundred micrograms of membrane homogenates resuspended in 0.1 M Na_2CO_3 (pH 9.6) was incubated overnight at 4 °C. Varying concentrations of RGS (□), SGR (○), or PWY (●), at the concentrations indicated, were also incubated overnight at 4 °C with 10 μg of mAb TL4 and measured by ELISA as described in Materials and Methods. Error bars are shown as standard deviations.

to inhibit binding of mAb TL4 to its membrane receptor. RGS peptide inhibited mAb TL4 binding to the receptor with an IC_{50} value of 6.3×10^{-4} M. In contrast, neither a tripeptide with the reverse orientation (SGR) nor an unrelated tripeptide (PWY) inhibited Ap_4A binding to its receptor (Figure 3). The ability of the free RGS peptide to inhibit antibody binding to the Ap_4A receptor indicates that sequences present in the phage PIII protein are not part of the epitope selected by mAb TL4 from the phage library. These data also suggest that the sequence RGS mimics an important

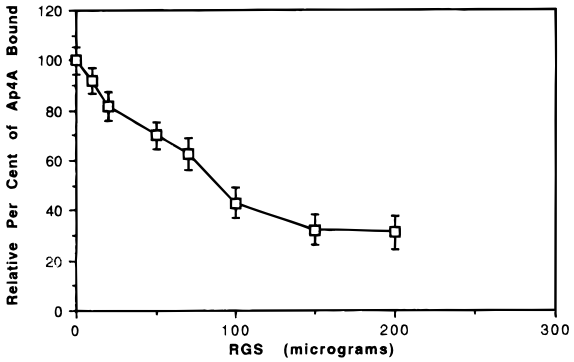

FIGURE 4: RGS peptide inhibition of Ap_4A binding to the Ap_4A membrane receptor. Membrane homogenates were isolated as previously described (Hilderman et al., 1991) and activated as described in Materials and Methods. Varying concentrations of RGS peptide at the concentrations indicated and 0.25 μM [^{3}H]Ap_4A (specific activity = 2000−5000 cpm/pmol) were incubated with 22 μg of activated membrane for 30 min at 20 °C. All samples were collected on glass fiber disks and counted as described (Hilderman et al., 1991). Error bars are shown as standard deviations.

Table 3: Inhibition of Ap_4A Binding to Its Receptor by Various Tripeptides[a]

peptide	Ap_4A bound (pmol/mg of protein)	relative percent
none	73.6	100.0
RGS	29.4	40.0
SGR	71.0	96.4
PWY	79.5	108.0

[a] Membrane activation and [^{3}H]Ap_4A binding assays were performed as described in Figure 4. Tripeptide (314 nmol) and 0.25 μM [^{3}H]Ap_4A (specific activity = 2000−5000 cpm/pmol) were incubated with 22 μg of membrane fractions for 30 min at 20 °C.

component of the mAb TL4 binding site on the membrane receptor.

RGS Peptide Inhibits Binding of Ap_4A to Its Receptor. Our rationale in using mAb TL4 as the selective agent to identify a peptide in the phage library was based on the ability of bound mAb TL4 to inhibit Ap_4A binding to its membrane receptor. Therefore, such a peptide would represent an important component of the Ap_4A recognition site of the receptor. Free RGS peptide did interfere with [^{3}H]Ap_4A binding to its receptor, with an an estimated IC_{50} value of 5.3×10^{-4} M (Figure 4). This IC_{50} value is similar to the value obtained for the RGS peptide inhibition of mAb TL4 binding to the Ap_4A receptor (Figure 3). As shown in Table 3, a tripeptide with the reverse orientation (SGR) did not inhibit Ap_4A binding to its receptor, nor did an unrelated tripeptide (PWY) inhibit Ap_4A binding. Furthermore, equal molar amounts of the amino acids R, G, or S or a combination of all three amino acids did not interfere with Ap_4A binding to its receptor (Table 4). These data strongly support the contention that the RGS peptide mimics at least part of the Ap_4A receptor.

