

# Role of transmembrane domains in the functions of B- and T-cell receptors

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## Abstract

The antigen receptors on the surface of B- and T-lymphocytes are complexes of several integral membrane proteins, essential for their proper expression and function. Recent studies demonstrated that transmembrane (TM) domains of the components of these receptors play a critical role in their association and function. It was specifically demonstrated that in many cases point mutations in the TM domains can partially or completely disrupt the receptor surface expression and function. Here we review studies of the TM domains of B- and T-cell receptors. Furthermore, we use a novel method, PHDtopology, to provide estimates of the exact locations and lengths of the TM domains of the subunit components of these receptors. Most previous studies used single residue hydrophobicity as a criterion for determining the position and length of the TM domains. In contrast, PHDtopology utilizes a system of neural networks and the evolutionary information contained in multiple alignments of related sequences to predict the location, length, and orientation of transmembrane helices. Present results significantly differ from most published estimates of the TM domains of the B- and T-cell receptor components, primarily in the length of the TM domains. These results may lead to modification of putative TM motifs and re-interpretation of the results of studies using mutated TM domains. The availability of PHDtopology on the Internet would make it a valuable tool in the future studies of the TM domains of integral membrane proteins. © 1998 Elsevier Science B.V. All rights reserved.

**Keywords:** Transmembrane domains; B- and T-cell receptors

## 1. Introduction

Most membrane proteins exist as part of multicomponent complexes [1]. One obvious advantage of forming complexes is the additional flexibility in regulating the cellular responses by modulating the association/dissociation parameters of the complexes, as well as the use of a few relatively uniform ‘adaptor’ proteins for transduction of the signals initiated by structurally variable external agents. Such a multicomponent receptor

design has an evolutionary advantage by allowing formation of new signal transduction systems using already available cellular components [2,3].

The family of antigen receptors named multichain immune recognition receptors (MIRR) [4] shares common structural and functional features. Members of this family include the B-cell receptor (BCR) complex, the T-cell receptor (TCR) complex, type 1 receptor for IgE, (FcεRI), and the high and low affinity receptors for IgG, FcγRI (CD64) and FcγRIII (CD16). It has been noted that MIRR also employ similar transmembrane (TM) signal transduction mechanisms [5]. Thus, both TCR and BCR associate with members of the src family of protein tyrosine kinases, which are activated by antigen-receptor interaction coupling it to down-

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stream signal transduction processes [5–9]. Indeed, with the exception of the TCR  $\zeta$  chain, general architecture of the BCR and TCR complexes is similar [10]. Each antigen-binding subunit of both receptors is non-covalently associated with two identical heterodimers (Ig- $\alpha$ - $\beta$ )<sub>2</sub> or (Ig- $\alpha$ - $\gamma$ )<sub>2</sub> in the BCR, and (CD3 $\epsilon$ - $\gamma$ )<sub>2</sub> or (CD3 $\epsilon$ - $\delta$ )<sub>2</sub> in the TCR [10]. Furthermore, a high structural homology is found among Ig- $\alpha$ , Ig- $\beta$  and Ig- $\gamma$ , and CD3 $\gamma$ , CD3 $\delta$  and CD3 $\epsilon$  [10].

It has been assumed that antigen-binding (TCR- $\alpha$ / $\beta$ , mIg) subunits have only very short (three to five amino acids) cytoplasmic tails, and it has been therefore postulated that it is the complex of these subunits with the associated molecules that acts as signal transducer. In contrast, the associated subunits contain long (48–180) amino acids cytoplasmic domains, which were shown to bind to the members of the src family of the tyrosine kinases initiating the signal transduction [11–14]. Consequently, understanding in detail the structural basis of association among the components of MIRR is a subject currently attracting considerable interest. The emerging consensus in the literature is that the critical role in MIRR association is played by the TM domains of the subunit components. The identification of the exact boundaries and determination of the length of the TM domains of these proteins are therefore required to study the functions of these domains. Nevertheless, most of the studies of the role of the TM domains in the function of MIRR relied for these parameters on earlier works, when amino acid sequences of the corresponding proteins were first published and the TM domains were identified usually by visual examination of the sequences or using a hydropathy scale, primarily that of Kyte and Doolittle [15]. More recent studies, however, have found the presence of topogenic signals-sequence patterns which correlate with the topology of the membrane-spanning segments [16–19], allowing significant improvement in predicting TM domains. One of the most accurate prediction methods currently available is PHDtopology, a system of neural networks using the evolutionary information contained in multiple sequence alignments to predict the location and orientation of transmembrane helices. The advantages of the method are: (i) a sustained, high level of overall prediction accuracy [20]; (ii) a thoroughly evaluated distinction between more or less reliably predicted segments [21]; and (iii) the ease of access via the Internet (via PredictProtein, [22]). In the following sections we will review the recent studies on the role of the TM domains of BCR, TCR and associated proteins in the function of these receptors. We will also present the results of determination of the location and length of these domains using the neural network protein prediction method.

## 2. Neural Network Protein Prediction Method (PHDtopology)

Most early prediction methods [23] have been based on hydrophobicity analyses of single sequences. Despite relatively high levels of accuracy, most methods have major problems: cores of TM helices are predicted better than their ends, and therefore TM helices are predicted as too long [20,23]. The breakthrough in structure prediction of the 1990s has originated from the use of evolutionary information [24]. Feeding multiple alignments into a system of neural networks (PHDhtm and PHDtopology; [20,21]) improves prediction accuracy by about ten percentage points (95% of residues predicted correctly). Furthermore, PHDtopology: (i) optimizes the accuracy in predicting segment ends; (ii) predicts TM helices at an average length similar to biochemically determined TM regions; (iii) accurately predicts the correct number, and locations of TM segments (for about 90% of the test proteins all TM regions are correctly predicted, compared to a level of <80% for advanced hydrophobicity-based prediction methods [19]); and (iv) prediction strength correlates extremely well with expected accuracy. The question arises whether the alignment-based predictions are sufficiently accurate. In fact, helix placement and topology of the cytochrome C oxidase [25] had been predicted more accurately by PHDtopology (prior to the 3D structure determination) than by experiments that were annotated in the Swiss-Protein sequence database [26] demonstrating that PHDtopology is clearly more valid as a basis for expert-based predictions than are single sequence-based methods of the 1980s [27].

