Soluble T-Cell Receptor Fragments — Guidance of Folding and Assembly

Christoph Wülfing
Andreas Plückthun

The TcR is the central recognition molecule in cellular immunity, yet our knowledge about its structure and interactions with the MHC/peptide complex and with its coreceptors is sparse. Therefore, the production of soluble TcR fragments is of considerable interest, in particular for biophysical studies. Here, Christoph Wülfing and Andreas Plückthun discuss soluble TcR fragments produced in *E. coli* and in eukaryotic systems, and analyze the success to date.

The Need for Soluble TcR Fragments and the Principal Options

The T-cell receptor (TcR) is a member of the immunoglobulin superfamily [1]. It is a heterodimer, consisting of either one α and one β chain, or one γ and one δ chain. The extracellular part of each chain consists of one variable and one constant domain and it is anchored to the membrane by a transmembrane helix. The structure is illustrated in Figure 1. The complete extracellular portion of the TcR is thus equivalent to an antibody Fab fragment. Indeed, all molecular modeling studies to date have indicated a convincing structural similarity between antibody and TcR domains [2]. The TcR is associated with the membrane-bound chains of the CD3 complex which mediates signal transduction by the TcR [1].

Because of its transmembrane nature, the TcR is difficult to purify. Since the antigen recognition specificity resides in the extracellular domains, most studies have the ultimate objective to define the structure and biophysical properties of these domains: binding constants, binding kinetics and effects on stability. Therefore, genetic means have to be employed to devise soluble TcR fragments.

For most biophysical studies, fairly large amounts of pure, highly soluble proteins are necessary. This is especially true for the two methods of three-dimensional structure determination, X-ray crystallography, and nuclear magnetic resonance (NMR). Only such high-resolution structure determinations will ultimately make the fine specificity of T cells for its peptide antigen and the effect of major histocompatibiliy complex (MHC) restriction understandable. Crystal structures are already available for several MHC/peptide complexes, and by now both MHC class I and MHC class II structures have been solved [3].

The rational modification of the interactions between the TcR and its antigen-complex would not only be an invaluable tool in the study of the mechanism of activation of T cells, but may ultimately also pave the way for new therapeutic principles in autoimmune disorders.

To obtain soluble TcR fragments with MHC/peptide recognition activity, two types of fragments have been produced to date (see Figure 1). The first type are fragments that contain the complete extracellular portion, in other words, both the variable and the constant domain of each chain. They should, therefore, constitute an independent structural unit, and they have been chosen for all eukaryotic expression strategies. For all expression studies in *Escherichia coli* reported to date, a second type of fragment has been used which contains only the variable domains (see Figure 1). The reason for this is that in the case of the related antibodies the expression of the so-called Fv or scFv fragments generally has given rise to higher yields of folded molecules than the expression of the Fab fragments [4].

All *E. coli* work and the large majority of the eukaryotic work has been done with α/β TcRs. Nevertheless, the γ/δ TcRs offer some interesting properties with respect to production in a soluble form (see below).

Folding, Assembly, and Secretion of TcRs

In order to express a recombinant molecule successfully, it is helpful, if not necessary, to understand its *in vivo* biosynthetic route. We will first summarize some of the particular problems that the TcR poses in this respect.

Evolution optimizes proteins for their function, but not for maximal thermostability, nor for most efficient folding or assembly [5]. If a protein has to fulfill a very complex function, as the TcR has to do in its interaction with several other molecules, it is subject to many constraints, so that its thermodynamic stability, folding, and assembly efficiency may be barely sufficient for efficient production at the level required *in vivo*, which in comparison to other membrane proteins is rather low. The *in vivo* production probably involves several kinds of assistance (for instance, in the form of chaperones [6] or stabilizing coreceptors), and recombinant overexpression without the concomitant overexpression of this assistance might result in inefficient folding or assembly, or low stability.

