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Role of Transmembrane Domain Interactions in the Assembly of Class II MHC Molecules

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Evidence is presented that suggests a role for transmembrane domain interactions in the assembly of class II major histocompatibility complex (MHC) molecules. Mutations in the transmembrane domains of the class II MHC α or β chains resulted in proteins that did not generate complexes recognized by conformation-dependent antibodies and that were largely retained in the endoplasmic reticulum. Insertion of the α and β transmembrane domains into other proteins allowed the chimeric proteins to assemble, suggesting a direct interaction of the α and β transmembrane domains. The interactions were mediated by a structural motif involving several glycine residues on the same face of a putative α helix.

Class II molecules of the MHC are membrane-bound glycoprotein complexes expressed primarily in cells of the immune system, where the class II molecules function to present antigenic peptides to T lymphocytes. Class II molecules are composed of two polymorphic transmembrane subunits, α and β, that associate to form a noncovalent heterodimer (*1*). Both chains are type I integral membrane proteins, each consisting of two NH_2-terminal extracellular domains, a single membrane-spanning sequence, and a short COOH-terminal cytoplasmic tail (*1*). Assembly of the heterodimer occurs in the endoplasmic reticulum (ER), where the heterodimer associates with a type II integral membrane protein known as the invariant (Ii) chain (*2*, *3*). After assembly, αβIi complexes are transported through the Golgi system and into a prelysosomal compartment, where the Ii chain is proteolytically degraded (*2*, *3*). At this location, the αβ dimers bind antigenic peptides; the dimers are then delivered to the cell surface (*3*). The high degree of conservation of the transmembrane domains of class II MHC α and β gene products among various haplotypes and animal species (Fig. 1A) has suggested an essential role for these sequences (*1*, *4*). Because of the known function of transmembrane interactions in the assembly of other multiprotein complexes, such as in the assembly of the T cell antigen receptor (*5*, *6*) and Fc receptors (*7*), we decided to examine whether the membrane-spanning sequences were required for efficient assembly of newly synthesized class II MHC molecules.

Normal or mutagenized α and β chains of the I-A^k haplotype (*8*) were expressed in COS-1 cells (*9*). Transfected cells were metabolically labeled for 15 min and class II chains immunoprecipitated (*9*) with conformation-independent or -dependent antibodies (Abs) (*10*) (Fig. 2). Coexpression of α and β chains resulted in coprecipitation of both chains by conformation-independent Abs to the β or α chains (Fig. 2A) or by conformation-dependent Abs that require correct pairing of α and β subunits for reactivity (Fig. 2B). Insertion of a Bgl II site in the α and β cDNAs near the boundary between sequences encoding the extracellular and transmembrane domains resulted in replacement of three codons in both α and β (*8*) but had no effect on association (Fig. 2). We used this restriction site in order to replace the transmembrane domains of α and β by the transmembrane domain of the α chain of the interleukin-2 receptor (the Tac antigen) (*11*). The resulting αTα and βTβ chimeras associated, as assessed by coprecipitation with conformation-independent Abs (Fig. 2A). However, conformational epitopes found in normal class II MHC complexes were not generated (Fig. 2B), suggesting that the transmembrane domains of the α and β chains are required for correct assembly of αβ heterodimers. The nature of the chimeric protein species coprecipitated with conformation-independent but not conformation-dependent Abs is unclear. One possibility is that they represent complexes that are improperly assembled or that have an abnormal conformation. An alternative explanation is that the apparent association of the chimeric proteins does not reflect the formation of specific αβ dimers but rather the aggregation of unassembled α and β species with each other or with other intracellular proteins.

