Evolution of interleukin-15 for higher E. coli expression and solubility

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Directed evolution was used to generate IL-15 mutants with increased solubility and cytoplasmic over-expression in Escherichia coli. A protein solubility selection method was used in which the IL-15 gene was expressed as an N-terminal fusion to chloramphenicol acetyltransferase (CAT) as reporter protein. Clones that grew in the presence of high concentrations of chloramphenicol were then screened by ELISA to assay the binding activity of the IL-15-CAT fusion to the IL-15Rα/β/γ complex. In vitro expression and solubility

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expression of IL-15. Bacterial expression of IL-15 results in the formation of mammalian cells (~283 µg/l in human kidney cell lines or insect cells) and its tendency to aggregate at high concentration. Bacterial expression of IL-15 results in the formation of inclusion bodies, which impose an additional step of solubilization and in vitro refolding of limited yield due to the presence of two disulfide bonds in IL-15 (Olsen et al., 2007).

In this report, we have used directed evolution in an attempt to generate IL-15 mutants with increased solubility and cytoplasmic over-expression in E. coli. We used a protein solubility selection method in which the IL-15 gene is expressed as an N-terminal fusion to chloramphenicol acetyltransferase (CAT) (Sieber et al., 2001; Seitz et al., 2007). This reporter protein enabled E. coli to grow on chloramphenicol (cam) when the fusion protein had sufficient solubility. Two variants of IL-15 were selected for their improved solubility and further characterized for their binding to IL-15Rα and their ability to stimulate the T-cell growth response. One of the mutants was shown to bind as strongly as native IL-15 to IL-15Rα and to act as an effective agonist of IL-15.

Keywords: agonist/bacterial expression/CAT selection/IL-15/solubility

Introduction

Interleukin-15 (IL-15) is a pleiotropic cytokine that plays a pivotal role in both innate and adaptive immunity (Alpdogan and van den Brink, 2005). IL-15 is a member of the four-α-helix bundle family of cytokines, and was first identified by its ability to mimic the function of interleukin-2 (IL-2), with which it is closely structurally related (Grabstein et al., 1994). Both cytokines can stimulate the proliferation and differentiation of natural killer (NK), T and B cells. However, IL-15 has been shown to have distinct functions in NK cell development, memory T cell homeostasis and innate immune responses (Carson et al., 1994; Zhang et al., 1998; Li et al., 2001).

The cell-surface receptor for IL-15 is trimeric and shares with IL-2 the IL-2Rβ/γ chain (γc) receptor complex for cell signaling. In addition, the two cytokines use a non-transducing α chain (IL-2Rα and IL-15Rα, respectively) as a cytokine-specific binding element. The α-receptors are characterized by the presence, in their extracellular part, of conserved structural motifs (so-called ‘sushi’ domains) which comprise the cytokine-binding site. The IL-15Rα chain binds IL-15 with high affinity (100 pM) (Anderson et al., 1995), and it is proposed that it is the IL-15/IL-15Rα complex that recruits the IL-2Rβ/γ signaling complex.

IL-15Rα has only one N-terminal sushi domain, which is necessary and sufficient for high-affinity IL-15 binding. IL-15 is believed to act as a soluble protein that interacts with the heterotrimeric IL-15Rα/IL-2Rβ/γ receptor expressed at the surface of target cells (cis-activation). However, other studies have suggested that IL-15 could also act in ‘trans’ (Dubois et al., 2002; Chirifu et al., 2007). Indeed, recent data have demonstrated that IL-15Rα from activated monocytes presents IL-15 in trans to IL-2Rβ/γ-expressing cells such as CD8+ memory T cells and NK cells (Mortier et al., 2008). This process would be one of the key mechanisms within the immunological synapse that lead to proper cell activation and proliferation.

In vitro and in vivo studies have highlighted the specific role of IL-15, which is produced by stromal and antigen-presenting cells, in the development of innate immune cells (NK, NK-T and γδ T cells), for the early steps of T and NK cell activation, and in the survival of memory CD8+ T cells. Accordingly, a number of diseases have been identified (inflammatory diseases, infectious diseases, transplant rejection, cancer and immunodeficiency) in which targeting the IL-15 system is of clinical relevance, and in which the development of agonists or antagonists is of potential benefit (Cheever, 2008; Bessard et al., 2009). The progress of such therapeutic benefit is dependent on the availability of IL-15 and its variants. Unfortunately, the production of IL-15 is limited by its very low expression level in human kidney cell lines or insect cells and its tendency to aggregate at high concentration. Bacterial expression of IL-15 results in the formation of inclusion bodies, which impose an additional step of solubilization and in vitro refolding of limited yield due to the presence of two disulfide bonds in IL-15 (Olsen et al., 2007).