DISCUSSION

Our ultimate goal is to develop rationally designed agonists and antagonist of the Ap_4A receptor to probe the physiological role of this important compound. To develop an inhibitor of Ap_4A binding, we have used an antibody generated to the Ap_4A receptor that inhibits Ap_4A binding (Walker & Hilderman, 1993) to screen a randomly generated hexapeptide library fused to the PII protein of the phage coat

Table 4: Inhibition of Ap_4A Binding to Its Receptor by Various Amino Acids[a]

amino acid or peptide	Ap_4A bound (pmol/mg of protein)	relative percent
none	75.1	100.0
RGS	30.1	41.4
R	74.8	99.6
G	77.7	103.5
S	74.5	99.2
R, G, and S	70.0	93.2

[a] Membrane activation and [^{3}H]Ap_4A binding assays were performed as described in Figure 4. Each amino acid or RGS (314 nmol) and 0.25 μM [^{3}H]Ap_4A (specific activity = 2000−5000 cpm/pmol) were incubated with 22 μg of membrane fractions for 30 min at 20 °C.

(Parmley & Smith, 1988). Our working hypothesis was that hexapeptides recognized by the antibody would contain sequences mimicking the binding domain of the Ap_4A receptor. We have succeeded in identifying a set of sequences with a common tripeptide motif, RGS. Synthetic RGS peptide binding to mAb TL4 is concentration dependent (Figure 2) and inhibits mAb TL4 binding to the Ap_4A membrane receptor (Figure 3). The RGS peptide also inhibits [^{3}H]Ap_4A binding to its membrane receptor (Figure 4). These data are consistent with the RGS tripeptide mimicking at least a portion of the receptor sequences essential to binding.

The use of random peptide libraries can lead to the isolation of ligand mimotopes for biologically active molecules that differ in structure from the binding site of the natural ligands, but mimics its specificity. An example is the peptide DLVWLL, which bears no sequence homology to acetylcholine receptor, but specifically binds to the anti-acetylcholine receptor mAb and blocks its biological activity (Balass et al., 1993). Other investigators have identified peptides from random peptide libraries that inhibit basic fibroblast growth factor (bFGF) binding to its receptor. These peptide sequences correspond to continuous protein sequences of bFGF (Yayon et al., 1993).

IC_{50} values of 10^{-7}−10^{-8} M for peptide mimotopes have been reported (Koivunent et al., 1993; Yayon et al., 1993); however, the IC_{50} value for DLVWLL inhibition of mAb binding to the acetylcholine receptor is 2×10^{-4} M (Balass et al., 1993). The high IC_{50} values for RGS peptide inhibition of mAb TL4 and [^{3}H]Ap_4A binding to the membrane receptor (6.3×10^{-4} and 5.3×10^{-4} M, respectively) might suggest that, like the DLVWLL sequence, the RGS sequence is mimicking not a linear sequence, but one formed by the juxtaposition of residues in the fully folded configuration of the receptor. To resolve this issue, we are determining the amino acid sequence of the Ap_4A receptor by sequencing full-length receptor cDNA clones. Regardless of whether the RGS sequence mimics an epitope formed by a linear sequence in the receptor or a sequence formed through folding of the receptor protein, the RGS peptide will be invaluable in probing the physiological significance of this ligand receptor interaction.

Investigators have demonstrated that specific positively charged amino acid residues (Arg^{265}, Arg^{292}, and His^{262}) in a P_{2u} receptor may be involved in ligand recognition (Erb et al., 1995). Molecular modeling of this P_{2u} receptor suggests that specific R and H residues may be interacting with phosphate groups of the nucleotide ligand. Furthermore, sequence alignment of various P_{2u} receptors has indicated that charged amino acids (K, R, and H) are conserved near the plasma membrane extracellular interface (Erb et al., 1995). These data are consistent with our observations that the RGS peptide is part of, or a mimic of, the Ap_4A binding region on the receptor. Characterization of the Ap_4A binding region, along with site-directed mutagenesis of the Ap_4A receptor, ultimately will identify which regions of the Ap_4A receptor are responsible for determining specificity and similarities to other nucleotide receptors.

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