The subcellular localization of the normal and chimeric protein species was analyzed by immunofluorescence microscopy with a conformation-independent Ab to the β chain. These studies revealed that

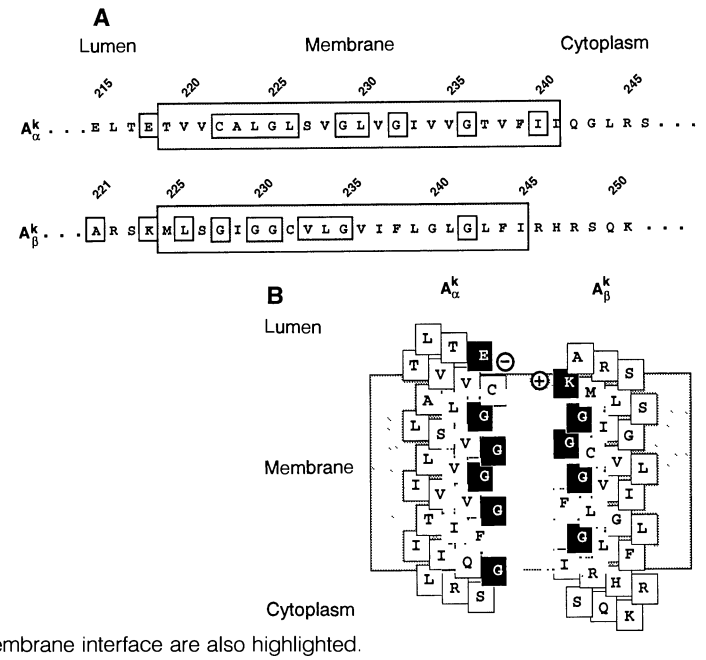

Fig. 1. (**A**) Sequences of transmembrane domains and adjacent regions of class II MHC chains of the I-A^k haplotype (*22*). Open boxes denote residues that are identical in >95% of all class II MHC chains examined [56 α and 124 β chain sequences (*24*)]. Amino acids are numbered from the initiator methionine of the normal A^k_α (*20*) and A^k_β (*21*) precursors. (**B**) Two-dimensional representations of I-A^k transmembrane domains, assuming an α-helical configuration (*13*). Conserved Gly residues on one face of the potential α helices are indicated by black boxes. Residues with charged side chains near the lumen-membrane interface are also highlighted.

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whereas a substantial amount of normal α-β complexes were localized to the cell surface (Fig. 3, A and C), species made of αTα and βTβ showed a reticular staining pattern characteristic of the ER (Fig. 3B) and were barely detectable at the cell surface (Fig. 3D). In addition, processing of newly synthesized chains into endoglycosidase H–resistant species was significantly reduced for the chimeric protein complexes (*12*). Thus, the inability to generate a population of correctly assembled complexes correlates with decreased transport out of the ER and to the cell surface.

An unusual feature of the class II α and β transmembrane domains is the high content and conserved position of Gly residues (Fig. 1A). If an α-helical structure for the transmembrane domains of these proteins is assumed (*13*), then five Gly residues in A^k_α and four in A^k_β would be aligned on the same face of the helix (Fig. 1B). To test whether these Gly residues were involved in mediating class II MHC assembly, we replaced Gly residues by Val residues in both α and β domains, and the assembly characteristics of the mutant proteins were analyzed (Fig. 2). Mutation of the Gly residues in normal α and β chains had the same effect as replacing the entire transmembrane domain; the mutant chains seemed able to associate (Fig. 2A) but did not bind conformation-dependent Abs (Fig. 2B). The mutant proteins were also localized to the ER by immunofluorescence microscopy analysis (*14*).

The Gly residues in the transmembrane domains could be required for proper folding of the individual subunits or for correct assembly of the complex. To test whether the α and β transmembrane domains were capable of interacting with each other, we constructed a chimeric protein in which the transmembrane domain of the Tac antigen was replaced by that of the class II α chain. Coexpression of this TαT chimera with the class II β chain resulted in assembly that was dependent on the presence of Gly residues in the α transmembrane sequence (Fig. 4A). In contrast, TβT chimeras, like the Tac antigen, showed little coprecipitation with the normal β chain (Fig. 4A). Thus, the transmembrane domain of the α chain can mediate specific interaction with the β chain even in the context of an unrelated protein.