In this report, we have used directed evolution in an attempt to generate IL-15 mutants with increased solubility and cytoplasmic over-expression in E. coli. We used a protein solubility selection method in which the IL-15 gene is expressed as an N-terminal fusion to chloramphenicol acetyltransferase (CAT) (Sieber et al., 2001; Seitz et al., 2007). This reporter protein enabled E. coli to grow on chloramphenicol (cam) when the fusion protein had sufficient solubility. Two variants of IL-15 were selected for their improved solubility and further characterized for their binding to IL-15Rα and their ability to stimulate the T-cell growth response. One of the mutants was shown to bind as strongly as native IL-15 to IL-15Rα and to act as an effective agonist.
agonist of IL-15. These results also show that a few mutations (≤5) can strongly enhance IL-15 solubility while keeping its biological activity.

**Materials and methods**

**Construction and cloning of the fusion gene**

An NdeI-6× His sequence and the thrombin cleavage site were added at the 5’ and 3’ ends, respectively, of the FlagXa IL-15 sequence of pETFlagXaIL-15 (Mortier et al., 2006) by two successive PCR reactions using first the primers FuILfor1 CATCACCACAAAGCTTTAGAAGGGCTTAAC and FuILrev1 CCGATCCACGGAGAAGAGAAGGCTTAAC and secondly FuILfor1 and FuILrev2 CTAACCCACCAGATCCACGCGCAGAACCAG. The reporter gene CAT encoding CAT was amplified from plasmid pACYC184 (New England Biolabs) with primers FuCATfor1 TCTGGTTTTCCGCGTGGAT and FuCATrev AGGTCCGTTAACTCGAGTTATTACGCCCCGC. The fusion gene Ilcat was obtained by overlap PCR from these two amplicons using primers FuILfor2 and FuCATrev (see above). This fusion gene Ilcat was then inserted in frame with the His sequence and the thrombin cleavage site into the HindIII/XhoI site of pILCAT as shown in Fig. 1.

**Construction of mutant libraries**

Two libraries of mutants IL-15 were generated by error-prone PCR from the plasmid pILCAT using the following conditions: the reaction mixture (50 μl), containing 20 μM of primer FuILfor1 and FuILrev2, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 7 mM MgCl2, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dTTP, 1 mM dCTP, 5 U of Taq polymerase (Promega) and 10 ng of pILCAT (with 0.5 mM MnCl2 for library 1 or without for library 2), was submitted, after denaturation for 5 min at 94°C, to 25 cycles of 94°C 45 s, 50°C 45 s, 72°C 1 min and achieved by a final extension at 72°C for 5 min. The generated amplicons were digested by HindIII and BamHI, ligated in plasmid pCAT and used to transform the E. coli strain DH5α.

**Selection of IL-15 mutants for solubility**

Before plating, the DH5α-transformed cells were grown for 1 h in LB liquid medium and 1 h in LB containing Ampicillin (Amp) 100 μg/ml and IPTG 1 mM.

For the first library, the cells were then grown overnight at 37°C on LB medium plates containing IPTG 1 mM, Amp 100 μg/ml and cam 50 μg/ml. The bacteria expressing wild-type IL-15-CAT fusion did not grow at this cam concentration. The obtained colonies were subsequently replated on Petri dishes containing LB medium, Amp, IPTG 1 mM and increasing concentrations of cam (50–300 μg/ml).

For the second library, DH5α cells were then grown overnight at 37°C on LB medium plates containing Amp 100 μg/ml and cam (100 or 200 μg/ml) without IPTG. The obtained colonies were then replated on Petri dishes containing increasing concentrations of cam (200–1500 μg/ml). The clones growing at cam concentrations above 200 μg/ml were retained for in vitro screening.

**In vitro screening of IL-15 mutants for solubility and functionality**

The selected clones were cultivated in 1.4 ml of LB medium at 37°C with Amp 100 μg/ml and cam 50 μg/ml. The expression of the fusion protein was induced after 3 h by addition of 100 μM IPTG and cultivated overnight at 30°C. The cells were harvested by centrifugation and lysed in buffer containing Tris 50 mM, NaCl 150 mM, pH 8, lysozyme 100 μg/ml, benzonase (1 U/ml), MgCl2 10 mM and PMSF 0.5%. After incubation for 15 min at room temperature, the suspension was submitted to three cycles of freezing and thawing and centrifuged. The soluble fraction was then tested by ELISA.

