Resolution of a Signal Transfer Region from a General Binding Domain in Gβ for Stimulation of Phospholipase C–β2

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Signaling by guanine nucleotide–binding proteins (G proteins) involves sequential protein-protein interactions. G protein–βγ subunit (Gβγ) interactions with phospholipase C–β2 (PLC-β2) were studied to determine if all Gβ contacts are required for signaling. A peptide encoding Gβ amino acid residues 86 to 105 stimulated PLC-β2. Six residues (96 to 101) within this sequence could transfer signals and thus constitute a core signal transfer region. Another peptide, encoding Gβ amino acid residues 115 to 135, did not substantially stimulate PLC-β2 by itself but inhibited Gβγ stimulation, indicating that residues 115 to 135 constitute a general binding domain. Resolution of signal transfer regions from general binding domains indicates that all protein-protein contacts are not required for signal transfer and that it may be feasible to synthesize agonists and antagonists that regulate intracellular signal flow.

Transmembrane signaling in G protein–coupled systems occurs through protein-protein interactions. Agonist-occupied receptors interact with G proteins to promote nucleotide exchange and subunit dissociation. The Gα subunits and the Gβγ complex interact with and regulate effectors (*1*). The Gβγ complex regulates numerous effectors including K^+ channels, adenylyl cyclase 2, PLC-β2, and Ca^{2+} channels. We designed experiments to test whether signal transfer through protein-protein interactions requires all of the contacts between protein partners for information flow.

Residues including amino acids 60 to 150 of Gβ have been implicated in effector interactions (*2–5*). Because a relatively large area of Gβ participates in effector interactions, we chose one effector, PLC-β2, and determined a minimal region of Gβ required for stimulation. We also determined whether there are regions of Gβ that take part in effector interactions but are not required for signal transfer. Two peptides (*6*) encoding amino acids 86 to 105 and 115 to 135 of Gβ can inhibit Gβγ regulation of AC1 and AC2 (*3*). We tested the effect of the Gβ 86-105 peptide on Gβγ stimulation of PLC-β2 (*7*). The Gβ 86-105 peptide stimulated PLC-β2 in both the absence and presence of Gβγ. The stimulation by maximal concentration of peptide was not additive with that by Gβγ (Fig. 1A). Substitution of Met for Asp at position 101 (M101N) renders this peptide inactive for interactions with AC2 and AC1 (*3*). The M101N peptide did not activate PLC-β2 (Fig. 1A), indicating that the 101 position could be important for interactions with PLC-β2. To determine if the stimulation resulted from direct interactions between the peptide and PLC-β2, we tested the binding of the Gβ 86-105 and Gβ 86-105 M101N peptides to PLC-β2 by fluorescent resonance energy transfer (FRET) (*8*). The Gβ 86-105 peptide bound to PLC-β2 with a dissociation constant of about 1 μM (Fig. 1B), whereas the M101N peptide had no measurable binding. The difference in the affinities for binding and activation is probably due to differences between the two assay systems. The activity measurements were done with PLC from cytosolic lysates, whereas the binding measurements were done with purified PLC. The binding experiment was done in the presence and absence of phospholipids with identical results, indicating that the binding of the peptide to PLC-β2 is independent of the binding of substrate.

Complementary charge interactions are often key determinants for protein-protein interactions. The Gβ86-105 peptide contains two charged residues, Lys^{89} and Arg^{96}, and one histidine, His^{91}. We evaluated the importance of each of these residues for the Gβ86-105 peptide stimulation of PLC-β2. Substitution of either Lys^{89} or Arg^{96}, but particularly Arg^{96}, decreased the affinity of the peptide for PLC-β2 but did not affect maximal stimulation (Fig. 2A). The $Lys^{89} \rightarrow$ Ala (K89A) substitution has a similar effect when made in the Gβ subunit through site-directed mutagenesis (*9*). When all three residues were substituted, the peptide did not stimulate PLC-β2 (Fig. 2B, top panel) and did not affect Gβγ stimulation of PLC-β2 (Fig. 2B, bottom panel). These results indicate that charge interactions may be crucial for both interactions and signal transfer from Gβγ to PLC-β2. To test whether charged peptides might nonspecifically activate PLC-β2, we measured the effects on PLC-β2 of an unrelated peptide, FLLT, which encodes region 660 to 688 of adenylyl cyclase 6 but has the same overall charge (+2 at pH 6.8 to 6.9) as the Gβ 86-105 peptide. The FLLT peptide had no measurable stimulatory effect (Fig. 2C). These results demonstrate that the stimulatory effects of the Gβ 86-105 peptide on PLC-β2 are not solely due to the charge of the peptide. To ascertain that the Gβ 86-105 peptide stimulation of PLC-β2 was selective, we tested stimulation of PLC-Xβ, an isoform of PLC-β from *Xenopus* that is stimulated