Structural features of the α and β transmembrane domains involved in mediating interactions were further delineated with a quantitative coprecipitation assay (6). In this assay, Tac chimeras containing normal or mutated forms of the class II α or β transmembrane domains were coexpressed with CD3δ (*15*) chimeras containing the class II α or β transmembrane domains and *Escherichia coli* β-galactosidase fused at the end of the cytoplasmic tail. After immunoprecipitation with Abs to the Tac antigen, we quantitated assembly by measuring β-galactosidase activity in the immunoprecipi-

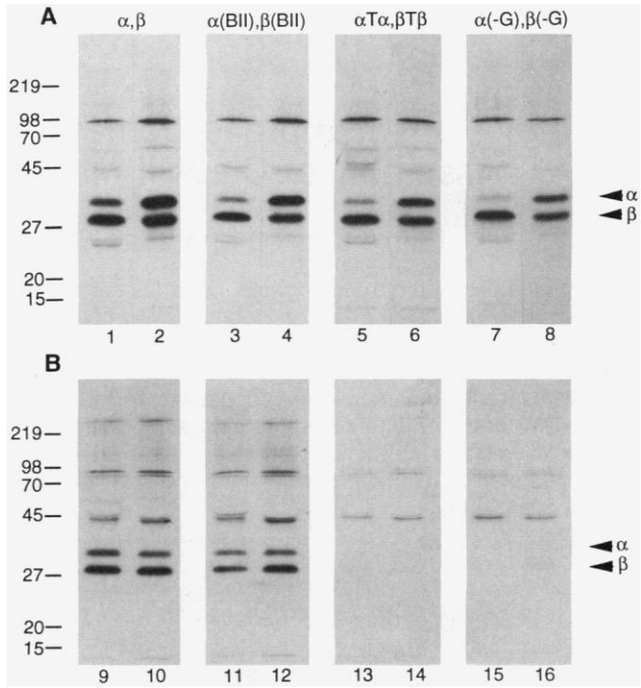

Fig. 2. Assembly of normal and mutant class II MHC chains. COS-1 cells cotransfected with plasmids encoding normal and mutant forms of class II α and β chains (*8*), as indicated in the figure, were pulse-labeled for 15 min with [^{35}S]methionine (*9*). Cells were then lysed, and labeled proteins were isolated by immunoprecipitation with (**A**) conformation-independent Abs 10-2.16 (anti-β, lanes 1, 3, 5, and 7) or FF282-4 (anti-α, lanes 2, 4, 6, and 8) or with (**B**) conformation-dependent Abs 11-5.2 (anti-α, lanes 9, 11, 13, and 15) or 40B (anti-αβ, lanes 10, 12, 14, and 16). Immunoprecipitates were analyzed by SDS-PAGE. Gels shown in (B) were exposed ten times longer than those shown in (A), as immunoprecipitation with conformation-dependent Abs resulted in weaker signals. Coexpression of the mouse Ii chain did not increase the amount of labeled complexes recovered with conformation-dependent Abs (*14*). Molecular size markers are shown at left in kilodaltons; α and β are indicated at right.