To screen mutants for their solubility, a sandwich ELISA test was used with Mab247 (anti-human IL-15 IgG, R&D system), dilution 1 μg/ml, as the capture antibody and a two-step detection system with a polyclonal rabbit anti-CAT IgG (Sigma) and a goat anti-rabbit IgG conjugated with peroxidase (Sigma). The epitope of Mab247 was located at the C-terminal part of IL-15 at the end of the D-helix (Bernard et al., 2004) and binding of Mab247 to IL-15 did not interfere with binding to IL-15Rα.

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**Fig. 1** Cloning region of pCAT vector (only important elements are represented). Fx, cleavage site of Factor X; T, cleavage site of thrombin.
To screen IL-15 mutants for their functionality, we performed a second ELISA test using the IL-15Rα Sushi domain, previously produced using Baculovirus/SF9 system (Bouchaud et al., 2008), as the capture element and a two-step detection system using Mab247 and a peroxidase-conjugated donkey anti-mouse IgG (Jackson) as the secondary antibody. The absorbance was determined at 450 nm with OPD substrate using an iEMS analyzer (Labsystem).

Subcloning, expression and purification of IL-15 mutants

The selected IL-15 sequences were subcloned in pET22b+ and the resulting recombinant vectors were used to transform Origami B cells.

The Origami B cells transformed by recombinant plasmids pET22b+ were cultivated in 21 of LB containing Amp 100 µg/ml, kanamycin (Kan) 50 µg/ml and tetracycline (Tet) 15 µg/ml at 37°C. At OD₆₀₀nm 0.7, 100 µM IPTG was added and cells were grown for 5 h at 30°C. The cells were harvested by centrifugation (6000 g, 20 min at 4°C) and the pellet was frozen at −20°C. The cell pellets were resuspended in 20 ml of lysis buffer containing Tris 50 mM, NaCl 500 mM, imidazole 10 mM, pH 7.5, in the presence of a cocktail of antiproteases (Complete Mini, EDTA-free, Roche) and disrupted using Emulsiflex-C5 (Avestin). The soluble fraction was recovered by centrifugation at 13000 g for 30 min at 4°C, then the soluble fraction was purified by Ni NTA metal chromatography (Qiagen) and gel filtration on Superdex 75 (GE Healthcare).

Western blot analysis

Soluble fractions of WT IL-15, IL-15 mutant 38 and IL-15 mutant 253 (100 µg protein) were separated by 15% SDS–PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). After saturation with PBS/5% milk, the membrane was incubated with primary antibody directed against HisTag (HisProbe H-15, Santa Cruz) and revealed with secondary antibody anti-rabbit-HRP (Jackson). Specific proteins were visualized with a chemiluminescence system using BM Chemiluminescence Blotting Substrate (Roche), according to the manufacturer’s instructions.

Surface plasmon resonance

The surface plasmon resonance (SPR) experiments were performed at 25°C with a BIAcore 3000 biosensor (GE Healthcare, Chalfont St Giles, UK). The IL-15Rα soluble domain (sIL-15Rα), previously produced using the Baculovirus/SF9 system (Bouchaud et al., 2008), was covalently linked to CM5 sensor chips using the amine coupling method in accordance with the manufacturer’s instructions, and the binding of increasing concentrations of IL-15 was monitored as previously described (Mortier et al., 2006). Competition BIAcore analysis was performed under mass transport limitations as described previously (Karlsson, 1994) using a CM5 sensor chip coated with 4000 Resonance Units of sIL-15Rα. Competition studies were carried out by co-incubation of IL-15 WT or IL-15 mutant with sIL-15Rα at increasing concentration for 1 h at 23°C before injection. Initial slopes (slopei) were determined from the first derivative at t = 0 of the equation of binding interaction in the absence of mass transport limitation:

$$R = R_{\text{max}} \left( \frac{k_{\text{on}} \times C}{k_{\text{on}} \times C + k_{\text{off}}} \right) \left(1 - e^{-((k_{\text{on}} \times C + k_{\text{off}})t)} \right)$$