## 3. TCR complex

The structural model of the TCR currently consists of four dimeric components: (i) the TCR- $\alpha\beta$  or - $\gamma\delta$  clonotypic chains (Ti) which are covalently linked via an extracellular disulfide bond and confer antigen specificity; (ii) the CD3- $\gamma\epsilon$  non-covalent dimer; (iii) the CD3- $\delta\epsilon$  non-covalent dimer; and (iv) a disulfide-linked homo- or heterodimer that is composed of any of the three chains:  $\zeta$ ,  $\eta$ , and the  $\gamma$  chain of the mast cell Fc $\epsilon$ RI [28,29]. The  $\gamma$ ,  $\delta$  and  $\epsilon$  subunits, expressed as non-covalently associated  $\gamma\epsilon$  and  $\delta\epsilon$  pairs [30–32] are related to the immunoglobulin superfamily [33], whereas the  $\zeta$ ,  $\eta$  subunits and the  $\gamma$  chain of Fc $\epsilon$ RI are members of a different protein family [34–36]. The TCR has to be completely assembled in the endoplasmic reticulum (ER) for efficient transport to and expression at the cell surface. Partially assembled complexes and unassembled subunits undergo degradation within the ER or in lysosomes [37].

		out	transmembrane domain	in
mouse	TcR- $\alpha$	TEKSFETDMNLFNLFQNL	SVMLRILLKLVAGFNLLMTLRLWSS	
human	TcR- $\alpha$	V-----T-----	I-F-----	
human	TcR- $\alpha^a$	V-----T-----	I-FL----I-----	
human	TcR- $\alpha^b$	V-----T-----	I-FG----I-----	
mouse	TcR- $\delta$	GPRVTVHTEKVNMSLTVLGLRLLFAKTIAINFLLTVKLVFF		
human	TcR- $\delta$	K-KAI-----	M-----V-V-----A-----L	
human	TcR- $\delta^c$	K-KAI-----	LM---I-V-V-----A-----L	

Fig. 1. Sequences of the C-terminal regions of human and mouse TCR- $\alpha$  and TCR- $\delta$ . Light shade: TM domains according to [52]. Dark shade: TM domains suggested by PHDtopology. The sequences used and their database accession numbers are given below. Mouse TCR- $\alpha$ : [53,54]; Swiss Protein # P01849, EMBL # X01133. Human TCR- $\alpha$ : [55]; PIR # S18893, EMBL # X63455. Mouse TCR- $\delta$ : [56]; EMBL # M37694. Human TCR- $\delta$ : [57]; PIR # S03421. <sup>a</sup> Mutated R/L, K/I in the TM domain, [41]. <sup>b</sup> Mutated R/G, K/I in the TM domain, [42]. <sup>c</sup> Mutated R/L, K/I in the TM domain, (computer mutation).

An unusual feature of the sequences of all Ti and CD3 chains is the conservation of charged residues in their transmembrane domains with basic residues present in the TM domains of Ti chains, and acidic residues in the TM domains of associated CD3 chains (Figs. 1–3). It has been proposed that these oppositely charged residues are responsible for the CD3-Ti association [28,34,38–40]. Subsequent studies confirmed that the assembly of TCR- $\alpha$  with CD3- $\delta$  or CD3- $\epsilon$ , or of TCR- $\beta$  with CD3- $\gamma$  and CD3- $\delta$ , is mediated by TM interactions involving charged amino acid residues [41–45], whereas assembly of TCR- $\beta$  with CD3- $\epsilon$  occurs at the level of their extracellular domains [44]. Manolios et al. [44], using chimeric proteins and point mutations, located the association of the TCR- $\alpha$  chain with CD3- $\delta$  and CD3- $\epsilon$  at an eight-amino acid motif within the TM domain of TCR- $\alpha$ . Mutations of the charged residues within this motif to leucine and similar point mutations of the TM CD3- $\epsilon$  and CD3- $\delta$  charged groups resulted in the abolition of assembly [44]. Three-leucine sequence in the TM domain of human TCR- $\alpha$  was apparently also required; deletion of a single leucine resulted in disruption of the interaction of this chain with CD3- $\delta\epsilon$  complexes.

The presence of the charged residues in the TM domains of the TCR components is critical for the association of TCR- $\alpha$  or TCR- $\beta$  with CD3, but not for the association of CD3 chains with each other. Proper assembly and surface expression of the TCR were prevented by mutations that changed the charge of both basic residues in the TCR- $\alpha$  or the single basic residue in the TCR- $\beta$  TM domain [41,46] without affecting the intracellular formation of disulfide-linked TCR- $\alpha\beta$  dimers [42,43,46–48]. Similarly, the changes of the charged residues of the CD3 TM domains abolished CD3-Ti association [43,48,49], but not the formation of CD3- $\gamma\epsilon$  and CD3- $\delta\epsilon$  dimers [43,44,50]. Thus, site-specific mutations of the negatively-charged TM amino acid residues of the CD3 chains to alanines abolished (for TCR- $\alpha$ -CD3- $\delta$  and TCR- $\beta$ -CD3- $\epsilon$ ) or diminished (for TCR- $\alpha$ -CD3 $\gamma$ ) the TCR-CD3 interactions [43]. The mutations had no effect on CD3-CD3 interactions

or upon synthesis, metabolism, or intracellular distributions of the CD3 components. Conversely, new subunit interactions can be induced by changing the charge of the TM residues. Manolios et al. [50] reported that the replacement of negatively charged TM residues in both CD3- $\gamma$  and CD3- $\delta$  with leucine allows for formation of stable  $\gamma\delta$  dimers not observed otherwise.