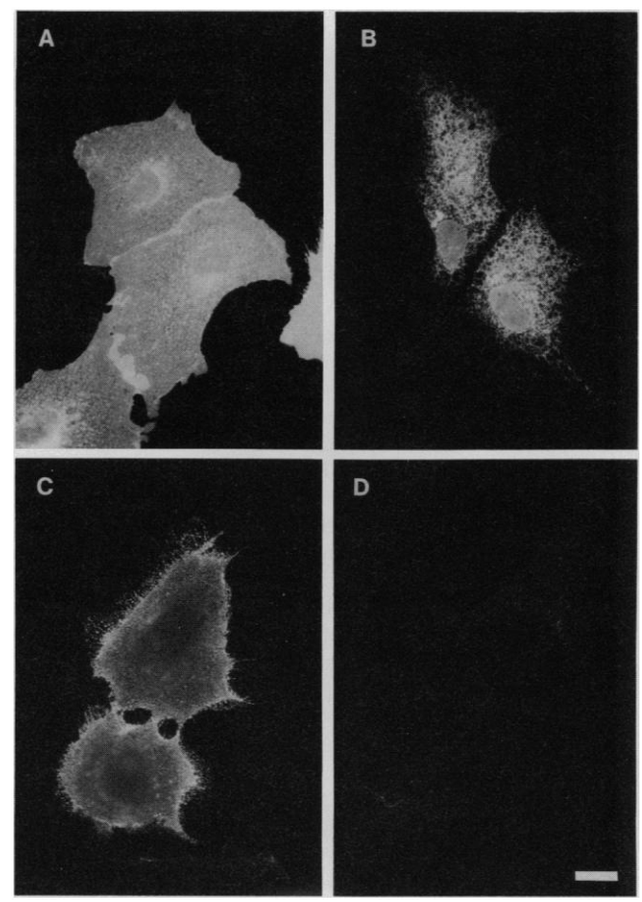

Fig. 3. Immunofluorescence microscopy localization of normal and chimeric class II MHC molecules (*25*). CV-1 cells were cotransfected with plasmids encoding either normal α and β chains (**A** and **C**) or chimeric αTα and βTβ chains (**B** and **D**). At 48 hours after transfection, cells were either fixed and permeabilized (A and B) or left intact (C and D) before incubation with the conformation-independent antibody 10-2.16. Ab binding was revealed with rhodamine-conjugated goat Abs to mouse immunoglobulins. Identical exposure times were used for photographs in (C) and (D). Bar, 5 μm.

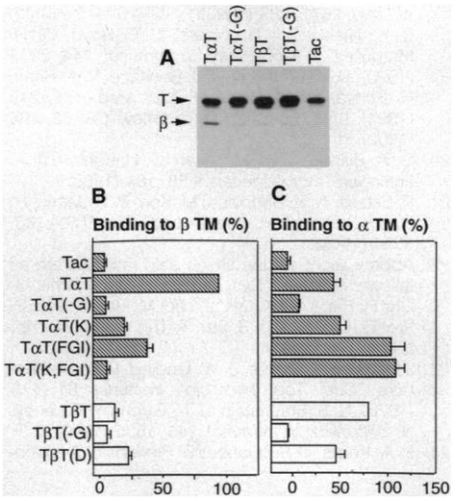

Fig. 4. Interaction between the transmembrane domains of class II MHC α and β chains. (**A**) COS-1 cells were cotransfected with DNAs encoding the normal class II β chain and mutants of the Tac antigen (*8*). Cells were pulse-labeled for 15 min with [^{35}S]methionine (*9*), and proteins were isolated by immunoprecipitation with a MAb to Tac (7G7). Immunoprecipitates were analyzed by SDS-PAGE. The positions corresponding to the different Tac species and class II β chains are indicated. (**B**) and (**C**) Delineation of residues involved in class II α-β transmembrane interactions. COS-1 cells were cotransfected with DNAs encoding mutants of the Tac antigen (*8*) and the chimeric proteins δ–β-Gal (B) or δ–α-Gal (C). At 48 hours after transfection, cells were lysed, and galactosidase activity was determined in whole cell lysates and immunoprecipitates with antibody to Tac, as described (*6*). The proportion of total galactosidase activity coprecipitated with the different Tac mutants is expressed as a percentage of a control determination included in every experiment. The control (arbitrarily defined as 100%) consisted of TαT association with δ–β-Gal in (B) and association of TβT with δ–α-Gal in (C). Results are the mean ± SD of values obtained in at least three independent experiments. TM, transmembrane.

tates. This assay confirmed that the α and β transmembrane domains are capable of associating with each other even when placed in the context of other integral membrane proteins (Fig. 4, B and C). Interactions detected with this assay were also abolished by the mutation of Gly into Val residues in either the α or β transmembrane sequences (Fig. 4, B and C).

The observed interactions tended to favor hetero- over homo-associations (Fig. 4, B and C). This selectivity would be difficult to explain if only Gly residues were involved in interactions. Additional elements were noted that might contribute to the observed pattern of association. First, there is a negatively charged residue in α (Glu^{218}) and a positively charged residue in β (Lys^{224}) near the boundary between the lumenal and transmembrane domains (Fig. 1B). Second, residues with bulky hydrophobic side chains potentially face Gly residues in the lower part of both the α and β transmembrane helices (Fig. 1B). Insertion of three codons that changed the interfacial Glu^{218} in α to Lys resulted in a marked decrease in binding to the β transmembrane domain (Fig. 4B). Conversely, a deletion of three codons that changed Lys^{224} in β into Asp reduced binding to the α transmembrane domain (Fig. 4C) (*16*). Changing the alternating Gly and bulky hydrophobic residues in the α transmembrane sequence into the configuration found in β (Gly^{236} → Phe, Phe^{239} → Gly, and Gly^{243} → Ile) also reduced the magnitude of interaction with β (Fig. 4B) and increased interaction with α (Fig. 4C).