Slope, obtained from each mixture injected were plotted against the concentration of the sIL-15Rα added. The concentration of IL-15 WT or IL-15 mutant was obtained by extrapolation of the minimal concentration of sIL-15Rα responsible for no interaction response. The same method was applied to determine the $K_d$ value of each mutant. IL-15 mutant and IL-15 WT were incubated at a fixed concentration (2 nM) with various concentrations of sIL-15Rα. In this case, slopes plotted against the concentration of sIL-15Rα were fitted by a non-linear regression with the following equation:

$$\text{Slope} = \frac{slope_0}{B \times (B - (K_A + A + B/2) + (0.25 \times (A + B + K_D)^2)} \left( -A + B \right))^{1/2}$$

where slopei is the initial slope at a given concentration of sIL-15Rα (B), slope0 the maximal slope in the absence of sIL-15Rα (B), A the total concentration of IL-15 WT or mutant, B the total concentration of sIL-15Rα, $K_A$ the association constant and $K_D$ the dissociation constant. The BiaEval 4.1 software was used to fit the data.

Binding assays

Human IL-15 was radiolabeled with ¹²⁵I-labeled iodine (with a specific radioactivity of ~8000 cpm/finmol) using a chloramine T method (Tejedor and Ballesta, 1982). To measure inhibition of IL-15 binding to IL-15Rα on TF-1 (ATCC CRL-2003), cells were starved for 1 h in medium without cytokine and washed twice in phosphate-buffered saline, 0.5% bovine serum albumin and 2% FCS. They were then seeded in a multi-well plate at 10⁶ cells/well in 50 µl and incubated for 60 min at 4°C in the presence of increasing concentrations of IL-15 WT or mutant. Then, a fixed concentration of iodinated IL-15 was added for 90 min at 4°C. Non-specific binding was measured in the presence of a 100-fold excess of unlabeled IL-15. Cell-bound and unbound fractions were determined, the non-specific binding component (<6%) was subtracted from total binding and a regression analysis of the binding data was achieved using a one-site equilibrium binding equation (Graphit; Erithacus Software, Staines, UK).

Proliferation assays

The proliferation-inducing activity of IL-15 and mutants was assessed by [³H] thymidine incorporation on Kit225 cells (Hori et al., 1987) or CTLL-2 (ATCC number: TIB-214™). Cells were maintained in the culture medium for 3 days, washed and starved for 16 h in the same medium without cytokine. They were plated at 10⁴ cells in 100 µl and cultured for 48 h in the medium supplemented with increasing concentrations of rIL-15, HisTag-IL-15 WT, IL-15 mutant 38 and IL-15 mutant 253. Cells were then pulsed for 16 h with 0.5 µCi/well of [³H] thymidine and harvested onto glass fiber filters before the cell-associated radioactivity was measured (betaPlate).
Results

Screening IL-15-CAT fusion for expression and solubility in E. coli cytoplasm

Recombinant IL-15 expressed in the cytoplasm of E. coli cells is mainly produced in an insoluble form as inclusion bodies (Olsen et al., 2007; Zhu et al., 2009). To increase IL-15 solubility and stability, we applied random mutagenesis followed by selection in vivo using a CAT reporter system (Sieber, 2003). Escherichia coli cells expressing fusions of a more stable and soluble IL-15 variant were assumed to exhibit increased resistance to cam. To generate improved variants of IL-15, the IL-15 gene was submitted to random mutagenesis using error-prone PCR. Two libraries were constructed with different numbers of mutations per gene by adding or not MnCl₂ (0.5 mM) to the PCR mixture. The resulting libraries were ligated in a pCAT plasmid upstream of the CAT gene (Fig. 1), and then used to transform DH5α strains. The first library, which contained up to five mutations per gene (Table I) as revealed by sequencing, was selected on successive medium plates containing IPTG (1 mM) and increasing cam concentrations (50, 100, 200 and 300 μg/ml). Cells transformed with WT IL-15-CAT fusion did not grow above 40 μg/ml cam. The second library (10⁴ clones), which contained only one or two mutations per gene, was screened without IPTG on successive medium plates containing cam concentrations of 200, 400, 800, 1200 and 1500 μg/ml. The over-expression of IL-15-CAT fusion was toxic for the E. coli cells so that better resistance to cam was observed when cells were grown in the absence of IPTG. In this condition, cells expressing WT IL-15-CAT fusion could grow with cam up to 150 μg/ml cam. The second library (10⁴ clones), which contained only one or two mutations per gene, was screened without IPTG on successive medium plates containing cam concentrations of 200, 400, 800, 1200 and 1500 μg/ml. The over-expression of IL-15-CAT fusion was toxic for the E. coli cells so that better resistance to cam was observed when cells were grown in the absence of IPTG. In this condition, cells expressing WT IL-15-CAT fusion could grow with cam up to 150 μg/ml cam and variants were selected on a plate containing up to 1500 μg/ml cam.