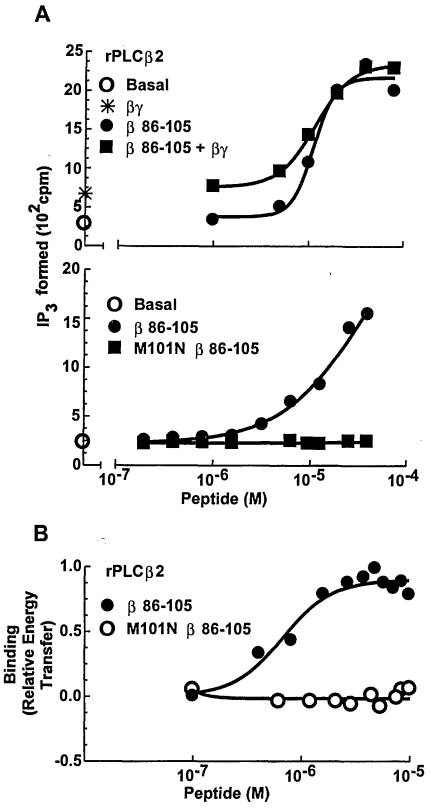

Fig. 1. (**A**) Effects of various concentrations of Gβ 86-105 peptide on PLC-β2 activity. (Top) Effects of Gβ 86-105 peptide on basal and Gβγ (100 nM)–stimulated PLC-β2 activity. (Bottom) Effects of Gβ 86-105 peptide and M101N Gβ 86-105 peptide on basal activity of PLC-β2. IP_3, inositol trisphophate. (**B**) Binding of Gβ 86-105 peptide and M101N Gβ 86-105 peptide to PLC-β2. The *y* axis is expressed as relative energy transfer, which was monitored by the loss of the Cascade Blue fluorescence by transfer to the nonfluorescent acceptor DABMI.

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poorly by Gβγ subunits under our assay conditions. The Gβ 86-105 peptide stimulated PLC-β2 robustly, but it had relatively little ability to stimulate PLC-Xβ (Fig. 2D). Thus, the Gβ 86-105 peptide selectively stimulates an isoform of PLC-β that is regulated by Gβγ subunits.

Mutants of Gβ in which Ser^{98} is mutated to Ala (S98A) stimulate PLC-β2 more extensively (*5*). We studied the effects of four types of substitutions at this position. When Ser^{98} was substituted with Ala (Fig. 3, top panel), there was about a twofold increase in the affinity for PLC-β2. This increase could be consistent with the site-directed mutagenesis experiment in Gβ (*5*). When both the serines were substituted with Arg (S97R and S98R), there was a fivefold increase in affinity of the peptide (Fig. 3, middle panel). In contrast, substitution with Asp (S97D and S98D) resulted in an inactive peptide, whereas substitution with Cys (S97C and S98C) resulted in reduced affinity (Fig. 3, bottom panel). These experiments indicated that the region around amino acids 96 to 101 was crucial for signal transfer. We tested several short peptides, including a 3–amino acid peptide encoding residues 96 to 98, a 6–amino acid peptide encoding residues 96 to 101, and a 13–amino acid peptide encoding residues 89 to 101. The 3–amino acid peptide did not stimulate PLC-β2, but the 6– and the 13–amino acid peptides did stimulate it (Fig. 4A). The Gβ 96-101 6–amino acid peptide had lower affinity than the Gβ 86-105 peptide (Fig. 4B). However, when the serines corresponding to positions 97 and 98 were substituted by Arg, Gβ 96-101 stimulated with an apparent activation constant (K_{act}) of 30 μM (Fig. 4B, top panel), as compared with 5 to 10 μM K_{act} for the Gβ 86-105 peptide (Figs. 1 to 3). When the serines were substituted with Asp, Gβ 86-105 did not stimulate PLC-β2 (Fig. 4B, bottom panel). Substitution of either Ser with Arg increased affinity of stimulation, and substitution at position 97 resulted in stimulation of PLC with a small (less than twofold) but reproducible and higher affinity than the substitution at position 98 (Fig. 4C). The efficacy of the S97R-substituted peptide appears to be similar to that for the full-length Gβ 86-105 peptide (Fig. 4C, bottom panel), albeit with lower affinity.