Cosson et al. [49] constructed a chimeric protein of the Tac antigen with its TM domain replaced by the corresponding domain of TCR- $\alpha$ . They found that the presence of the two basic residues in TCR- $\alpha$  and a single acidic residue in the CD3- $\delta$  TM domains are necessary for interactions between these subunits. Replacement of these two basic residues with uncharged ones abolished the interaction whereas replacement of other residues within the TM domain was not effective. Moreover, replacement of both basic residues of TCR- $\alpha$  with a single positively charged arginine restored the association; the optimal interaction was observed when the arginine residue was placed in the same position of depth in the bilayer as that of the corresponding negatively charged aspartic acid in the CD3- $\delta$  TM domain [49]. The authors suggested that the association could be due either to an ion-pair or a hydrogen bond formation.

Another function apparently determined by the charged residues of the TM domain is the retention and degradation of the corresponding protein in the ER. Indeed, charged amino acid residues of TCR- $\alpha$  and - $\beta$  TM domains are required for both assembly and surface expression of complete TCR complexes [41,42,46,47,51]. Bonifacino et al. [51] suggested that the same TM sequences contain information for assembly and for retention/degradation of the receptor components. Therefore association with another TM domain results in masking of the determinant for retention/degradation and allows for the surface expression of the protein. Bonifacino et al. [51] introduced the term ‘determinant for retention and degradation in the ER’ to denote short TM sequences responsible for these biological functions.

		out	transmembrane domain	in
mouse	TcR- $\beta$	ASYQQGVLSATILYEILLGKATLYAVLVSTLVVMAMVKRKNS		
human	TcR- $\beta$	V-----	-----A-L-----	DF
human	TcR- $\beta^a$	V-----	-----E-----A-L-----	DF
human	TcR- $\beta^a$	V-----	-----S-----A-L-----	DF
human	TcR- $\beta^a$	V-----	-----Q-----A-L-----	DF
human	TcR- $\beta^a$	V-----	-----R-----A-L-----	DF
human	TcR- $\beta^a$	V-----	-----L-----A-L-----	DF
mouse	TcR- $\gamma^b$	LQLQFTITSAYTYLLLLKSVIYLAIISFSLRRTSVCCNEKKS		
mouse	TcR- $\gamma^c$	--F--S-----	-----G-----	
human	TcR- $\gamma^d$	-L--L-N-----	-----M-----V-F--TCC--G--AF--GE--	
human	TcR- $\gamma^e$	-L--L-N-----	-----V-F--TCC--G--AF--GE--	

Fig. 2. Sequences of the N-terminal regions of human and mouse TCR- $\beta$  and TCR- $\gamma$ . Light shade: TM domains according to [52]. Dark shade: TM domains suggested by PHDtopology. Mouse TCR- $\beta$ : [38,58]; Swiss Protein # P01852. Human TCR- $\beta$ : [55]; PIR # S18894, EMBL # X63456. <sup>a</sup> Lysine in the TM domain mutated according to Morley et al. [46]. Mouse TCR- $\gamma$ : <sup>b</sup> clone C7.5 [59], Swiss Protein # P03985; <sup>c</sup> clone C10.5 [59], Swiss Protein # P01853. Human TCR- $\gamma$ : <sup>d</sup> clone pT $\gamma$ -1 [60], Swiss Protein # P03986; <sup>e</sup> clone M13k [61], EMBL # Y00790.

#### 4. Identification of the TM domains of TCR components

The common prediction of 27 residues in the TM helix of the TCR  $\alpha$  chain results in a cytoplasmic tail of five residues (Fig. 1, light shade). The neural network system PHDtopology [21] revealed that in most cases the lengths of the TM domains have probably been overestimated by non-alignment-based prediction methods. In the case of TCR- $\alpha$ , the PHDtopology analysis yields 18 residues in the TM helix, leaving seven residues for the cytoplasmic tail (Fig. 1, dark shade). The position and the length of the TM domain of TCR- $\alpha$  seem to be insensitive to the changes between the mouse and the human chain, or to the mutations of charged TM domain residues (Fig. 1, dark shade). Correct identification of TM domains is crucial in the search of a motif in these domains responsible for a specific function. Thus, Campbell et al. [52] have suggested a new TM motif (conserved antigen receptor transmembrane motif, CART) to be responsible for the MIRR subunit association. Campbell et al. [52] have based their conclusions on TM segments predictions based on earlier publications, and/or simple hydropathy analyses (Fig. 1, light shade). PHDtopology predictions, however, place some of the residues constituting the proposed TM-CART motif outside the membrane.

The situation is similar in the case of TCR- $\delta$  where the TM region predicted by PHDtopology is also considerably shorter than that assumed in [52] (Fig. 1). In order to test the potential effect of amino acid substitutions in the TM domain of TCR- $\delta$ , we performed computer mutations: changing specific residues and analyzing the resulting sequence using PHDtopology. It is interesting that the simulated mutation in the human TCR- $\delta$  TM domain (R/L, K/I), analogous to the TCR- $\alpha$  mutations studied in [41], caused changes in both the length and the location of the TM domain (Fig. 1).