This selection process is known to give numerous false-positive clones due to the loss of part of the insert (Seitz et al., 2007) or the appearance of a stop codon and re-initiation of translation in the reading frame variant (Sieber et al., 2001). Thus, an additional screening process, based on ELISA tests, was carried out on the clones that were selected at high cam concentrations (~400 clones). The first ELISA test was designed to assay the amount of soluble IL-15-CAT fusion expressed in the cell extracts. It used a sandwich assay in which an anti-IL-15 antibody (Mab247) was used to capture the fused IL-15-CAT, which was then revealed by a polyclonal anti-CAT antibody. The second ELISA test aimed to assay the functional activity of IL-15-CAT fusion by using the IL-15Rα Sushi domain (Bouchaud et al., 2008) as the capture reagent and Mab247 for detection. Figure 2 presents the results of this second-step screening process applied to the variants from the two libraries. As expected, variants that exhibited a higher expression level and solubility were found among the clones that were grown at high cam concentration (M254, M266, M276). However, most of the variants selected at high cam concentration had lost their binding activity against IL-15Rα. The sequencing of clones M254, M266 and M276 revealed that a large part of the IL-15 gene was deleted while the CAT gene remained (Table I).

Solubility of selected IL-15 variants

Only clones (M38, M40, M56 and M253) that had retained significant binding to IL-15Rα were further analyzed by sequencing and subcloning the gene without the CAT fusion. The M38 sequence was corrected by replacing the amber codon TAG by the normal CAG codon for Gln (mutation K41Q). After subcloning, only M38 and M253 clones were further analyzed. The IL-15 protein variants were expressed in E. coli Origami B. This strain, which has inactive thioredoxin reductase (Trx-) and glutathione reductase (gor-), was chosen to allow the oxidation of the two IL-15 disulfide bonds within the cytoplasm (Derman et al., 1993). The control expression of IL-15 in standard BL21 strains showed a lower expression of soluble and active IL-15 than in Origami B (data not shown). However, the WT IL-15 was also found to mainly in the insoluble fraction of the cell extract (95%) in Origami B (Fig. 3). The soluble form was barely detected by western blot and its concentration in the soluble fraction was as low as 50 μg/l in cell culture (Fig. 3). In contrast, M38 and M253 mutants were well expressed (600–950 μg/ml) with a good proportion (52% and 80%, respectively) in the soluble, cytosolic fraction. Surprisingly, the M38 variant gave better expression results in Origami B strain than those expected from the screening results on the fusion protein (Fig. 2). This difference was probably due to both a strain effect and a difference in the expression format. However, we cannot exclude that an inefficient suppression of the non-sense codon TAG by suppressor-tRNAs would result in lower expression of the fusion protein in DH5α strains. The estimated soluble concentration of IL-15 variants in Origami B cell culture was 12- to 18-fold higher, respectively, than for WT IL-15. It is interesting to note that the most soluble variant, M253, contains only one mutation in the sequence (F103L).

Binding of IL-15 variants to IL-15Rα

In the next step, the two soluble variants of IL-15 were assayed for biological activity and first for their ability to bind in vitro the IL-15Rα soluble domain (sIL-15Rα). The soluble cell extracts were purified by metal chelate affinity chromatography (Ni-NTA gel) followed by a gel filtration. The resulting purified fractions of WT IL-15 and variants M38 and M253 were assayed for their IL-15Rα binding. The different forms of IL-15 were first compared for their ability to compete with radiiodinated IL-15 binding (used at a fixed concentration of 100 pM) to IL-15Rα expressed by the human cell line TF-1 (Farner et al., 1997). Only the M38 variant

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<th>Table I. Sequence of selected mutants</th>
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<td>IL-15 variant</td>
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chloramphenicol concentration at which the IL-15-CAT fusion proteins were selected.

Suppressive stop codon.
completely inhibited IL-15 binding to TF1 cells (Fig. 4). Its inhibitory power (IC50 = 150 pM) was close to that of native IL-15 (IC50 = 90 pM), indicating that the five mutations of this variant did not significantly alter the binding to IL-15Rα.