Amino acids 96 to 101 of Gβ constitute a core signal transfer region (STR) for activation of PLC-β2. Other regions of Gβ that interact with PLC-β2 may contribute to the overall affinity of the interactions but not be involved in signal transfer. If this were the case, then a peptide encoding such a region should inhibit Gβγ stimulation of PLC-β2 but not stimulate PLC-β2 by itself. The Gβ 115-135 peptide inhibits Gβγ modulation of both AC2 and AC1 (*3*). This peptide evoked a small (~20% over basal) stimulation of PLC-β2 at saturating concentrations. But, when added with Gβγ, the peptide substantially (~80%) inhibited Gβγ stimulation of PLC-β2. When the conserved tyrosine at position 124 was substituted, the peptide was inactive (Fig. 5A). The Gβ 115-135 peptide inhibited PLC-β2 with an apparent K_{act} of 5 μM (Fig. 5B). Thus, we conclude that the

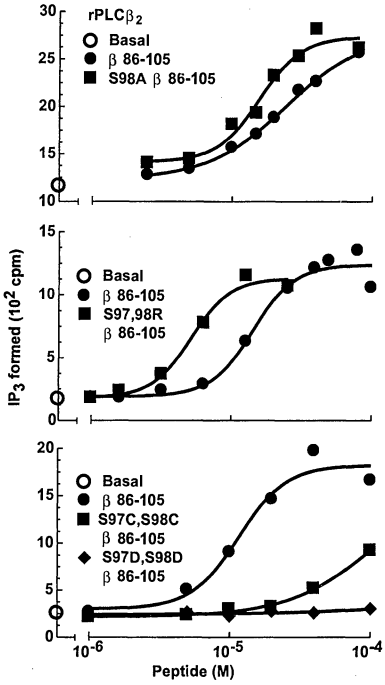

Fig. 3. Effects of various concentrations of Gβ 86-105 peptide and (top) S98A Gβ 86-105 peptide, (middle) S97R and S98R Gβ 86-105 peptides, and (bottom) S97D and S98D and S97C and S98C Gβ 86-105 peptides on PLC-β2 activity.