The involvement of specific chaperones in the assembly of the TcR in the endoplasmic reticulum (ER) (i.e., TcR-associated protein (= TRAP or ω chain) [7, 8], calnexin [9], and Hsp70 (= antibody heavy chain binding protein, or BiP) [10]) was suggested, but the involvement of chaperones in the *in vivo* folding has not been directly proven to date. However, the existence of these proteins in large quantities in the ER [11], as well as the involvement of Hsp70 (BiP), Hsp90 (Grp94) [12], and protein disulfide isomerase (PDI) [13] in the folding of antibodies, suggests that some or all of these proteins might play a crucial role. Thus, folding problems for TcR expression in *E. coli*, which is devoid of this help, are to be expected and do, in fact, occur [14]. Furthermore, high-level expression, in particular in prokaryotic systems, might be expected to lead to substantial folding problems, simply due to the quantities of aggregation-prone intermediates produced and the nature of aggregation as a second-order process. Assembly problems, maybe due to insufficient levels of TRAP or calnexin in non-T cells, are found in most eukaryotic systems (for instance, see [15]), although the final product, the extracellular soluble TcR, is stable.

To facilitate the correct pairing of chains, and thus circumvent assembly problems, the chains can be forced into close proximity using a single-chain TcR (scTcR) (see Figure 1). For production of soluble constructs, this approach has so far only been tested in prokaryotes (see [14, 17], for example). In mammalian cells, an scTcR anchored to the membrane as a fusion to the ζ chain induced the production of lymphokines upon exposure to relevant target cells, thus qualitatively demonstrating functionality [18]. In contrast to the constant domains, the linker of an scTcR does not force the variable domains into a particular orientation, so that they might still be able to reversibly "open" and "close" or form dimers or larger oligomers. Such reactions have been observed for antibody scFv fragments [19]. Since the scTcRs consist of the variable domains only, the variable–constant domain interfaces become exposed to the solvent, potentially impairing the solubility and the stability of folding intermediates and the recombinant molecule. This longitudinal interaction between constant and variable domains may be quite specific, because in contrast to antibodies, a domain switch (generating Vα-Cβ and Vβ-Cα chains) did not produce molecules which localized to the surface [20]. Alternatively, other proteins may recognize part of both the variable and constant domain of either the α or β chain and thus lose interactions in the domain-switch construct.