These results were confirmed by kinetic analysis of the binding of IL-15 variants to sIL-15Rα by SPR. $K_d$'s for WT IL-15 and M38 were estimated by slope affinity solution at $2.5 \times 10^{-10}$ and $3.0 \times 10^{-10}$ M, respectively.

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**Fig. 2** Results of second-step screening process of IL-15-CAT fusions when expressed in E. coli DH5α. The vertical axis (solubility test) corresponds to the ELISA absorbance of cell extracts incubated in plates coated with Mab247, revealed with an anti-CAT polyclonal antibody. The horizontal axis (functionality test) corresponds to the ELISA absorbance of cell extracts incubated in plates coated with IL-15Rα and revealed with Mab247. Filled squares represent clones selected at cam $<400 \mu$M; filled circle, clones selected at cam $800 \mu$M; filled triangle, clones selected at cam $1200 \mu$M; open square, clones selected at cam $1500 \mu$M.

**Fig. 3** Representative experiments of total expression (1) and soluble expression (2) of WT IL-15 and mutants M38 and M253 in Origami B. Total homogenates were separated by SDS–PAGE and immunoblotted with anti-HisTag antibodies. Relative amounts of IL-15 were estimated with a densitometer and the value was reported in the table. The quantity of expressed soluble and functional IL-15 was determined by active-site titration using SPR and soluble IL-15Rα as competitor after IMAC and size-exclusion chromatography purification.
Taken together, these results show that the M38 variant has kept the binding properties of native IL-15 to IL-15Rα, while the F103L mutation of the M253 variant has a dramatic effect on IL-15Rα affinity.

Proliferation of Kit225 cells in response to IL-15 mutants

In order to further evaluate the activity of the soluble IL-15 variants, they were compared with the native IL-15 for their capacity to induce the proliferation of the human lymphoma cell line Kit225, which expresses endogenous IL-15Rα, IL-15/IL-2Rβ and IL-15/IL-2Rγ chains. Figure 5A shows representative data for the activity of WT IL-15, and M38 and M253 variants toward Kit225 cells at 37°C. The proliferative responses of WT IL-15 and M38 were observed in the 10 pM–1 nM concentration range, thus reflecting activation of the high-affinity IL-15Rα/β/γ complex. M38 exhibited a slightly lower proliferative activity than native IL-15, in agreement with their small difference in binding affinities to IL-R15α (Fig. 4). In contrast, the M253 variant, which lost its binding capacity to IL-15Rα, did not induce high-affinity proliferation of Kit225 cells. It remained, however, able to support proliferation in the 1–100 nM range, reflecting activation of the IL-15Rβ/γ complex.

Interestingly, when CTLL-2 cells expressing mouse IL-15Rα, IL-15/IL-2Rβ and IL-15/IL-2Rγ were used in proliferation assays, the M38 variant was 10-fold less potent than WT IL-15, with EC50 of ~50 and ~7 pM, respectively (Fig. 5B). This suggests that some mutations of the M38 variant would specifically affect the binding of human IL-15 to the mouse (versus human) receptors.

Discussion

The low solubility and the poor expression level of IL-15 have long delayed the appearance of structural information on this cytokine and its interaction with its receptors. Some insight has been gained only recently from the crystal structure of the IL-15/IL-15Rα complex (Chirifu et al., 2007; Olsen et al., 2007). Nevertheless, such structural data were obtained after denaturation of the precipitated proteins expressed in E. coli cell-free reaction and a complex refolding scheme. Such processes preclude any IL-15 development for therapeutic use. Therefore, we focused on a strategy that could lead to IL-15 variants with improved solubility, while keeping their biological activity. For this purpose, we used a combination of random mutagenesis and successive screening for biological function.

To evolve IL-15 for increased solubility and stability, we chose an approach in which the randomized IL-15 gene was fused to the CAT gene, and selected for IL-15-CAT fusion proteins that allowed their host cells to grow in the presence of high concentrations of cam. As already pointed out by others (Maxwell et al., 1999; Sieber et al., 2001; Seitz et al., 2007), this selection scheme generates numerous false positives, which necessitates additional screening tests to confirm the improved solubility. Among false-positive clones, some arose from plasmids with extensively truncated IL-15 mutants in frame with the CAT gene, generating CAT proteins with
short N-terminal extensions that did not compromise its high solubility. Although the most soluble clones, as detected by ELISA tests, were found among the strains that grew at high cam concentrations, most of the variants expressed by these clones had lost their binding activity toward IL-15Rα. Most of the active IL-15 variants as revealed by ELISA test involving the sushi form of IL-15Rα (Bouchaud et al., 2008) were obtained from clones growing at intermediate cam concentrations. This illustrates the importance of combining the two screening procedures (solubility and functionality) in order to optimize the selection of variants.