Although the mutagenesis analysis performed is not exhaustive, the results described above are consistent with a direct interaction involving Gly-rich faces of the putative α helices (*17*). The mutations shown to affect assembly involve transmembrane residues with either no side chains (Gly) or nonpolar side chains (Phe and Ile). Thus, the nature of the interaction between the α and β transmembrane domains is probably distinct from other interactions shown to involve potentially charged transmembrane residues (*5–7*). Molecular packing of amino acid side chains away from the lipid environment and polar interactions between the polypeptide backbones could provide the driving forces for this type of association. In addition, electrostatic interactions between interfacial charged residues would also contribute to the attraction of the transmembrane helices. According to this model, specificity would arise from the spatial configuration of some of the Gly residues and complementing side chains on the interacting surfaces, as well as from charged residues at the lumen-membrane interface.

The subtle nature of this putative interaction motif raises the prospect that other proteins may be similarly engaged in transmembrane associations. Glycophorin A is another protein for which there is evidence for a role of transmembrane domain interactions in dimerization (*18*). The glycophorin A transmembrane sequence contains four Gly residues (*18*), some of which may be implicated in α-helical pairing. The observed perturbation of class II MHC biogenesis by substitution of nonpolar transmembrane residues emphasizes the importance of this type of interaction in the assembly of multiprotein complexes in vivo. Whereas the lumenal domains of class II MHC subunits have an intrinsic ability to associate (*19*), the role of transmembrane domain interactions would be to promote the formation of correctly assembled complexes in the ER, thereby increasing the expression of functional molecules.

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8. Chimeric and mutant proteins were derived from the mouse $I\text{-}A^k_\alpha$ (*20*) and $I\text{-}A^k_\beta$ (*21*) chains. In α(BII) and β(BII), a Bgl II site was inserted on the lumenal side of the transmembrane domain with polymerase chain reaction (PCR) mutagenesis [R. Higuchi, B. Krummel, R. K. Saiki, *Nucleic Acids Res.* **16**, 7351 (1988)]. In αTα and βTβ, we replaced the transmembrane domains of the α and β chains by that of the Tac (T) antigen (*11*) by subcloning double-stranded oligonucleotides at the Bgl II site (chimeric proteins are denoted by a three-letter name, in which each letter represents the lumenal, transmembrane, and cytoplasmic domains in order). We obtained mutants of the Tac antigen in a similar manner by introducing a Bgl II site on the lumenal side of the Tac transmembrane domain. In TβT and TαT, the transmembrane domain of the Tac antigen was replaced by those of A^k_β and A^k_α, respectively. Various mutations of TαT and TβT were made by PCR mutagenesis. TαT(−G) and TβT(−G) correspond to mutants of TαT and TβT, respectively, in which the transmembrane Gly residues highlighted in Fig. 1B were replaced by Val residues. In TαT(K) and TβT(D), the charged residues at the lumen-membrane interface were changed from Asp to Lys and from Lys to Asp, respectively. In TαT(FGI), the alternating Gly and bulky hydrophobic residues in the lower part of the putative-interacting face (Gly, Phe, and Gly) (Fig. 1B) were changed into residues found in the analogous region of the β transmembrane domain (Phe, Gly, and Ile). Sequences of the transmembrane domains and adjacent regions of the various normal and mutant proteins are as follows: α, ELTETVVCALGLSVGLVGIVVGTVFIIQGLRS; α(BII), ELTDLTVCALGLSVGLVGIVVGTVFIIQGLRS; α(−G), ELTDLTVCALVLSVVLVVIVVVTVFIIQVLRS; αTα, ELTDLQVAVAACVFLLIAVLLLSGLTWQRS; β, QSESARSKMLSGIGGCVLGVIFLGLGLFIRH; β(BII), QSDLSRSKMLSGIGGCVLGVIFLGLGLFIRH; β(−G), QSDLSRSKMLSVIGVCVLVVIFLGLVLFIRH; βTβ, QSDLQVAVAACVFLLIAVLLLSGLTWQRH; Tac, FTTDLQVAVAACVFLLIAVLLLSGLTWQRRQ; TαT, FTTDLTVCALGLSVGLVGIVVGTVFIIQGLRRQ; TαT(−G), FTTDLTVCALVLSVVLVVIVVVTVFIIQVLRRQ; TαT- (K), FTTDLTRSKCALGLSVGLVGIVVGTVFIIQGLRRQ; TαT(FGI), FTTDLTVCALGLSVGLVGIVVFTVGIIQILRRQ; TαT(K,FGI), FTTDLTRSKCALGLSVGLVGIVVFTVGIIQILRRQ; TβT, FTTDLSRSKMLSGIGGCVLGVIFLGLGLFIRRQ; TβT(−G), FTTDLSRSKMLSVIGVCVLVVIFLGLVLFIRRQ; and TβT(D), FTTDLSVMLSGIGGCVLGVIFLGLGLFIRRQ (*22*). Mutated residues are underlined. All constructs were cloned into the mammalian expression vector pCDM8 [B. Seed, *Nature* **329**, 840 (1987)].