If the expression of a protein is highly regulated, as the expres-

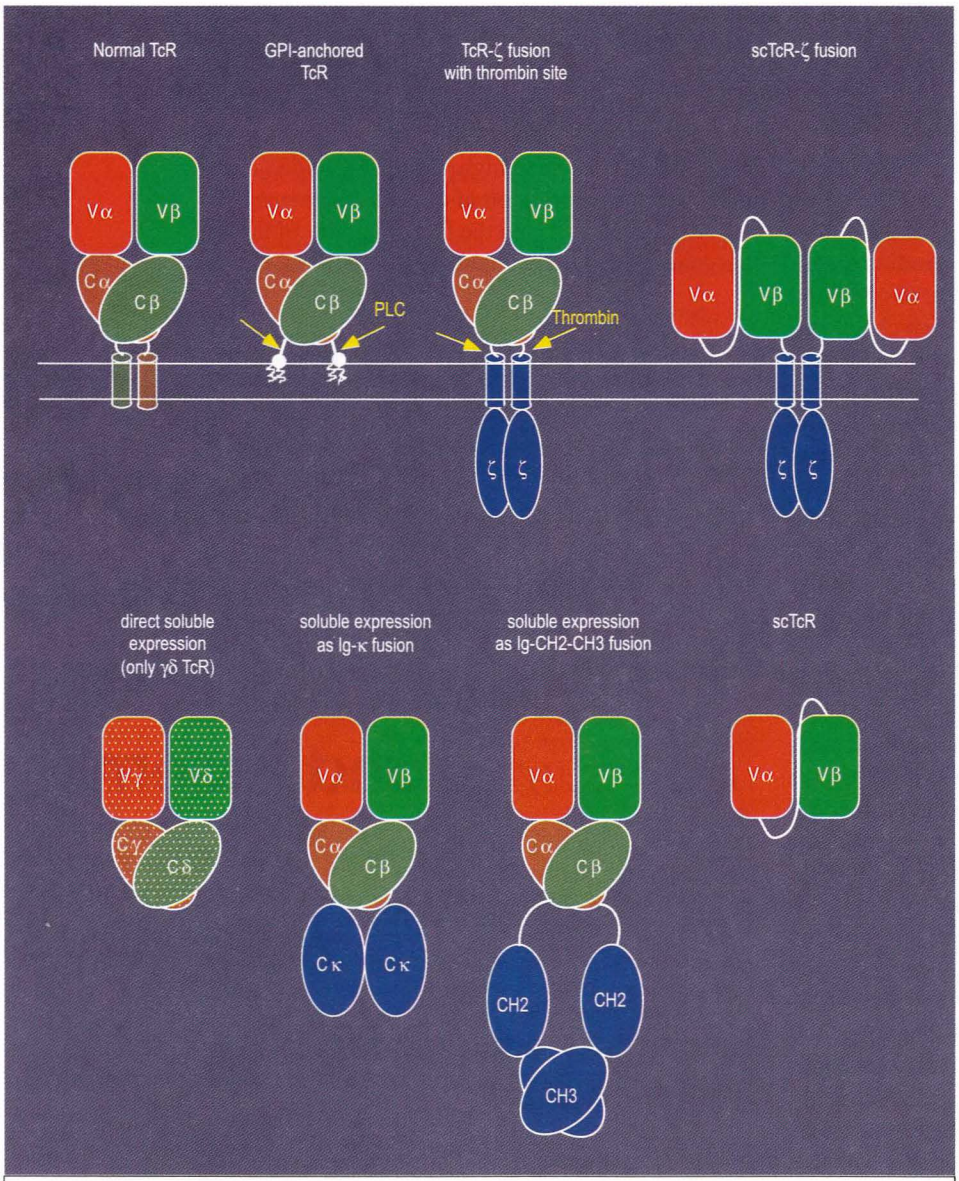

Figure 1. Different TcR fragments produced, with labels corresponding to Tables 1–3. At the top left is the natural α/β TcR. The TcR domains are colored red and green and labeled. Non-TcR parts are shown in blue. Engineered enzymatic cleavage sites are indicated by a yellow arrow.

sion of the TcR/CD3 complex is, the intracellular transport [21] and/or the stability of this protein might be purposefully limited as a means of regulation. The α chain seems to be unusually labile to proteolysis [22]. Limitations along the secretory pathway might influence TcR fragment expression in eukaryotic systems [21], whereas the intrinsic proteolytic lability might limit the stability of TcR fragments. One specific problem of TcR fragment expression in *E. coli* is that there is no glycosylation, which can contribute significantly to the stability and folding efficiency of a protein [23–25].

Expression Systems

An overview of the published expression systems is given in Tables 1–3. Since these tables are largely self-explanatory, the following paragraphs do not repeat a description of the features of the specific systems, but rather try to introduce the underlying strategies and discuss some advantages and disadvantages of the different types of approaches, as well as some criteria for evaluating them. We include a more detailed evaluation of the prokaryotic, and only a shorter one of the eukaryotic systems, because of our own practical experience.

Evaluation of the Folding State

Whatever method is used, the characterization of the folding state of the recombinant material is of crucial importance. If the TcR fragments are produced as soluble secreted material, there exists a built-in quality check: in *E. coli* secretion systems misfolded TcR material is rapidly degraded (see below), while in eukaryotic systems nonassembled material is retained in the ER and subsequently degraded [21]. The appearance of soluble TcR fragments in the periplasm of *E. coli* (in the range of 1 mg/l culture), or correct surface localization or secretion in eukaryotic systems, is a good indication that the material is correctly folded. However, it is not proof, and the folding state must be confirmed by other methods. Much worse is the situation for methods using *in vitro* refolding. Due to the low yield of refolding of immunoglobulin-like molecules, the presence of misfolded soluble mate-