Among the selected variants, M253 was the most soluble although it contains only one mutation (F103L). This variant was almost completely (80%) expressed as a soluble form within the cytoplasm of Origami strains. The mutation F103L is located on helix D of IL-15 (Fig. 6) and, in native IL-15, Phe-103 makes a strong stacking with the only other Phe (Phe-43) of IL-15 on helix B. These two phenylalanines are located in the hydrophobic core of IL-15 and are probably involved in the stability of the four-helix bundle structure of this cytokine (Olsen et al., 2007). The replacement of Phe-103 by a Leu maintains the hydrophobic character of the environment but breaks the stacking between aromatic rings, which probably disturbs the final 3D structure of IL-15. Such effects would explain the lower affinity of the M253 variant toward IL-15Rα or the other receptor chains. However, the much higher solubility of this variant suggests that the formation of the stacking between Phe-103 and Phe-43 in native IL-15 is critical during the folding process and would be responsible for the formation of inclusion bodies in E. coli cells. This result suggests that correlated mutation at position 43 could compensate the F103L mutation and possibly result in functional and soluble IL-15. Interestingly, the Phe-103 is highly conserved in IL-15 from mammals, but F103L mutation is observed in IL-15 sequences of Takifugu rubripes (NCBI accession NP001028220) and Tetraodon nigroviridis (NCBI accession AAR25702) with residue at position 43 (Y and F) nearly conserved.

The variant M38, which contains five mutations (K41Q, L45S, I67T, N79Y, E93A), exhibits high solubility while keeping a biological activity only slightly lower than native human IL-15. The mutations L45S and E93A are located in helix B and in the CD loop, respectively, and are near the binding interface with IL-15Rα, which could explain why this variant has a slightly lower affinity to IL-15Rα. However, due to the small effect on binding, these two residues might not greatly contribute to the binding strength. Furthermore, the mutation L45S, which substitutes a hydrophobic residue at the IL-15 surface by a hydrophilic residue, might be partially responsible for improved IL-15 solubility. Similarly, a better production yield in 293-EBNA cell supernatant has already been observed for the mutation L45D (Bernard et al., 2004). The non-conservative mutation I67T is surprising since it is located in helix C and points out toward the hydrophobic surface of helix B rather than toward IL-15Rβ. Interestingly, the two mutations K41Q and I67T are both observed in IL-15 sequences from Ovis aries (NCBI accession NP8001009734), Bubalus bubalis (NCBI accession Q4GZL1) and Bos taurus (NCBI accession NP776515). The other mutations, N79Y, which is present in Pig IL-15, and E93A, correspond to surface residues located on loops and it is difficult to define their contribution to the improved solubility of M38.

The variant M38 has almost kept the biological activity of wild-type IL-15 on cells bearing human IL-15R. This is probably because the five mutations in M38 affect residues whose side chains are mainly exposed outside the IL-15/IL-15R interfaces, therefore having no significant effect on the native structural conformation of the molecule, nor its receptor binding capacity. Nevertheless, the variant M38 is less potent on cells bearing mouse IL-15R. By analyzing the locations of the mutations in the molecule (Fig. 6), we can speculate that some of them could affect the human versus mouse receptor binding specificity by selectively reducing the binding to mouse IL-15Rα or β. Indeed, residues of the α-chain implicated in the interaction with IL-15 are conserved between the human and mouse (Chirifu et al., 2007; Olsen et al., 2007), whereas variation in the amino acid residue at position 41 of IL-15Rβ (human: Arg, mouse: Leu) has already been reported as responsible for the loss of the agonistic potency of the mutant IL-15N72D on cells bearing mouse IL-15R (Zhu et al., 2009). The mutation at position 67 of the variant M38, being the closest to the IL-15Rβ interface, is therefore probably responsible for the species differences observed in this study.

Taken together, these results show that M38 can be considered as an agonist of IL-15 that is more easily expressed in bacteria in a soluble form.

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