The TM domains of the TCR- $\beta$  and TCR- $\gamma$  chains are presented in Fig. 2. The TM helices predicted by PHDtopology are again shorter and the cytoplasmic tail is longer than those used by Campbell et al. [52]. In the case of the mouse TCR- $\beta$ , one of the original publications suggested different TM domain: -ILLG...MAMV- [58], and even longer TM domain (-SYQQ...VMAM-) was suggested by Hendric et al. [38]. We have further tested the effects of the single substitutions of the charged lysine residue within human TCR- $\beta$  TM domain, experimentally studied by Morley et al. [46]. Fig. 2 shows that whereas the K/E mutation does not affect the TM domain, K/S, K/Q, or K/R mutation lengthen it by one, and a single residue K/L mutation even by two residues. It is therefore possible that the biological changes observed by Morley et al. [46] were at least partially due to the changed lengths and/or locations of the TM domains.

There is no consensus in the literature about the precise locations of the TM regions of different human and mouse TCR- $\gamma$  clones (Fig. 2). The alignment and the TM helices given in Fig. 2 (light shade) correspond to those assumed by Campbell et al. [52] who have used this alignment to derive the CART motif. In the original publications of the TCR- $\gamma$  sequences the mouse TM domain was suggested to be -YLLL...FSLL- [59], and the human domain -MYLL...LGRT- [60]. Yet different length and location are suggested by PHDtopology which, importantly, allows for longer cytoplasmic tails (Fig. 2).

The TM domains of the mouse and human CD3 proteins are shown in Fig. 3. Clevers et al. [28] suggested identical lengths for the CD3- $\epsilon$ , - $\gamma$  and - $\delta$  chain TM domains (Fig. 3, light shade). The results of PHDtopology, however predict different locations of the TM domains (Fig. 3, dark shade). The lengths of the domains obtained from PHDtopology are shorter and, moreover, different between the mouse and the human proteins. Thus, in the case of CD3- $\epsilon$ , the length of the

	out	transmembrane domain	in
mouse CD3 $\epsilon$	EYCVEVDL	TAVAI I I IVDICITLGLLMVIY	YWSKNRKA
human CD3 $\epsilon$	-N-M-M-VMS	-T-V-----G--LLV-----	
human CD3 $\epsilon^a$	-N-M-M-VMS	-T-V-A---G--LLV-----	
mouse CD3 $\gamma$	ENCIELNIGTISGFIFA	EVISIFFLALGVYLI	AGQDGV
human CD3 $\gamma$	Q-----AA-----	L---IV---V--V--F-----	
human CD3 $\gamma^b$	Q-----AA-----	L--AIV---V--V--F-----	
mouse CD3 $\delta$	QNCVELDSGMTAGVIF	IDLIATLLALGVYCF	AGHETG
human CD3 $\delta$	Q-----PA-V--I-VT-V-----	F-----	
human CD3 $\delta^c$	Q-----PA-V--I-VTAV-----	F-----	
mouse CD3 $\zeta$	QSFGLLDPKLCYLLD	GILFIYGVII	ITALYLRAKFSRSA
human CD3 $\zeta$	-----	-----L--F--V-----	
human CD3 $\zeta^d$	-----	-----A-----L--F--V-----	

Fig. 3. Sequences of the TM regions of human and mouse CD3 chains. Light shade CD3- $\epsilon$ , $\gamma$ , $\delta$ : TM domains according to [28]; CD3- $\zeta$ : TM domains according to [62]. Dark shade: TM domains suggested by PHDTopology. Mouse CD3- $\epsilon$ : [63], Swiss Protein # P22646. Human CD3- $\epsilon$ : [40], Swiss Protein # P07766. Mouse CD3- $\gamma$ : [64–66], Swiss Protein # P11942. Human CD3- $\gamma$ : [67,68], Swiss Protein # P09693. Mouse CD3- $\delta$ : [66,69], Swiss Protein # P04235. Human CD3- $\delta$ : [39,69,70], PIR # A94706. Mouse CD3- $\zeta$ : [34,35], Swiss Protein # P24161. Human CD3- $\zeta$ : [62], Swiss Protein # P20963, EMBL # J04132. <sup>a</sup> Mutated D/A in the TM domain, according to Hall et al. [43]. <sup>b</sup> Mutated E/A in the TM domain, according to Hall et al. [43]. <sup>c</sup> Mutated D/A in the TM domain, according to Hall et al. [43]. <sup>d</sup> Mutated D/A in the TM domain (computer mutation).

mouse TM domain is 20, and that of the human is 21 amino acids. A single mutation D/A in the human CD3- $\epsilon$ , studied by Hall et al. [43], shortens the domain by one residue (Fig. 3).

In the case of CD3- $\gamma$ , the TM domains of the human and mouse proteins are identical; a single E/A mutation [43] shortens the TM sequence by one residue (Fig. 3). Other mutations of the same residue (E/Q, E/K, E/L) [45] did not affect the boundaries and the length of the TM. There is also no difference in the location of the TM domains between the mouse and the human CD3- $\delta$ ; in this case a single D/A mutation [43] extends the domain by one residue (Fig. 3). A stable location and length of the TM domains of CD3- $\zeta$  are indicated by the findings that neither a change from the human to the mouse sequence, nor a simulated D/A mutation had any effect (Fig. 3). The PHDTopology program indicated that the TM domain of these chains is 3 residues shorter than suggested by Weissman et al. [62] (Fig. 3, light shade).

In all examined cases PHDTopology results were significantly different from those deduced from a single residue hydrophathy sequence analysis, placing some residues suggested to be part of the CART motif outside the membrane. Moreover, CD3- $\gamma$  also contains CART, [52] and yet does not assemble with TCR- $\alpha$  [44], demonstrating that the presence of this motif is not sufficient for subunit assembly. An additional reservation about the significance of the CART motif comes from the study of Rajagopalan et al. [71], who have shown that the retention of the TCR/CD3 components in the ER is a function of the interactions of the unassociated chains with calnexin. By analogy with the arguments made by Grupp et al. [72], for the BCR (see

below) it is likely that calnexin does not recognize a protein-specific amino acid sequence motif, but rather a glycosylation site.