Table 1
Comparison of different *E. coli* scTcR expression publications: Part I

TcR used and expression strategy	Yield in mg/l culture (not purified)	Characterization	Purification	Application using purified scTcR	Ref.
Secreted scTcR RFL 3.8 with 5 aa exchanged *lac* control	5–10 (insoluble)	Binding to fluorescein (which is recognized both directly and bound to MHC class II) on a column	Isolation of insoluble material + (HPLC in urea) refolding + fluorescein affinity column	Characterization of fluorescein binding by site-directed mutagenesis (46)	(35)
Secreted Vα and Vβ chain and scTcR 1934.4 (+ affinity tag) *lac* control	Vα: 10 (soluble) Vβ: 1 (soluble) scTcR: 0.5–1 (purified)	CD spectrum (scTcR)	Isolation of insoluble material + (HPLC in urea) refolding + fluorescein affinity column	Crystallization of the α-chain (47)	(16, 17)
Secreted scTcR 2C λ p_L control	?	Sandwich ELISA	Isolation of insoluble material + refolding + antibody-affinity column (only small scale)	None	(36)
Cytoplasmic scTcR 2B4 (with small parts of the Cα,Cβ) T7 control	65–100 (after screening for high level expression clone) (insoluble)	ELISA with different antibodies, size exclusion chromatography	Isolation of inclusion bodies + refolding	None	(34)
Cytoplasmic scTcR T7 control	1–30 (insoluble)	ELISA with different antisera (unclear whether conformation specific)	Isolation of insoluble material + IMAC on Ni-NTA	ELISA to detect autoantibodies (scTcR is coated)	(49)
Secreted scTcR *lac* control	10 (insoluble)	CD spectrum (scTcR) BIAcore (qualitative) Anti-Vβ17 Ab does not bind	Isolation of insoluble material + refolding + IMAC on Ni-NTA	BIAcore (qualitative)	(32)
Secreted scTcR CR15, P14, 8-10/2 (+ affinity tag), *lac* control, CR15 with coexpressed DsbA and σ^{32}	CR15: 1 mg; P14: 1 mg; 8-10/2: μg range (all soluble)	Binding to immuno-affinity columns	Antibody affinity column (only small scale)	Production of bivalent scTcR (14)	(14)

The first column "authors" refers to the publication. The second column "TcR used and expression strategy" indicates first whether the expression was periplasmic (secretion) or cytoplasmic, second, which TcR clone was used and whether this TcR was engineered, and third, which promoter was used. The third column "yield" gives the yield reported in the publication. This means, when not stated otherwise, yield in cell extracts estimated by, for example, Western blot. The fourth column "characterization" briefly describes if and how the correct folding of the scTcRs was monitored; antibodies used in different immunological techniques are all claimed to be conformation specific. The fifth column "purification" indicates how purification of the scTcRs was achieved, if not stated otherwise on a large scale. The last column "application" gives applications for which the scTcRs or the expression system have been successfully used.

rial is a certainty after *in vitro* refolding. Such material has generally been observed, and the misfolded and correctly folded material must be separated. The success of such a separation has to be monitored in every case.

The only methods that can definitely answer the question of correct folding are methods involving the specific biological recognition function of the TcR [26–29], such as the competitive inhibition of T-cell activation by soluble TcR fragments [27]. The most direct is to investigate the specific recognition function of the TcR fragments *in vitro* by determining the binding constant and stoichiometry of the TcR fragments to the MHC/peptide complex using surface plasmon resonance (as in the BIAcore instrument) [29, 30]. The interactions of a TcR fragment with MHC and superantigen have also been studied this way [31]. Material produced in *E. coli* by *in vitro* refolding [32] has been qualitatively demonstrated to contain a small amount of active protein which is able to bind to the MHC/peptide complex. In contrast, there is evidence from indirect methods (see below) that the material produced in *E. coli* in soluble form is correctly folded, although direct proof of its biological function is still awaited.

The method used most often for the evaluation of the folding state is the recognition of the recombinant material by conformation-specific antibodies. Assuming that there is only one thermodynamically stable correct fold, the reaction of epitopes that are part of this fold would indicate with reasonable certainty the existence of "the" correct fold. It appears that *in vivo*, nonfunctional TcR is degraded or at least truncated, whereas *in vitro*, metastable soluble forms, perhaps even small aggregates, do appear.