9. Transfection of COS-1 or CV-1 cells (American Type Culture Collection) was done by calcium phosphate precipitation [F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973)]. At 48 hours after transfection, cells were metabolically labeled for 15 min at 37°C with [^{35}S]methionine (0.5 mCi/ml) (*5*). Labeled cells were removed from plates, solubilized in lysis buffer [0.5% (w/v) Triton X-100, 0.3 M NaCl, and 50 mM tris-HCl at pH 7.4], and centrifuged for 15 min at high speed. Supernatants were added to Abs bound to protein A–Sepharose beads. After incubation for 1 hour at 4°C, immunoprecipitates were washed five times with wash buffer [0.1% (w/v) Triton X-100, 0.3 M NaCl, and 50 mM tris-HCl at pH 7.4] and one time with phosphate-buffered saline (PBS). Samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions on 13% acrylamide gels.
10. Immunoprecipitations were done with the following Abs: 10-2.16, a mouse monoclonal antibody (MAb) that binds both unassembled and assembled forms of A^k_β (*23*); FF282-4, a rabbit antiserum that reacts with the cytoplasmic tail of A^k_α, independent of assembly [A. J. Sant, L. R. Hendrix, J. E. Coligan, W. L. Maloy, R. N. Germain, *J. Exp. Med.* **174**, 799 (1991)]; 11-5.2, a mouse MAb to A^k_α associated with certain A^k_β haplotypes (*23*); and 40B, a mouse MAb that recognizes certain assembled $A_\alpha A_\beta$ heterodimers [M. Pierres, F. M. Kourilsky, J. P. Rebouah, M. Dossetto, D. Caillol, *Eur. J. Immunol.* **10**, 950 (1980)]. For simplicity and on the basis of their pattern of reactivity, Abs 10-2.16 and FF282-4 are referred to in the text as conformation independent, and 11-5.2 and 40B are referred to as conformation dependent. However, the actual nature of the epitopes recognized by these Abs is not known. Tac protein constructs were immunoprecipitated with 7G7, a mouse MAb to an extracellular epitope of the human Tac antigen [L. A. Rubin, C. C. Kurman, W. E. Biddison, N. D. Goldman, D. L. Nelson, *Hybridoma* **4**, 91 (1985)].
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12. Processing of newly synthesized αβ and αTα-βTβ complexes in CV-1 cells was analyzed by pulse-chase metabolic labeling and immunoprecipitation with the MAb to A^k_β (10-2.16). After 4 hours of chase, the amounts of α and αTα having one endoglycosidase H–resistant, *N*-linked oligosaccharide chain were 51 and 13%, respectively.
13. Most hydrophobic transmembrane sequences are thought to adopt an α-helical structure in lipid bilayers [D. M. Engelman, T. A. Steitz, A. Goldman, *Annu. Rev. Biophys. Biophys. Chem.* **15**, 321 (1986); S. J. Singer, *Annu. Rev. Cell Biol.* **6**, 247 (1990)]. Despite the demonstrated helix-breaking potential of Gly residues in water-soluble peptides [K. T. O'Neil and W. F. DeGrado, *Science* **250**, 646 (1990)], the presence of several Gly residues in transmembrane domains has been shown to be compatible with α-helical folding. For instance, transmembrane domains A and B of bacteriorhodopsin and ME of the *Rhodobacter sphaeroides* photosynthetic reaction center have four to five Gly residues and are known to be α helices by physical methods [R. Henderson *et al.*, *J. Mol. Biol.* **213**, 899 (1990); D. C. Rees, H. Komiya, T. O. Yeates, J. P. Allen, G. Feher, *Annu. Rev. Biochem.* **58**, 607 (1989)].
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16. Similar results were obtained by point mutation of Arg^{222} and Lys^{224} in β to Glu and Asp, respectively.
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22. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trip; and Y, Tyr.
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25. Fixed or permeabilized cells were studied by immunofluorescence microscopy as described [J. S. Bonifacino, P. Cosson, N. Shah, R. D. Klausner, *EMBO J.* **10**, 2783 (1991)]. Nonpermeabilized cells were incubated for 1 hour at 4°C with culture medium containing MAb 10-2.16, followed by a 1-hour incubation at 4°C with a rhodamine-conjugated Ab to mouse immunoglobulins (1:200 dilution). Cells were then fixed with 2% formaldehyde and prepared for fluorescence microscopy.
26. We thank R. Klausner, R. Germain, D. Engelman, M. Lemmon, G. Storz, J. Humphrey, and M. Toledano for discussions and comments on the manuscript, and R. Germain, J. Salamero, and E. Long for gifts of reagents. Supported by a fellowship from the European Molecular Biology Organization (to P.C.).