## 5. BCR complex

BCR consists of a membrane-anchored immunoglobulin (mIg) expressed on the surface of mature B-cells in association with a 32-kD phosphoprotein Ig- $\alpha$ , and Ig- $\beta$  that exists as two differentially processed forms, a 39-kD and a 37-kD [5,7,73]. The 39 kD protein is now sometimes called Ig- $\beta$ , and the 37 kD protein Ig- $\gamma$  [73]. Ig- $\alpha$  and Ig- $\beta$  form disulfide-linked dimers. Ig- $\alpha$  and Ig- $\beta$  have recently been designated CD79a and CD79b, respectively. Both Ig- $\alpha$  and Ig- $\beta$  have structural homologies to CD3 chains [73]. Ig- $\alpha$  and Ig- $\beta$  are non-covalently associated with mIg and it is generally thought that transmission of the TM activation signal requires an intact BCR complex [74,75]. Venkitaraman et al. [76] further demonstrated that the same *mb-1* gene product (Ig- $\alpha$ ) associates with all five Ig classes; the Ig- $\alpha$  may, however have different glycosylations for each class.

Another important function of associated proteins, such as Ig- $\alpha$  and Ig- $\beta$  for the BCR, may be to release newly synthesized receptors from intracellular retention sites [72]. Strict requirement for the presence of Ig- $\alpha/\beta$  for the expression of the IgM on the cell surface was demonstrated [77–79]. IgD and IgG, however, apparently do not have this requirement [76]. Thus, similarly to TCR, the absence of Ig- $\alpha/\beta$  unmask the intracellular retention sites in the TM domain of mIgM.

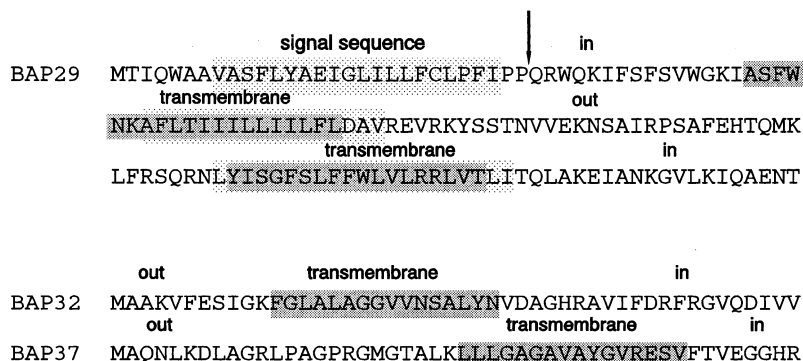


Fig. 4. Sequences of the N-terminal regions of mouse BAPs. Light shade: TM domains according to Adachi et al. [82]. Dark shade: TM domains suggested by PHDTopology. Signal sequence for BAP29 was suggested by SIGNALP program [88]. BAP29: [80,81]; EMBL # X78684. BAP32: [80]; EMBL # X78682. BAP37: [80]; PIR # S46996.

Terashima et al. [80] described three new IgM-associated proteins: BAP32, BAP37 and BAP41. The proteins associated with IgM, but not IgD, suggesting that they may be responsible for different function of these surface receptors. Terashima et al. [80] have suggested that the TM domain of mouse IgM determines the interaction with BAP37 and BAP32/prohibitin. The same group also reported two other BAPs: BAP29 and BAP31 which associate with IgD [81]. The co-purification of BAP29 and BAP31 with IgD also requires the presence of the TM region of the IgD [81,82]. Like other BAPs, BAP29 and BAP31 have hydrophobic N-terminals, but here with a possibility of forming 'two' TM helices (Fig. 4).

Similarly with the TCR, the TM domain of the BCR determines both the signal transduction properties of the receptor and its ER retention. Most reports express the opinion that the antigen response and the ER retention of the BCR are a function of the mIgM-Ig- $\alpha/\beta$  association which, in turn, requires the presence of specific residues within the TM domain.

It has been shown that the polar residues in the mIg TM are important for both signal transduction and transport of IgM to the cell surface [83–87]. On the basis of the studies where systematic point mutations were performed in various parts of the TM sequence, it has been suggested that the -YSTTVT- part of the TM sequence (Fig. 5) is the critical region responsible for both the association of the BCR with Ig- $\alpha/\beta$  and hence the signal transduction [74,94]. Residue substitutions in this region were extensively investigated in a number of recent studies. The reported results often depended on the nature of substitution, the system used (i.e. mouse or human), and on the biological responses tested.

Pleiman et al. [94] studied the effects of the point mutations  $S_{20}/A$ ,  $Y_{23}/F$ ,  $T_{28}/V$  or  $K_{33}/I$  (Fig. 5) of mouse  $\mu$  chain transfected into K46 murine B lymphoma cells. As an antigen, Pleiman et al. [94] used (nitrophenyl)<sub>12</sub>-BSA, monoclonal rat anti-mouse IgM