It must be stressed that the existence of a unique stable fold is very difficult to prove. The use of antibodies for the determination of the correctly folded state can therefore not be the final criterion of functionality. In order to strengthen the antibody binding results, it might be desirable to use as many antibodies as possible. Clonotypic antibodies, which by definition recognize an epitope formed by two different chains, may be more informative than antibodies recognizing only parts of one chain. If recognition by antibodies is used to quantify the per-

Table 2
Comparison of different *E. coli* scTcR expression publications: Part II

Authors	Major advantages	Major disadvantages
Novotny et al. (35)	• Isolation of sufficient amounts of material to allow the study of the antigen binding	• Very unusual antigen: instead of peptide plus MHC, fluorescein is recognized • Could not express original sequence, required modeling and alteration of the sequence • Long purification procedure (HPLC + refolding + affinity column) • Purification not general
Ward (16, 17)	• Isolation of sufficient amounts of material to allow crystallization • Fast purification (1-column procedure, no refolding)	• Sequence dependent, does not seem to be generally applicable (see (14))
Soo Hoo et al. (36)		• Small yields (as judged from the data shown) • Long purification procedure (isolation of insoluble material + refolding + affinity column)
Kurucz et al. (34)	• Very high yields	• Long purification procedure (isolation of insoluble material + refolding) • No separation of correctly folded and misfolded material after refolding reported • High variation of the expression level among different clones with the same construct, indicating an uncharacterized mutation in the chromosome or the plasmid • Seems to be sequence dependent
Lake et al. (49)	• Isolation of sufficient amounts of material to allow the scTcR to be coated in ELISA	• Long purification procedure (isolation of insoluble material + refolding) • No separation of correctly folded and misfolded material after refolding reported, folding yield unclear
Hilyard et al. (32)	• Isolation of sufficient amounts of material to allow the study of the antigen binding	• Only small fraction refolded (no reaction with clonotypic antibody) • Very low BIAcore signals, only qualitative study
Wülfing and Plückthun (14)	• Offers some principal understanding of the scTcR secretion process • Potentially fast purification (no refolding)	• Native secretion more widely applicable than Ward (17), but still not general • The particular TcR sequences investigated could not be purified with IMAC

BIAcore: instrument to measure molecular interactions using surface plasmon resonance.
IMAC: immobilized metal ion affinity chromatography.

In this comparison, the achievements reported in the publications and potential promise for further work are compiled. Since the publications use different TcR sequences (and most publications include only one sequence), caution should be exercised in generalizing the results (see text).

centage of correctly folded material, it is important to use liquid phase methods (such as sandwich ELISA, immunoprecipitation, immunoaffinity chromatography) as opposed to direct ELISAs (that is, coupling the TcR fragment to a solid surface) in order to avoid surface mediated denaturation [33]. Other methods like circular dichroism [17] and characterization of the recombinant material on a size exclusion column [34] give only unspecific accessory information, such as the predominant secondary structure elements or the presence of monomers in the refolded material.

Introduction to Prokaryotic Expression

Expression in prokaryotic systems (thus far only *E. coli* has been reported for TcR fragments) offers a large number of potential advantages: bacteria are very easy to handle and grow very quickly. Consequently, mutated variants can be produced quickly. A large number of heterologous proteins have been produced in high yields. Furthermore, bacteria are easy to manipulate. This might be important, for example, for metabolic labeling in NMR studies. The absence of glycosylation is a major advantage for crystallization. Furthermore, the high transformation efficiency is an important advantage for all random mutagenesis or selectable library experiments.