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Phase Coupling by Synaptic Spread in Chains of Coupled Neuronal Oscillators

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Many neural systems behave as arrays of coupled oscillators, with characteristic phase coupling. For example, the rhythmic activation patterns giving rise to swimming in fish are characterized by a rostral-to-caudal phase delay in ventral root activity that is independent of the cycle duration. This produces a traveling wave of curvature along the body of the animal with a wavelength approximately equal to the body length. Here a simple mechanism for phase coupling in chains of equally activated oscillators is postulated: the synapses between the cells making up a "unit oscillator" are simply repeated in neighboring segments, with a reduced synaptic strength. If such coupling is asymmetric in the rostral and caudal directions, traveling waves of activity are produced. The intersegmental phase lag that develops is independent of the coupling strength over at least a tenfold range. Furthermore, for the unit oscillator believed to underlie central pattern generation in the lamprey spinal cord, such coupling can result in a phase lag that is independent of frequency.

An important feature of the neural activity that gives rise to locomotion in the lamprey is a rostral-to-caudal intersegmental delay that scales with the cycle duration, remaining equal to approximately 1% of the cycle per spinal cord segment at all swimming frequencies (*1*) (Fig. 1, a and b). Synaptic or conduction delays do not scale in this way, so the intersegmental timing must result from more complex interactions in the neural system. Although the mathematical analysis of the central pattern generator as a chain of coupled nonlinear oscillators (*2*) (Fig. 1c) has successfully predicted some unexpected behavior of the in vitro preparation of the lamprey (*3*), it gives no indication of what sort of neuronal interactions could mediate the coupling. It has been postulated (*4*) that the intersegmental phase lag in the lamprey is due to increased excitability of the most rostral segment, but experimental evidence indicates no such excitation in the lamprey (*5*). Buchanan (*6*) has shown by computer simulation that synaptic contacts between simulated unit oscillators can give rise to appropriate phase lags. In this report I suggest a form of intersegmental coupling with a simple, biologically plausible developmental rule: whatever synaptic contacts each neuron makes in its own segment, it must also make in neighboring segments but with a smaller synaptic strength. I will show by computer simulation that, with such "synaptic spread" between equally activated oscillators, a constant intersegmental phase lag can result as long as the connections between oscillators are asymmetric.

The detailed structure of the segmental oscillators in the lamprey is not known. However, a small network model (Fig. 1d) consisting of identified neurons that are rhythmically active during "fictive locomotion" (*7*) (neural activity with the same patterns as those recorded during locomotion) has produced oscillations with phase relations among the three cell types that are

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