(B76, Jackson Lab), or polyclonal goat anti-mouse IgM antibodies. Each point mutation resulted in complete abrogation of the (nitrophenyl)<sub>12</sub>-BSA-induced protein tyrosine phosphorylation and  $Ca^{2+}$  mobilization. The clustering of the receptors by the monoclonal antibodies led to a restoration of the signal transduction in the cases of the peripheral (with respect to the -YSTTVT- sequence)  $S_{20}/A$  and  $K_{33}/I$  mutations, but not when the central  $Y_{23}/F$  or  $T_{28}/V$  were mutated. None of these point mutations disrupted the association of  $\mu$  chain with the Ig- $\alpha/\beta$  [94]. It is important to note that when Pleiman et al. [94] used polyclonal antibodies to induce activation, the signal transduction via the mutated receptor was restored in all cases of the point mutations. Pleiman et al. [94] suggested that the polyclonal antibodies are better activators than monoclonal antibodies because they induce more extensive receptor clustering. Indeed, the results could be understood using the two step model: antigen-induced receptor aggregation followed by the Ig- $\alpha/\beta$ -induced signal transduction. In this model, aggregation of a fully functional receptor is induced by monovalent antigens; each of the residues  $S_{20}$ ,  $Y_{23}$ ,  $T_{28}$ , and  $K_{33}$  is required for this process. Substitution of  $S_{20}$ ,  $T_{28}$ , or  $K_{33}$  results in a decreased propensity of the receptor to aggregate. However, if  $Y_{23}$  is not substituted, a monoclonal anti-

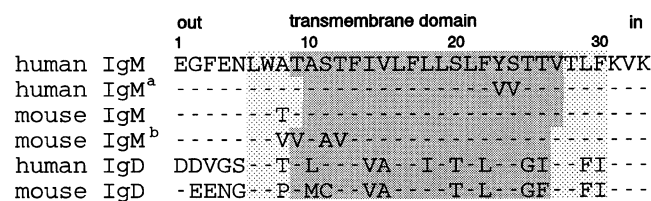


Fig. 5. Sequences of the C-terminal regions of human and mouse IgM and IgD. Light shade: TM domains according to [89,90]. Dark shade: TM domains suggested by PHDTopology. Human IgM: [91]; PIR # S15590. Mouse IgM: [89,92]; Swiss Protein P01873. Human IgD: [93]; EMBL # K02882. Mouse IgD: [90]; EMBL # J00450. <sup>a</sup> Mutated  $Y_{23}S_{24}/VV$  in the TM domain [84]. <sup>b</sup> Mutated TTAST/VVA AV in the TM domain [83].

body, is still capable of inducing the receptor aggregation and signal transduction. In the absence of this critical residue, the receptors lose their intrinsic capacity to undergo antigen binding-induced aggregation, but can be aggregated by a polyclonal antibody leading to Ig- $\alpha/\beta$ -mediated cell activation. When the association between the  $\mu$  chain and Ig- $\alpha/\beta$  was completely disrupted by the Y<sub>23</sub>S<sub>24</sub>/VV mutation, stimulation by polyclonal antibodies no longer activated the cells [84]. Blum et al. [95], and Stevens et al. [96] have also reported that substitutions of Y<sub>23</sub>/F, S<sub>24</sub>/A, or both (Y<sub>23</sub>S<sub>24</sub>/FA) in murine chains did not affect the association with Ig- $\alpha/\beta$  or the receptor function when polyclonal antibodies were used as aggregating agents. However, in some experiments, specifically when the tyrosine phosphorylation capacity of the mutants was examined, Stevens et al. [96] employed monoclonal rat anti-mouse  $\mu$ , and, contradictory to the results of Pleiman et al. [94], they did observe fully efficient tyrosine phosphorylation by the Y<sub>23</sub>S<sub>24</sub>/FA mutant. The reasons for the discrepancy are not clear; although the employed monoclonal antibody is not specified in [96], the supplier is the same as in [94].

The mutation of the same Y<sub>23</sub>S<sub>24</sub> residues into VV resulted in a sharp decrease in both Ig- $\alpha/\beta$  association and anti-mIg-induced signaling [96]. Stevens et al. [96] observed that Y<sub>23</sub>S<sub>24</sub>/FA mutation, changing the phenolic hydroxyl group maintained a similar size and shape of the side chains, whereas the Y<sub>23</sub>S<sub>24</sub>/VV mutation altered both, and changed hydrophilicity of the residues. Since the mouse  $\mu$  chain Y<sub>23</sub>S<sub>24</sub>/FA mutants retained their full ability to both bind Ig- $\alpha/\beta$  and transduce the signal when stimulated by polyclonal antibodies, it was concluded that size and shape, rather than hydrophilicity of these residues are critical for the association with other subunits [96]. The murine  $\mu$  chain Y<sub>23</sub>S<sub>24</sub>/VV mutants still retained some capacity for signal transduction and association with Ig- $\alpha/\beta$ , whereas identical human chain mutants studied by Sanchez et al. [75], and Shaw et al. [84] failed completely in both (see below). Stevens et al. [96] attributed these differences with [75,84] to the use of different species of  $\mu$  chain: mouse chain was used in [96], whereas the other two groups studied the human chains with identical mutations. The difference between the murine and human  $\mu$  chains was also probably responsible for the discrepancy between the results of Williams et al. [83], and Grupp et al. [72]. Williams et al. [83] used a mouse  $\mu$  chain and concluded that -TTAST- patch (Fig. 5) of the TM region is involved in retention; whereas Grupp et al. [72], using human  $\mu$  chain, found the responsible residues to be Y<sub>23</sub>S<sub>24</sub>. It is interesting that TM domains of mouse

and human  $\mu$  chains are highly conserved with only one substitution in the TM sequence: in mouse alanine<sub>8</sub> is replaced by threonine (Fig. 5). Stevens et al. [96] speculated that this substitution, by reducing the number of hydroxylated residues, may decrease the affinity of the interaction between human  $\mu$  chains and Ig- $\alpha/\beta$  to the extent that the additional disruption by the Y<sub>23</sub>S<sub>24</sub>/VV mutation is sufficient to abolish interaction with both retention proteins and accessory heterodimers. An alternative explanation is that the sequence differences in the extracellular domains between the murine and human  $\mu$  chains may contribute to reduced interactions between the human C $\mu$ 4 domain and extracellular regions of murine accessory proteins [96].

Shaw et al. [84], and Mitchell et al. [97] tested the mutants of human  $\mu$  chains transfected into murine cells for signal transduction induced by the clustering of the mIg, and monitored Ca<sup>2+</sup> transients and antigen presentation by the transfectants to T-cells. It is difficult to directly compare the results in [84,97] with those in [94–96] because of the different antigens used; Pleiman et al. [94] used both mono- and polyclonal anti- $\mu$  antibodies, [96] primarily, and [95] exclusively polyclonal anti- $\mu$  antibodies. Shaw et al. [84], and Mitchell et al. [97], on the other hand, used PC (phosphorylcholine)-specific mIgM transfectants and either PC-OVA or (Fab')<sub>2</sub> of anti- $\mu$  polyclonal antibodies for stimulation. It is likely that both antigens employed in [84,97] are efficient receptor aggregating agents, in which case the results should be directly comparable to those with polyclonal antibodies. Indeed, it was reported that single substitutions in the TM domain, including Y<sub>23</sub>/F, do not affect the signal transduction properties of the receptor [84,97]. However, both single substitutions performed, Y<sub>23</sub>/F and S<sub>24</sub>/A, as well as Y<sub>23</sub>S<sub>24</sub>/FA resulted in the failure of antigen presentation by the cells [84,97]. It was concluded that the association with the Ig- $\alpha/\beta$  is not sufficient for the antigen presentation in the context of the MHC II molecule; this function is critically dependent on the presence of the Y<sub>23</sub> which could be responsible for the association of the  $\mu$  chain with an as yet unidentified chaperon-like molecule that traffics it to the class II-rich processing vesicle [97]. An alternative possibility is a presence of a specific sequence in the TM domain that retains internalized mIg within appropriate intracellular organelles [97].

It was shown that human  $\mu$  chain Y<sub>23</sub>S<sub>24</sub>/VV mutant, transfected into murine cells does not associate with the Ig- $\alpha/\beta$  subunits and completely fails to transduce intracellular signals upon antigen binding [72,84,97]. When expressed in Ig- $\alpha$ -negative cells the Y<sub>23</sub>S<sub>24</sub>/VV mutants are observed on the cell surface, whereas the wt  $\mu$  chains are not [72]. Similarly, mutation of Y<sub>23</sub>S<sub>24</sub> to VV

in human  $\mu$  chains, expressed in A20 mouse lymphoma cells, allowed surface expression of mIgM in the absence of association with Ig- $\alpha/\beta$  dimer [74,75]. This further confirms the hypothesis that the Y<sub>23</sub>S<sub>24</sub> residues signal the retention of the  $\mu$  chain in the ER; replacing or masking these residues by the association with Ig- $\alpha$  allows for the surface expression of the protein. A role for a chaperone molecule calnexin in this process was suggested by Grupp et al. [72]. They further argue that because calnexin chaperons a wide variety of proteins [98], it is unlikely that any consensus sequence motif can be responsible for the calnexin binding, and it is mediated by carbohydrate moieties. In this model, the Y<sub>23</sub>S<sub>24</sub>/VV mutation alters the glycosylation of the  $\mu$  chain, thereby weakening its binding to calnexin [72]. In the wild type  $\mu$  chain, the dissociation may only be achieved by the binding of Ig- $\alpha$ , with or without Ig- $\beta$  [72].

Different results were recently reported by Wu et al. [99], who used murine mIgM and mIgD expressed in murine lymphocytes. It was demonstrated that TM regions of the  $\mu$ - and  $\delta$ -chains account for their differential surface expression in the absence of Ig- $\alpha/\beta$  [99]. Specifically, Wu et al. [99] focused on the effect of G<sub>25</sub>F<sub>26</sub> versus T<sub>25</sub>T<sub>26</sub> difference between mouse IgD and IgM. The  $\delta$  chain G<sub>25</sub>F<sub>26</sub> residues were substituted by  $\mu$  chain TT residues. Either one of the single mutations was sufficient to abolish Ig- $\alpha/\beta$ -independent surface IgD expression; the expression was rescued by coexpression with Ig- $\alpha$  [99]. Conversely, substituting the  $\mu$  chain T<sub>25</sub>T<sub>26</sub> residues by  $\delta$  chain GF residues resulted in the expression of the mutant IgM on the surface [99]. In direct contradiction to the results with human mIg [72], Wu et al. [99] found that both, wild type  $\delta$  chain and the mutants interacted with calnexin; moreover, inhibition of calnexin association with IgM or IgD did not lead to surface expression of normally retained mIgs, demonstrating that calnexin is not necessary for the ER retention. The reason for the discrepancy between the results of Grupp et al. [72], and Wu et al. [99] is still unclear; although it may, in principle, be due to the intrinsic differences between the murine and the human systems. The clarification of this issue is important: the involvement of calnexin, with its lack of specificity, would further undermine the relevance of a 'TM motif' to the receptor function and association with other TM proteins.

## 6. Identification of the TM domains of BCR components

Adachi et al. [82] identified three TM regions in BAP29 (Fig. 4, light shade) and acknowledged the possibility that the first one can be a leader sequence. The results using PHDtopology and the signal sequence

identification program SIGNALP [88] indeed indicate the presence of a signal sequence and two TM domains in BAP29 (Fig. 4, dark shade); however the precise locations of the domains differs from those suggested by Adachi et al. [82]. Terashima et al. [80] have suggested amino-terminal sequences, composed of 25, mostly hydrophobic, residues of BAP32 and BAP37 as possible membrane anchors. Indeed, our results using PHDtopology indicate the presence of TM domains in both BAP32 and BAP37 (Fig. 4), although the latter was given a lower degree of confidence ( $> 0.5$ ). We tested the possibility that the putative TM domains of BAP32 or BAP37 are parts of signal sequences; however the SIGNALP program did not identify a well-defined cleavable signal sequence in either of these proteins.