The major disadvantage is that folding of the TcR fragments, which are devoid of their cellular milieu and helper proteins, becomes a nontrivial problem. If the TcR fragments are produced in a misfolded, insoluble form and purified before refolding *in vitro* [32, 34–36, 49], the *in vivo* folding problem is avoided at the price of an *in vitro* folding problem. This strategy may allow, though probably in a sequence-dependent way, the production of very large amounts of material as insoluble inclusion bodies [34]. The alternative route has been taken by Ward [16, 17] and ourselves [14] in directing the TcR fragments to the periplasm. This compartment allows the formation of disulfide bonds, necessary for the correct folding of immunoglobulin domains [4]. This strategy also is the basis for *in vivo* screening methods [37]. The problem is that the periplasm as a folding environ-

Table 3
Comparison of different eukaryotic TcR fragment expression publications

TcR used and expression strategy	Yield	Characterization	Purification	Application	Major advantages	Ref.
α/β TcRs						
GPI-linked TcR 2B4, in CHO-cells	0.5 mg/week with hollow fiber reactor (10^{10} cells)	Overlapping binding of antibodies MHC + peptide binding (30)	Cleavage with PI-PLC + immunoaffinity columns (sequential anti-α- and anti-β-chain)	Determination of the TcR affinity constant (28, 30)	Functionality verified by affinity constant	(15)
GPI-linked TcR 2C, in BW5147 thymoma cells	? ("large amounts")	Immunoprecipitations after cleavage with PI-PLC MHC + peptide binding (29)	Immunoaffinity column (anti-TcR, clonotypic) (29)	Determination of the TcR affinity constant (29)	Functionality verified by affinity constant	(46)
C_κ fusion of both chains; KB5-C20, in X63-Ag 8.653 myeloma	up to 1 mg/ml in medium	Immunoprecipitations	Immunoaffinity column (anti-TcR, clonotypic)	None		(44)
C_κ fusion of both chains; 14.3.d, in J558L myeloma	10 mg/ml in medium (clone dependent)	Inhibition of TcR activation Material homogenous on DEAE Sephacel	Immunoaffinity column (anti-C_κ)	Determination of the TcR affinity constant, β-chain crystallization (22)	Functionality verified by affinity constant High yields (isolation of several 100 mg reported)	(27)
CD3 ζ-chain fusion; 2B4, in rat basophil RBL-2H3 cells + two other TcRs	? ("large amounts, more than GPI-linked material")	Signal transduction through the hybrid receptor after binding of specific MHC + peptide	Cleavage of the fusion at the introduced thrombin site + immunoprecipitation (only small scale)	Application of method to human αβ TcR to characterize complexes with MHC and superantigen (31)	Method seems to be generally applicable Functional assay of membrane-bound material (signal transduction via the ζ-chain fusion part)	(26)
γ/δ TcRs						
IgG1 (hinge C_{H2} C_{H3}) fusion T195/BW, transient in COS-7	0.6 mg/10^7 cells	Immunoprecipitation	Protein A affinity column	None		(45)
Translational stop before membrane G9, in CHO cells	1–5 mg/ml in medium	Sandwich ELISA (as IRMA)	Immunoaffinity column (anti-Vγ)	None		(40)

The first column "TcR used and expression strategy" indicates first which strategy was used, second, which TcR clone was used, and third, which cell line was used for the expression. Cell lines were stably transfected if not mentioned otherwise. The second column "yield" gives the yield reported in the publication. Claims by the authors without any numbers in the publication are given in parentheses. The third column "characterization" briefly describes how the correct folding of the TcR fragments was monitored; antibodies used in different immunological techniques are claimed to be conformation specific. The fourth column "purification" indicates how purification of the scTcRs was achieved. The column "application" gives applications for which the TcR fragments have been successfully used. Some major advantages of those methods which produce biologically active material are mentioned in the next to last column. Since the publications use different TcR sequences (and most publications include only one sequence), care should be taken in generalizing the results (see text).

ment is considerably different from the ER (as recently reviewed by Wülfing and Plückthun [38]), and low folding yields can be observed, again in a sequence-dependent manner [14, 16, 17]. Several authors [32, 35, 36] have thus attempted to refold the insoluble protein from the periplasm.