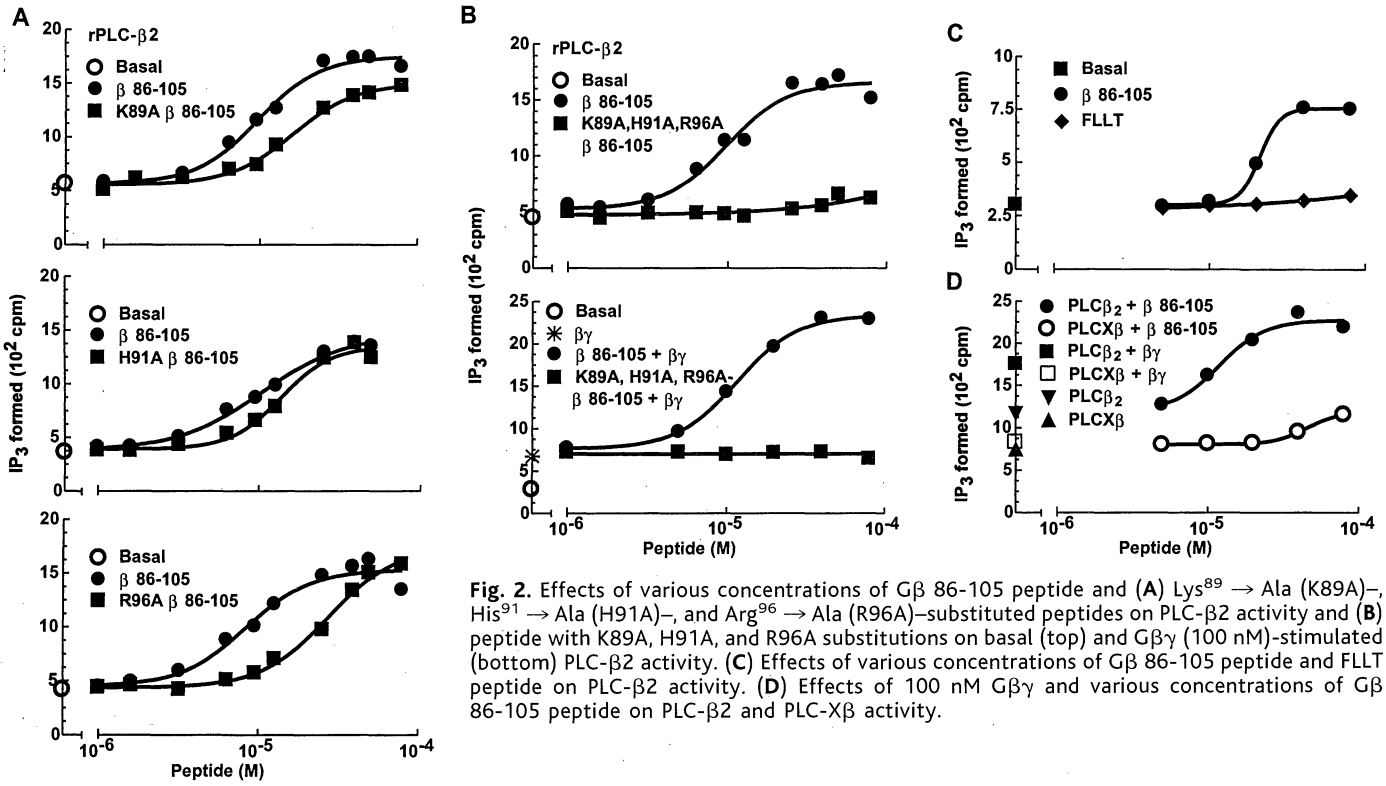

Fig. 2. Effects of various concentrations of Gβ 86-105 peptide and (**A**) Lys^{89} → Ala (K89A)–, His^{91} → Ala (H91A)–, and Arg^{96} → Ala (R96A)–substituted peptides on PLC-β2 activity and (**B**) peptide with K89A, H91A, and R96A substitutions on basal (top) and Gβγ (100 nM)-stimulated (bottom) PLC-β2 activity. (**C**) Effects of various concentrations of Gβ 86-105 peptide and FLLT peptide on PLC-β2 activity. (**D**) Effects of 100 nM Gβγ and various concentrations of Gβ 86-105 peptide on PLC-β2 and PLC-Xβ activity.

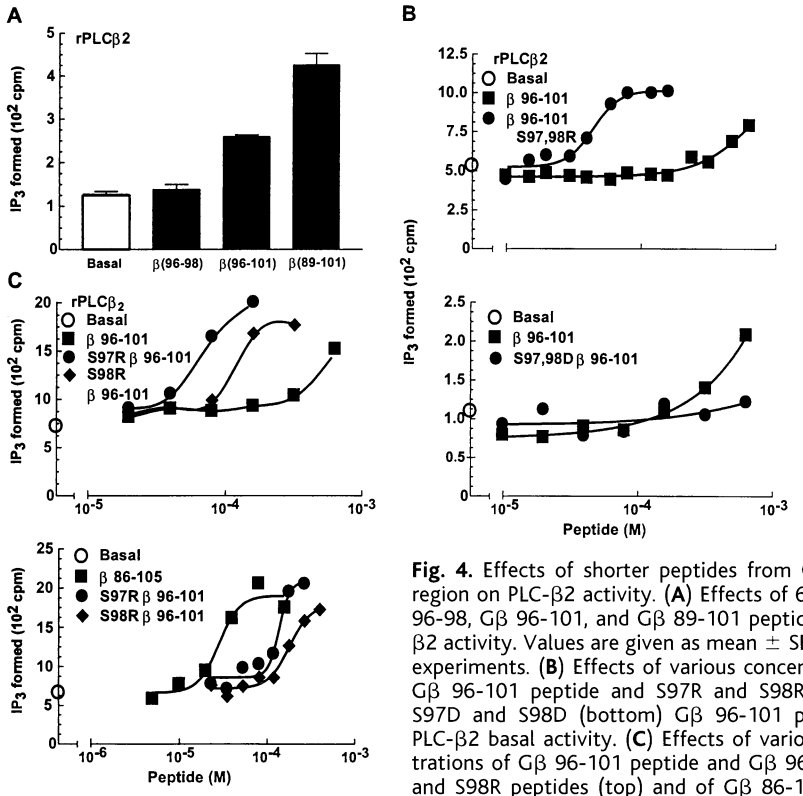

Fig. 4. Effects of shorter peptides from Gβ 86-105 region on PLC-β2 activity. (**A**) Effects of 600 μM Gβ 96-98, Gβ 96-101, and Gβ 89-101 peptides on PLC-β2 activity. Values are given as mean ± SEM of three experiments. (**B**) Effects of various concentrations of Gβ 96-101 peptide and S97R and S98R (top) and S97D and S98D (bottom) Gβ 96-101 peptides on PLC-β2 basal activity. (**C**) Effects of various concentrations of Gβ 96-101 peptide and Gβ 96-101 S97R and S98R peptides (top) and of Gβ 86-105 peptide and Gβ 96-101 S97R and S98R peptides (bottom) on PLC-β2 basal activity.

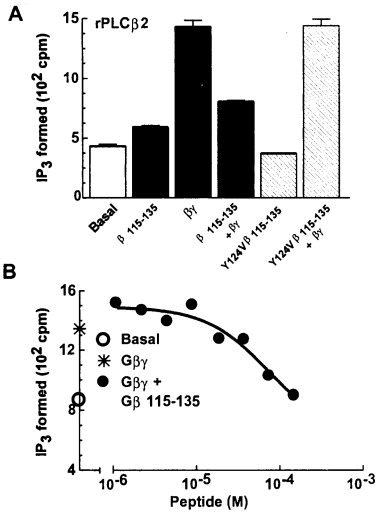

Fig. 5. Effects of Gβ 115-135 peptide on PLC-β2 activity. (**A**) Effects of 30 μM Gβ 115-135 peptide and $Tyr^{124} \rightarrow Val$ (Y124V) Gβ 115-135 peptide on basal and Gβγ (100 nM)-stimulated PLC-β2 activity. Values are given as mean ± SEM of three experiments. (**B**) Effect of various concentrations of Gβ 115-135 peptide on Gβγ-stimulated PLC-β2 activity.

region of Gβ containing amino acids 115 to 135 constitutes a general binding domain (GBD) that is not required for signal transfer.

Our studies demonstrate that all of the contacts between two proteins are not required for signal transfer. In the case of Gβγ and PLC-β2, our data show that a relatively short stretch of six amino acids, 96 to 101, appears to be sufficient to transfer the signal, that is, activate the enzyme. Substitution of residues within the six–amino acid peptide produces a more potent peptide than the naturally occurring sequence. The naturally occurring residues in STRs may not be optimized for this particular set of interactions. Such suboptimal interactions may be one mechanism to achieve regulated reversibility. It should also be noted that the Gβ 86-105 peptide does not stimulate AC2 in the presence of Gαs(3), suggesting that there may be different STRs on Gβ for different effectors. Gβ 115-135 minimally stimulates PLC-β2 but is effective in inhibiting Gβγ stimulation of PLC-β2. This indicates that the 115 to 135 region of Gβ is part of a GBD that participates in interactions with PLC-β2.

What is the relevance of such a functional resolution between STRs and GBDs within the overall interactions area? From the perspective of protein engineering, it offers a built-in capability to regulate the affinity of interaction between the protein partners and thus make reversibility feasible. Peptide hormones have distinct address and message regions (*10*) that mediate binding interactions with receptors and activation of intracellular signaling pathways, respectively. This functional resolution of peptide hormones has been used for the design of peptidomimetic antagonists (*11*). Similarly, our resolution of an STR from GBD for interactions between intracellular proteins provides an approach to identifying molecular interactions relevant for the development of agonists and antagonists at intracellular protein interaction sites. The interactions between the STR peptide and PLC-β2 could form the basis for synthesis of agonists that mimic receptor-dependent activation of PLC-β2. In contrast, the interactions between the GBD peptide and PLC-β2 would form the basis for synthesis of antagonists that block receptor-dependent activation of PLC-β2. Signaling pathways are major targets for therapeutic agents. Up to now, agonists and antagonists have largely been focused on extracellular receptor sites; our studies indicate that it may be feasible to design agonists and antagonists directed at the interface between signaling components inside the cell.

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6. Peptides were synthesized on an Applied Biosystems peptide synthesizer (model 431A) and purified by high-pressure liquid chromatography on acetonitrile gradients. Purified peptides were lyophilized and stored at −20°C. When needed, peptides were dissolved in HED buffer [10 mM Hepes (pH 7.0), 1 mM EDTA (pH 8.0), and 1 mM dithiothreitol (DTT)]. The identity of peptides was verified by mass spectrometry.
7. Recombinant PLC-β2 was expressed in High 5 insect cells by infection with recombinant baculovirus. Three to 4 days after infection, the cells were lysed by pressure in a par bomb to 600 psi. The lysate was then centrifuged, and the cytosolic fraction was collected. About 10 to 15 μg of protein of cytosolic fraction was used per 100 μl of reaction volume. Phospholipid substrate is a mixture of [^{3}H]phosphatidylinositol-4,5-bisphosphate ([^{3}H]PIP_2) and unlabeled phospholipids. Unlabeled phospholipids (Sigma, P-6023) were crude lipids from bovine brain. The total diphosphoinositide and triposphoinositide content was 20 to 40%. The remainder was a mixture of phosphatidlyinositol and phosphatidylserine. Phospholipids were sonicated in 10 mM Hepes (pH 7.0) to form micelles. A total of 0.01 μCi of [^{3}H]PIP_2, corresponding to about 7000 cpm, and 5 μg of unlabeled mixed phospholipids was used per reaction. The PLC assay was done as previously described (*12*). Briefly, substrate, PLC-β2, peptide, and Gβγ subunits were mixed on ice in 100 μl of buffer containing 10 mM Hepes (pH 7.0), 1 mM DTT, 100 mM KCl, 10 mM NaCl, 2 mM EGTA, 1 mM EDTA, and 1 mM $MgCl_2$. Reactions were started by the addition of 25 μl of 5 mM $CaCl_2$ and incubated at 32°C for 15 min. Reactions were stopped by the addition of 1 ml of CMH (chloroform:methanol:H_2O mixed 100:100:1 by volume) and 250 μl of 10 mM EDTA. After extraction, aqueous phase (400 μl) was counted in a Beckman scintillation counter. All experiments were repeated at least three times with very similar results. Typical experiments are shown.
8. Recombinant PLC-β2 was expressed in Sf9 insect cells and purified and fluorescent studies were done as described (*13*). To label PLC-β2 with the amine-reactive probe Cascade Blue acetyl azide (Molecular Probes, Eugene OR), we raised the pH to 8.0 and

added a fourfold excess of probe from a freshly prepared concentrated dimethylformamide solution. The reaction was kept on ice for 30 min before extensive dialysis in 20 mM Hepes (pH 7.2), 0.16 M NaCl, 1 mM DTT, and 2 mM EGTA. Peptides were labeled with DABMI (4-dimethylaminophenylazophenyl-4′-maleimide), a nonfluorescent energy transfer acceptor, in the presence of an equimolar amount of dye in the absence of reducing agents. The reaction was allowed to proceed for 30 min at room temperature and quenched with 5 mM DTT. The final labeling ratios, as determined by absorption, were 1:1 for Cascade Blue–PLC-β2 and 0.8 for the two DABMI peptides. Fluorescence spectra were taken on an ISS-PC1 (ISS, Champaign, IL) photon-counting spectrofluorometer in a 3 mm by 3 mm cuvette with excitation at 380 nM and scanning from 400 to 560 nM. The FRET experiment was done under the same solution conditions as the PLC assay and the labeled peptide could still activate PLC. In fluorescent studies done in the presence of membranes, the lipid concentration was high enough so that all PLC was membrane-bound (*14*).

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Chlamydia Infections and Heart Disease Linked Through Antigenic Mimicry

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Chlamydia **infections are epidemiologically linked to human heart disease. A peptide from the murine heart muscle–specific α myosin heavy chain that has sequence homology to the 60-kilodalton cysteine-rich outer membrane proteins of *Chlamydia pneumoniae*, *C. psittaci*, and *C. trachomatis* was shown to induce autoimmune inflammatory heart disease in mice. Injection of the homologous *Chlamydia* peptides into mice also induced perivascular inflammation, fibrotic changes, and blood vessel occlusion in the heart, as well as triggering T and B cell reactivity to the homologous endogenous heart muscle–specific peptide. *Chlamydia* DNA functioned as an adjuvant in the triggering of peptide-induced inflammatory heart disease. Infection with *C. trachomatis* led to the production of autoantibodies to heart muscle–specific epitopes. Thus, *Chlamydia*-mediated heart disease is induced by antigenic mimicry of a heart muscle–specific protein.**

Cardiovascular diseases are the major cause of death in Western societies. Various risk factors have been associated with the pathogenesis of heart diseases, including increased cholesterol levels, smoking, stress, high blood pressure, obesity, and hyperglycemia (*1*). Bacterial infections may be a causative event in the development of heart diseases (*2*, *3*). *Chlamydia* infections cause pneumonia, conjunctivitis in children, and are a primary cause of sexually transmitted diseases and female infertility (*4*). The mechanism by which *Chlamydia* causes cardiovascular disease is unknown (*5*).

Inflammatory heart diseases and dilated cardiomyopathy in humans can be reproduced in mice by immunization with heart muscle myosin (*6*). Cardiac myosin–induced autoimmune myocarditis is dependent on $CD4^+$ T cells that recognize a heart muscle–specific peptide in association with self major histocompatibilty complex (MHC) class II molecules (*7*). Various peptides of the α myosin heavy chain protein have been identified that can induce autoimmune myocarditis in mice (*8*, *9*).

Immunization with a 30–amino acid peptide (amino acids 614 to 643) of the cardiac-specific α myosin heavy chain molecule [αmhc(614–643)] induces severe inflammatory heart disease in BALB/c mice (*8*). The first 16 amino acids [αmhc(614–629), SLKLMATLFSTYASAD] constituted a dominant autoaggressive epitope that was designated M7Aα (Table 1 and Fig. 1A) (*10*). In contrast, the homologous region of the β myosin heavy chain isoform, designated M7Aβ, did not induce disease (Table 1 and Fig. 1B). The introduction of single amino acid substitutions into M7Aα further revealed that the residues xxxMAxxxSTxxx (where x is any amino acid) were important for the pathogenicity of M7Aα in vivo (*11*). These immunogenic amino acids are conserved between murine and human α myosin heavy chains, and injection of the human M7Aα homolog into BALB/c mice also induced inflammatory heart disease (*11*).

After identification of the crucial pathogenic amino acids within the M7Aα peptide, we screened public databases for viral and bacterial sequences containing the MAxxxST motif (*12*). Peptide sequences from the 60-kD cysteine-rich outer membrane protein (CRP) from different serovars of *C. trachomatis* matched the M7Aα motif and were designated ChTR1 (serovar E), ChTR2 (serovar C), and ChTR3 (serovars L1, L2, and L3) (*13*).

Table 1. Sequence alignment of *Chlamydia* peptides, the immunogenic mouse M7Aα motif, and the nonimmunogenic mouse M7Aβ motif. Prevalence and severity of inflammatory heart disease as determined with these peptides are indicated. Six-week-old BALB/c mice were immunized twice at a 7-day interval with the indicated peptides (50 μg per mouse] in FCA and analyzed 21 days after the initial immunization for the presence and severity of myocarditis. Histological grading of severity was as follows: 0, no infiltration in heart muscle; 1, up to 5% of histological cross section is infiltrated; 2, 6 to 10%; 3, 11 to 20%; 4, >20%. Mean values of disease severity ± SD are indicated (*6*, *10*).

Peptide	Amino Acid Sequence	Prevalence	(%)	Severity
M7Aα (614-629):	SLKLMATLF STYASAD	18/21	(86%)	2.9 ± 0.7
ChTR1 (25-40):	VLETSMAEFTSTNVIS	12/15	(80%)	1.4 ± 0.4
ChTR2 (25-40):	VLETSMAESLSTNVIS	7/8	(88%)	1.3 ± 0.5
ChTR3 (25-40):	VLETSMAEFI STNVIS	7/8	(88%)	1.1 ± 0.6
ChPN (25-40):	GIEAAVAESLI TKIVA	6/10	(60%)	1.1 ± 0.2
ChPS (25-40):	KIEAAAAESLATRFIA	5/10	(50%)	1.0 ± 0.0
ChTR p11 (1-14):	MGSMAFHKSRLFLT	4/8	(50%)	1.0 ± 0.0
M7Aβ (614-629):	SLKL LS NLFANYASAD	0/19	(0%)	

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