In the original study describing the discovery of the membrane-bound form of mouse IgM, Rogers et al. [89] proposed the TM segment as a 25-uncharged amino acid stretch between the charged residues on putative extracellular and intracellular sides (Fig. 5, light shade). They supported these conclusions by the single-residue hydrophobicity analysis of Segrest and Feldmann [100]. Most of the subsequent studies of the BCR function, including those which investigated the effects of the substitutions in the putative TM domain on the receptor function, relied on Rogers et al. [89] for the definition of the TM domain. Thus, Cheng et al. [90] deduced the murine IgD TM domain by homology with Rogers et al. [89]. Specifically, it has usually been assumed that the cytoplasmic parts of mIgM or mIgD are very short and contain only three amino acids [73] (Fig. 5), which are conserved between humans and mice [101].

However, the size of the cytoplasmic domains of  $\mu$  and  $\delta$  chains is unclear: PHDtopology indicates a six residue cytoplasmic domain for both  $\mu$  and  $\delta$  chains (Fig. 5). The longer cytoplasmic tail is consistent with the recent suggestion that the function of the mIgM substructure extends beyond antigen binding and passive association with Ig- $\alpha/\beta$  dimers, and the cytoplasmic domain may interact with and activate some yet undefined effectors that play a critical primary role in signaling [7]. To support this conclusion Cambier et al. [7] cite the data which suggest association of the protein tyrosine kinase Syk with the cytoplasmic tail of  $\mu$  chain, consistent with the longer tail. It has also been shown that the Ig- $\alpha/\beta$ -independent membrane IgD is efficiently internalized after its clustering, demonstrating that it has full capacity to interact with the corresponding cytoplasmic components responsible for internalization [102,103] and opening a possibility that the cytoplasmic portion of the mIgD can by itself mediate the signal transduction. This would require cytoplasmic tails of the  $\mu$  and  $\delta$  chains to be longer than the usually assumed three amino acids KVK sequence (Fig. 5).

```

mouse Ig- $\alpha$   NPVPRPFLDMGEGTKNRIITAEGIILLFCAVVPGLLLFRKRWQNEKF
human Ig- $\alpha$   Q-P-----L
                                     [Light shade]
                                     [Dark shade]

mouse Ig- $\beta$   GFSTLDQLKRRNTLKDGIILIQTLIIILFIIVPIFLLLDKDDGKAGME
human Ig- $\beta$   -----A---Q-----M-----S-----
                                     [Light shade]
                                     [Dark shade]

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Fig. 6. Sequences of the TM regions of human and mouse Ig- $\alpha$  and Ig- $\beta$  aligned according to Reth [73]. Light shade: TM domains according to Reth [73]. Dark shade: TM domains suggested by PHDtopology. Mouse Ig- $\alpha$ : [104,105]; Swiss Protein # P11911. Human Ig- $\alpha$ : [106–108]; PIR # A46477. Mouse Ig- $\beta$ : [109]; PIR # A31403. Human Ig- $\beta$ : [110]; PIR # A46126.

Indeed, [7,94] argue that the sequence motif they were studying actually is ‘cytoplasmic’ which better explains their data. They also note that using the Klein instead of Kyte and Doolittle [15] algorithm places the –YSTTVT– sequence into the cytoplasm. As in the case of the TCR, the longer cytoplasmic tail predicted by PHDtopology places one (T<sub>28</sub>, in mIgM) or two (V<sub>27</sub>T<sub>28</sub>, in mIgD) putative members of CART [52] outside the TM domains (Fig. 5).

It has been suggested that two not mutually exclusive mechanisms may operate in the BCR activation: an allosteric mechanism or a receptor aggregation-dependent transactivation phenomenon [7]. Different antigens may activate either of these two mechanisms. Thus the thymus-dependent antigen, more likely to operate via an allosteric mechanism, is sensitive to the mutations in TM and cytoplasmic domains of  $\mu$  chain, presumably by allosteric activation of  $\mu$  chain-associated Syk. The anti-receptor antibodies and thymus-independent antigens may activate Ig- $\alpha/\beta$ -associated Src kinases directly by virtue of clustering-mediated transactivation [7].

None of the single-residue mutations discussed above alters the location of the TM domains of mouse or human mIgM, as indicated by PHDtopology (data not shown). However, the Y<sub>23</sub>S<sub>24</sub>/VV mutation shortens the TM region by one residue (Fig. 5). The TTASt/VVA AV mutation of the mouse  $\mu$  chain [83], shifts the location of the TM domain by one residue, elongating the cytoplasmic domain (Fig. 5).

The TM domains of human and mouse Ig- $\alpha/\beta$  are shown on Fig. 6, where the chain alignments and the light shades indicate their positions as suggested by Reth [73], and the dark shades correspond to the results of PHDtopology. Again, as in the most cases, PHDtopology indicates a shorter TM sequence.

## 7. Conclusions

An emerging consensus in the studies of signal transduction mechanisms by both BCR and TCR is that their TM domains and associated proteins are critical for the proper signal transduction and surface expression. It is thus surprising that little attention was paid to the identification of the exact locations and lengths of the TM domains. In most cases TM regions have

been deduced by visual inspection of the corresponding sequences and/or by single-residue hydrophobicity analyses. However, at times of rapidly growing knowledge of evolutionary relationships, basing predictions on single sequences is outdated. Currently available computer resources allow for a more in-depth analysis of the TM sequences which, in most cases, results in significant differences from the previously assumed locations. These results can lead to modification of the putative TM motifs and re-interpretation of the studies of the mutated TM domains. Furthermore, the TM analysis algorithms like PHDtopology can be used prior to the residue mutation studies allowing to choose among the mutations those that would not introduce any changes into the location and length of the TM domains. Such an approach would considerably improve the analysis of the effects of the sequence substitutions.

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