Evaluation of the *E. coli* Systems

E. coli has a heat-shock system that very efficiently removes misfolded, aggregated material [39]. In wildtype strains, very low yields of TcR fragments are seen, even for strategies producing insoluble material [36]. In strains that are devoid of the major heat-shock protease Lon and the heat-shock sigma-factor σ^{32}, insoluble, aggregated scTcR accumulates [14]. Thus, the symptom (total degradation) but not the cause (poor folding) is cured in these strains, and still no significant amount of soluble full-length TcR is seen. We have shown that folding problems indeed limit the production of those scTcRs investigated in *E. coli*. Therefore, one important goal is to improve *in vivo* folding yields [14]. We try to achieve this by using the plasmid-based coexpression of components of the folding apparatus of *E. coli*. This strategy allows us to produce soluble scTcRs which otherwise could be obtained only in minimal quantities (CR15 scTcR [14]). In a few TcRs (such as 1934.4 [16, 17] and P14 [14]), the intrinsic folding capability allows for efficient folding of the scTcRs even without additional folding assistance. We have argued [14] that this might be related to the number of charges in the intrinsically more unstable α-chain. Producing correctly folded material is rewarded by very simple purification schemes [16, 17]. The expression of soluble TcR fragments is thus the method of choice for us. However, understanding has not yet advanced to a point where the method is highly efficient for any sequence of choice.

If the secretion strategy does not produce the folded protein in the periplasm in satisfactory yields, there is another possible approach to avoid the degradation of the insoluble material: the production of TcR fragments at a sufficiently high rate that the protein forms inclusion bodies and becomes largely inaccessible to proteases [34]. This strategy seems very straightforward, but Kurucz and colleagues [34] have reported that different expression clones carrying the same construct vary considerably in expression yields, only one giving the yields reported. This seems to indicate that the *E. coli* genome or the plasmid has to acquire some uncharacterized mutation to allow the production of this scTcR at a high yield. Furthermore, one of us tried to apply the strategy of Kurucz and colleagues [34] to another, closely related TcR sequence and found that the expression plasmids could not be maintained in *E. coli* without deletion of the TcR sequence (Wülfing and Davis, unpublished results). The instability might be — again in a sequence-dependent way — related to folding intermediates that titrate certain chaperones or other cell proteins. At this point, it seems impossible to predict whether a TcR fragment that is produced in an insoluble state will form inclusion bodies at high yields (such as [34]), be degraded (such as [36]), or be so toxic that it cannot be produced at all.

In summary, the choice of a prokaryotic expression system seems to depend upon the sequence of the scTcR. The more hydrophilic it is, the more likely a soluble approach is to succeed [14, 16, 17]. Factors governing the success of an inclusion body approach still have to be delineated.

Introduction to Eukaryotic Expression

All eukaryotic expression experiments with soluble TcR fragments have made use of the secretory pathway emulating the normal assembly pathway of the complete membrane-bound TcR in the T cell. The material produced is functional [26–29]. Eukaryotic cells are usually less convenient to handle than bacterial cells, and a slow growth rate and lower transformation efficiencies make them more cumbersome for the characterization of mutants. Most published protocols use stable transfection as the method to introduce the recombinant genetic information, that is, integration of the genetic information into random sites in the genome of the host cell line. Since the achievable transcriptional activity depends on integration in particular locus control regions, expression levels between different clones vary significantly, making it necessary to screen for high expressing clones, and making any comparison of the intrinsic merits of various strategies very difficult.

Three different strategies have been pursued in eukaryotic systems. In the simplest case, a translational stop codon was introduced behind the second extracellular TcR domain, in order to obtain the extracellular part of the TcR as a soluble fragment directly. Success has only been reported with a γ/δ TcR [40]. Attempts to produce α/β TcR fragments in an analogous fashion failed, although the mechanistic reason has not been elucidated [41]. There is speculation that γ/δ TcRs share greater similarities with antibody Fab fragments than do the α/β TcRs [42, 43], although this is not readily apparent when comparing framework sequences.

In the second strategy, the extracellular portion of the TcR has been fused to antibody constant domains to facilitate the apparently difficult heterodimer assembly (for example [27, 44, 45]). This leads to the secretion of the fusion protein, but the assembly yield of the heterodimer still seems to be very sequence dependent [27, 41].

With the third strategy, the TcR fragments have been expressed on the surface of eukaryotic cells with an artificial membrane linkage (GPI [15, 46], CD3 ζ-chain [26]) and have then been cleaved by an externally added enzyme in order to obtain soluble material. These approaches seem to facilitate the assembly of the heterodimer, suggesting that the assembly of the TcR is somehow guided by the transmembrane domain, which is missing in the soluble constructs [21]. For reasons of limited space on the surface of a cell, membrane-bound expression may result in expression yields that are lower than the

yields of secretion systems. Using membrane-bound expression, the remaining drawbacks of eukaryotic systems are their more tedious handling and lower yields.

Evaluation of Eukaryotic Systems

For eukaryotic expression systems, specific MHC/peptide recognition of the recombinant material has been demonstrated for secreted [27] and membrane-bound material [26, 28, 29]. Nevertheless, the success of the secretion of α/β TcR fusions with immunoglobulin constant domains shows strong sequence dependence [41]. Furthermore, the yields from different clones carrying the same expression vector varied from none to considerable secretion [27]. Apart from the typical eukaryotic variations due to different transcription levels, this may again hint at folding or assembly problems. Overcoming inefficient folding and assembly thus seems to be the central theme in eukaryotic expression, too. Using membrane-bound recombinant TcR fragments this goal now seems to be achieved [15, 26, 46]. Engel and coworkers [26] are the only authors that have so far reported the successful production of more than one TcR heterodimer in eukaryotes.

General Evaluation of Prokaryotic and Eukaryotic Systems

At this point, it is still difficult to compare the different approaches, even within each category. This is mainly due to the fact that, for most systems, only one TcR sequence has been studied. It is therefore very difficult to evaluate whether the different approaches are generally applicable, which of course is the crucial question for anyone trying to construct soluble TcR fragments. The doubts on general applicability are particularly large for those approaches that use "standard" expression technology [17, 27, 34] because of the already existing examples where this has not been successful. Taking into account the importance of soluble TcR fragments, it is more than surprising that so few publications exist that use these generally available techniques, and it suggests that several other researchers might have unsuccessfully tried these approaches with their sequences. Our results, in fact, indicate that the two standard *E. coli* approaches [17, 34] are very sequence dependent [14] (see above). Similar problems have been reported with the standard eukaryotic approach [41]. In our opinion, therefore, generally applicable approaches will have to take into account the problems of the natural biogenesis of the TcR [14, 26].

A comparison of prokaryotic with eukaryotic approaches is also still difficult. The main reason for this is that different types of fragments have been produced in the two systems. Eukaryotic systems have so far used Fab analogs, and the biological activity of the eukaryotically produced fragments has been shown. In *E. coli,* by contrast, only Fv analogs have so far been produced. Judging from the experience with antibody-fragment expression [4], it seems likely that TcR Fab analogs will be produced only in lower amounts than Fv analogs in *E. coli.* Another question is whether scTcRs form stable entities at all. From their expression behavior, we tend to think that the answer to this question will prove to be positive, even though the exceptionally high sensitivity to proteolysis of one of the scTcRs studied by us [14] leaves some doubts whether all scTcRs are stable. To date, the biological functionality of the recombinant material produced in *E. coli* has only been demonstrated qualitatively. Judging from the recognition of the recombinant material by conformation-specific antibodies, we are optimistic that functional TcR fragments can be produced in *E. coli.*

Summary

The TcR is a receptor whose expression is highly regulated and which has to interact with several other molecules. Expressing parts of it as recombinant material thus poses special challenges regarding folding, assembly, and transport. This was reflected by the fact that standard expression strategies (for example [17, 27, 34]) worked only for specific TcR sequences and could not be generalized. Understanding the specific difficulties in TcR biogenesis has now led to more general expression strategies [14, 15, 26]. After numerous initial problems, eukaryotic systems for the expression of Fab analogs of TcRs are now well established. *E. coli* expression systems are now well understood, but still await the ultimate proof of biological functionality. As soon as this functionality can be shown, the ease of use of bacterial systems might make them the systems of choice.

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Christoph Wülfing is with the Department of Microbiology and Immunology, Beckman Center, Stanford University, Stanford, CA 94305-5428, USA. Andreas Plückthun, to whom correspondence should be sent, is at the Biochemisches Institut der Universität Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland.