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Preparation and characterization of mouse IL-22 and its four single-amino-acid muteins that act as IL-22 receptor-1 antagonists

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Recombinant mouse interleukin 22 (mIL-22) and its variants encoding four muteins (Y51A, N54A, R55A and E117A) were expressed in *Escherichia coli*, refolded and purified to homogeneity as monomeric proteins by one-step ion-exchange chromatography. The binding of IL-22 and its four muteins to immobilized mIL-22 receptor α1 extracellular domain (mIL-22 Rα1-ECD) exhibited similar affinity, indicating that the single-amino-acid mutations do not affect its binding properties. Similarly, no differences were found in binding to IL-22 binding protein expressed on the surface of yeast cells, although the affinity of all five proteins to the binding protein was higher than that to IL-22 Rα1-ECD. In an *in vitro* bioassay, recombinant mIL-22 stimulated signal transducer and activator of transcription-3 phosphorylation in HepG2 cells, whereas the four muteins were completely (Y51A) or almost completely (N54A, R55A and E117A) devoid of this agonistic activity. Furthermore, the agonistic activity of mIL-22 could be inhibited in a dose-dependent manner by the four muteins with almost identical efficiency. mIL-22 and its Y51A mutein were pegylated by methoxy polyethylene glycol-propionylaldehyde-20 kDa, yielding a mixture of mono (75–80%) and double (20–25%) pegylated proteins. The pegylated proteins showed lower affinity (50 and 25%) toward immobilized mIL-22 Rα1-ECD than their non-pegylated analogs. Wild-type pegylated IL-22 exhibited 5- to 10-fold lower activity in the HepG2 bioassay than its non-pegylated counterpart. Preparation of recombinant mIL-22 antagonists provides new tools for the study of IL-22 activity and of eventual therapeutic means for attenuating its negative effects.

Keywords: antagonist/HepG2 cells/interleukin 22/rational mutagenesis

Introduction

Interleukin 22 (IL-22) plays a potent proinflammatory role in several infectious, autoinflammatory and autoimmune diseases. IL-22 plays a pathogenic role in *Toxoplasma gondii* infection, as IL-22-deficient mice show disease amelioration as compared with their wild-type (wt) counterparts (Munoz *et al.*, 2009). In chronic T-cell-mediated skin inflammation, and particularly in psoriasis, IL-22 has a potent proinflammatory effect. IL-22 levels, in both mice and humans, are greatly elevated within the psoriatic lesions and in the serum, and are in direct correlation to disease severity (Wolk *et al.*, 2006; Haider *et al.*, 2008). Mice transgenic for IL-22 develop spontaneous skin lesions resembling psoriasis (Wolk *et al.*, 2009); in contrast, in several models of dermal inflammation, IL-22-deficient mice develop less severe immune-mediated skin pathology (Zheng *et al.*, 2007; Ma *et al.*, 2008). In fact, IL-22 has been convincingly shown to be a major downstream effector molecule in psoriasis, accounting for the alterations in keratinocyte differentiation and hyperproliferation that stem from T-cell infiltration and results in the formation of psoriatic skin lesions (Wolk *et al.*, 2009). Recently, IL-22 has been suggested to induce other proinflammatory cytokines in skin lesions, such as IL-36 (Carrier *et al.*, 2011). Similarly, IL-22 has been shown to play a pathogenic proinflammatory role in rheumatoid arthritis: IL-22-knockout mice featured a milder form of collagen-induced arthritis (Geboes *et al.*, 2009), while in humans, high levels of IL-22 were found in both the synovial spaces and blood of patients with rheumatoid arthritis (Shen *et al.*, 2009). Similarly, serum IL-22 levels are significantly correlated with clinical and serological severity markers in patients with Sjogren's syndrome (Lavoie *et al.*, 2011), and elevated IL-22 levels in patients with atopic dermatitis have been recently suggested to correlate with disease markers, coupled to locally elevated CCL27 levels within the skin lesions in these patients, possibly contributing to TH2 cell recruitment and progression of inflammation (Hayashida *et al.*, 2011). There is mounting evidence of a link between IL-22 signaling and tumor formation and progression. IL-22 has been suggested to induce lymphoma growth, whereby cells were shown to secrete IL-22 and aberrantly express IL-22 receptor (IL-22R), and the resultant autocrine IL-22 signaling in these cells led to signal transducer and activator of transcription (STAT)-3-dependent enhancement of cell proliferation (Bard *et al.*, 2008). In a recent study, IL-22 levels were shown to be significantly elevated in hepatocellular carcinoma tissues in mice, while IL-22-deficient mice featured slower tumor progression. A direct link between local overexpression of IL-22 and a tendency toward tumorogenesis was recently discovered when mice transgenically overexpressing IL-22 in adipose tissues featured a dramatic tendency toward the development of liposarcomas in the

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periepididymal fat pad following feeding with a high-fat diet, suggesting that a combination of metabolic events such as obesity, coupled with IL-22's proinflammatory effects, may lead to tumor formation (Wang *et al.*, 2011). Such tumor-promoting and other negative effects may be therapeutically exploited by developing a potent and specific IL-22 antagonist, featuring the beneficial effects of reducing several tumor types' resistance to chemotherapy. Interestingly, IL-22 was also shown to be central in the innate and adaptive immune response to both intestinal autoinflammation and pathogenic infection, by a variety of mechanisms including regulation of secretion of antimicrobial factors and promotion of intestinal regeneration and wound healing (Sugimoto *et al.*, 2008; Zenewicz *et al.*, 2008).

IL-22 is a 179-aa-long type II cytokine which, based on similarities in gene sequence, secondary protein structure and receptor use, is a member of the IL-10 cytokine subfamily along with IL-19, IL-20, IL-24 and IL-26 (Pestka *et al.*, 2004; Sabat, 2010). Following discovery of this cytokine's mouse and human homologs in 2000 (Dumoutier *et al.*, 2000a,b; Xie *et al.*, 2000), it has been shown to feature unique roles in exhibiting both pro- and anti-inflammatory activity, as well as in regulating diverse innate anti-microbial functions. The IL-22 protein has been crystallized (Nagem *et al.*, 2002a,b) and, like all other members of the IL-10 family, contains four major and two minor α-helices (Logsdon *et al.*, 2004). The IL-22 signal is transduced by sequential interactions with its receptor IL-22R1 and subsequently with IL-10R2 (Kotenko *et al.*, 2001). The latter subunit stabilizes the preformed interaction and forms a tertiary complex, composed of IL-21R1, IL-10R2 and IL-22, allowing signal transduction (Logsdon *et al.*, 2002, 2004). It should be noted that IL-20 and IL-24 also bind to IL-22R1 leading to their subsequent heterodimerization with IL-20R2 or IL-24R2 (Sabat, 2010). In addition to its interaction with IL-22R1, IL-22 also interacts with soluble IL-22 binding protein (IL-22BP) existing in mammalian sera (Bleicher *et al.*, 2008). Most recently, the four amino acids of IL-22 (Y51, N54, R55 and E117) required for formation of the tertiary complex with IL-10R2 were identified (Yoon *et al.*, 2010). Based on those findings, we hypothesized that such IL-22 Ala muteins will not only be inactive, but will also act as antagonists. To test this hypothesis, we expressed, refolded and purified to homogeneity mouse (m) IL-22 and its four muteins in *Escherichia coli*, tested their activity in two binding assays using immobilized extracellular domain of IL-22R1 fused to Fc (ECD of IL-22R1) and IL-22BP expressed on yeast cell surface and in an *in vitro* cell-based (HepG2 human cells) bioassay, and validated their activity as IL-22 antagonists.

Materials and methods

Materials

Highly pure high-performance liquid chromatography-purified DNA primers were ordered from Syntezza (Jerusalem, Israel); Gibco™ RPMI-1640 medium and Gibco™ Dulbecco's modified Eagle's medium (DMEM) were from Invitrogen (Carlsbad, CA, USA); fetal bovine serum (FBS) and Pen-Strep solution (5×10^4 U/ml penicillin, 50 mg/ml streptomycin), were from Biological Industries Ltd (Beit Haemek, Israel); Superdex™ 75 HR 10/30 column and Q-Sepharose were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden); antibiotic–antimycotic solution (5×10^4 U/ml penicillin, 50 mg/ml streptomycin, 0.125 mg/ml fungisone), NaCl and Tris base were purchased from Bio-Lab Ltd (Jerusalem, Israel). Bacto-tryptone, bacto-yeast extract, glycerol, EDTA, HCl, Triton X-100 and urea were purchased from ENCO Diagnostics Ltd (Petah-Tikva, Israel), and molecular markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Bio-Rad (Hercules, CA, USA). Methoxy polyethylene glycol (PEG)-propionylaldehyde-20 kDa (mPEG-propionyl-ALD-20 kDa) was purchased from Jenkem Technology Inc. (Allen, TX, USA) and recombinant mIL-22R1 extracellular domain (mIL-22R1-ECD) was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Peroxidase-conjugated streptavidin was from Jackson ImmunoResearch (West Grove, PA, USA) and 3,3′,5,5′-tetramethylbenzidine (TMB) was from Dako (DakoCytomation, Copenhagen, Denmark). Plasmid pCT302 and yeast strain EBY100 of *Saccharomyces cerevisiae* were provided by Dr E.T. Boder from the University of Tennessee, Knoxville, Tennessee. Monoclonal antibody (mAb) 9e10 was purchased from Covance (Emeryville, CA, USA); fluorescein isothiocyanate (FITC)-labeled F(ab')2 goat anti-mouse IgG was from Chemicon (Temecula, CA, USA) and streptavidin–phycoerythrin (Strep–PE) conjugate was from Pharmingen (San Jose, CA, USA). The following reagents were prepared in our lab: synthetic dextrose-casamino acids (SD-CAA) medium consisted of 20 g/l dextrose, 6.7 g/l Difco yeast nitrogen base, 5 g/l bacto casamino acids, 5.4 g/l Na_2HPO_4, and 8.56 g/l NaH_2PO_4, sterilized; synthetic galactose casamino acids (SG-CAA) media was as for SD-CAA, but with 20 g/l galactose instead of dextrose. Luria bertani (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, sterilized), terrific broth (TB) (80 g/l tryptone, 160 g/l yeast extract, 33.3 g/l glycerol, sterilized), all other reagents were of analytical grade.

Construction of IL-22 mutant expression vectors (Y51A, N54A, R55A and E117A)

The DNA template used for the construction of IL-22 mutants was wt IL-22 ordered from Entelechon GmbH (Regensburg, Germany) and cloned in the prokaryotic expression vector pMon using NcoI and HindIII sites as described previously for the preparation of leptin mutants (Raver *et al.*, 2002). The expression plasmid encoding wt IL-22 was then modified with the Stratagene QuikChange mutagenesis kit according to the manufacturer's instructions using two complementary primers (Table I) for preparation of four mIL-22 mutants. The procedure consisted of 18 polymerase chain reaction cycles as specified by the manufacturer's manual and the use of *Pfu* polymerase for the reaction. The mutated construct was then digested with restriction enzyme DpnI, which is specific to methylated and hemi-methylated DNA, to digest the template and to select the mutation-containing synthesized DNA. The vector was then transfected into XL1 competent cells. Minipreps from two colonies of each mutant were sequenced and confirmed to contain the desired mutation without any undesired misincorporation of nucleotides. Mon105 competent cells were then transformed with the desired mutated plasmid and used for expression.

Table I. Primers used for the preparation of mIL-22 mutants

Primer	Primer sequence[a]	Modified restriction site[b]	SIN[c]
Y51A-5′	CTAATTTTCAACAGCCA**GCG**ATCGTCAACCGCACAC	PvuI (+)	1
Y51A-3′	GTGCGGTTGACGATCGCTGGCTGTTGAAAATTAG	PvuI (+)	2
N54A-5′	CAGCCATATATCGTC**GCG**CGCACCTTTATGC	PauI (+)	3
N54A-3′	GCATAAAGGTGCGCGCGACGATATATGGCTG	PauI (+)	4
R55A-5′	CAGCCATATATCGTCAA**CGC**AACGTTTATGCTGGC	AclI (+)	5
R55A-3′	GCCAGCATAAACGTTGCGTTGACGATATATGGCTG	AclI (+)	6
E117A-5′	CGTACATGCAGG**C**AGTGGTACCGTTCCTGACT	KpnI (+)	7
E117A-3′	AGTCAGGAACGGTACCACTGCCTGCATGTACG	KpnI (+)	8

[a]Sense primer—5′; anti-sense primer—3′; all mutations are in bold letters.
[b]Successful mutations were monitored appearance (+) of the respective restriction site (underlined).
[c]SEQ ID NO.

Expression, refolding and purification of wt IL-22 and IL-22 mutants

The recombinant wt IL-22 and its mutants, having an additional Met–Ala at their N terminus (Met is cleaved by the bacteria) (Obukowicz *et al.*, 1992), were each expressed in 5 × 0.5 l of TB culture medium at 37°C in 2.5 l flasks. When the absorbance at 600 nm reached 0.9, nalidixic acid was added up to 50 μg/ml, bacteria were grown for an additional 4 h, centrifuged and frozen. Inclusion bodies were then prepared as described previously (Gertler *et al.*, 1998; Raver *et al.*, 2002) and frozen. Thawed inclusion bodies obtained from 1 l of bacterial culture were solubilized in 400 ml of 4.5 M urea and 40 mM Tris base containing 1 mM cysteine and adjusted to pH 11.3 with NaOH. After 2 h of stirring at 4°C, two volumes of double distilled water were added and stirring was continued for an additional 1.5 h. Then the solution was dialyzed against 10 l of 10 mM Tris base pH 10 for 60 h with six external solution exchanges, and applied to a Q-Sepharose column (30-ml bead volume) pre-equilibrated with 10 mM Tris base pH 10. The absorbed protein was eluted in a stepwise manner (50, 100, 150 and 200 mM NaCl in 10 mM Tris base, pH 10). Fractions (50 ml) were collected and protein concentration was determined by absorbance at 280 and 260 nm. The monomer content of each fraction was determined by gel filtration on an analytical Superdex 75 HR 10/30 column. Fractions containing monomeric IL-22 were pooled, dialyzed against $NaHCO_3$ (adjusted to pH 10) to ensure a 4 : 1 (w/w) protein-to-salt ratio and lyophilized.

Determination of purity and monomer content

SDS–PAGE was carried out according to Laemmli (Laemmli, 1970) in a 15% polyacrylamide gel under reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Gel-filtration chromatography was performed on a Superdex 75 HR 10/30 column for determination of wt IL-22 and its mutants with 0.2-ml aliquots of the Q-Sepharose-column-eluted fractions, and on a Superdex 200 HR 10/30 column for determination of pegylated wt IL-22 (PEG-IL-22) and its pegylated mutein PEG-IL-22 (Y51A). Both columns were pre-equilibrated and developed using Tris-NaCl (TN) buffer (25 mM Tris-HCl, 300 mM NaCl, pH 8) at room temperature. The columns were calibrated with leptin (16 kDa), chicken leptin-binding domain (chLBD, 24.5 kDa), leptin–chLBD complex (40.5 kDa), bovine serum albumin (BSA, 66 kDa), and bovine IgG monomer (150 kDa) and dimer (300 kDa).

Determination of circular dichroism spectra

The circular dichroism (CD) spectra in millidegrees were measured with an AVIV model 62A DS circular dichroism spectrometer (Lakewood, NJ, USA) using a 0.020-cm rectangular QS Hellma cuvette. The spectrometer was calibrated with camphorsulfonic acid. The absorption spectra were measured with an AVIV model 17DS UV–visible IR spectrophotometer using a 1.000-cm QS cuvette and correction for light scattering. Lyophilized protein was dissolved in water, dialyzed against 10 mM MOPS-[3-(*N*-morpholino)propanesulfonic acid] buffer, pH 8, for 20 h, and then centrifuged at 11 000 *g* for 10 min. The CD measurements were performed at 25.0°C as controlled by thermoelectric Peltier elements to an accuracy of 0.1°C. The CD spectra were measured in five repetitions resulting in an average spectrum for each protein. Standard deviation of the average CD signal at 222 nm was in the 5% range.

Pegylation of wt mIL-22 and mIL-22 (Y51A) mutant

mPEG-propionyl-ALD-20 kDa was used for pegylation under conditions that favor pegylation of the N-terminal amino group. mIL-22 or mIL-22 (Y51A) (144 mg) was dissolved in 40 ml of 0.1 M Na-acetate buffer (pH 5) and centrifuged at 35 000 *g* for 10 min to remove the insoluble material. Then 0.965 ml of 1 M $NaBH_3CN$ was added to a final concentration of 20 mM and the dissolved protein was conjugated with 0.72 g mPEG-propionyl-ALD-20 kDa dissolved in 7.2 ml of 1 mM HCl. After 20 h of stirring at 4°C, 58 μl of acetic acid (17 M) was added. The solution was stirred for a few seconds, filtered and applied in three consecutive portions of 16 ml onto a Superdex 200 HR 10/30 preparative column, pre-equilibrated with TN buffer pH 10. Fractions containing the mono-pegylated protein as determined by analytical gel filtration and SDS–PAGE analyses were pooled, dialyzed against $NaHCO_3$ to ensure a 4 : 1 protein:salt (w/w) ratio and lyophilized. Protein concentrations (mg/ml) were determined by absorbance at 280 nm using extinction coefficients of 0.26 for a 0.1% (w/v) solution of PEG-IL-22 protein and 0.18 for PEG-IL-22 (Y51A) protein. These values apply to the protein part of the pegylated product.

Biotinylation of mIL-22

mIL-22 (1 mg/ml) was dissolved in phosphate-buffered saline (PBS) pH 7 and incubated with a 10-fold molar excess of the biotinylation reagent sulfo-NHS-LC-biotin for 40 min at room temperature. Excess non-reacted biotin was removed by dialyzing against PBS.

Binding assay using the ECD of mIL-22R1 as a ligand

Biotinylated wt mIL-22 served as the ligand in all competitive-binding experiments, and wt mIL-22 and the respective mIL-22 muteins served as competitors for binding to recombinant mIL-22 R1-ECD. Polystyrene 96-well microtiter plates were coated overnight at 4°C with 100 μl mIL-22 Rα1-ECD (0.5 μg/ml) in PBS pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% w/v Tween 20) and blocked for 2 h at room temperature with PBS containing 3% (w/v) skimmed milk. All further incubations were also carried out at room temperature. Wells were washed again once with PBST and incubated with different concentrations of unlabeled IL-22 mutants (50 μl/well, in three replicates) for 30 min, and then 50 μl of 35 μg/ml biotinylated IL-22 was added to each well for another 90 min. Then the wells were washed three times with PBST and incubated with streptavidin-horseradish peroxidase (1 : 10 000 dilution) in PBST for 1 h. Wells were washed three times with PBST and the reaction was quantified at 450 nm by ELISA Micro-Plate Reader ELx808 (Bio-Tek Instrument Inc., Winooski, VT, USA) using TMB according to the manufacturer's instructions.

Preparation of yeast expressing IL-22BP and evidence of their functionality

Expression plasmid and yeast cells expressing IL-22BP on their surface were prepared by the methodology used previously for preparation of leptin library (Shpilman *et al.*, 2011). A respective protein of interest (IL-22BP in our case) is displayed on the surface of yeast as a protein fused to the Aga2p mating agglutinin protein after subcloning to pCT302 plasmid (Boder *et al.*, 2000) for expression. The Aga2p protein is tethered in the cell wall via disulfide bridges to the Agalp protein, in a yeast strain EBY100 in which Agalp expression is inducible from a single, integrated open reading frame downstream of the GAL1-10 promoter. The schematic fusion topology on the yeast surface is depicted in Supplementary Fig. S1. The presence of the fusion protein on the cell surface may be detected by its ligand-binding. Using fluorescent activated cell sorter (FACS), we documented that IL-22BP expressed on yeast surface interacts with biotinylated IL-22. Interaction with anti-c-myc mAb indicated that the full-size protein is expressed in frame (Supplementary Fig. S2).

Binding assay using yeast expressing IL-22BP

One colony of *S.cerevisiae* strain EBY100 constructed to express IL-22BP was taken from an SD-CAA agar plate and grown with shaking in SD-CAA medium at 30°C for 24 h. Cells were then diluted to $OD_{600}= 1$ (corresponding to $\sim 10^7$ cells) and were induced for 22 h by exchanging the medium with SG-CAA. Then 2×10^6 yeast cells (0.2 OD_{600}/ml) were aliquoted per tube. The cells were washed twice with 500 μl and 1000 μl cold FACS buffer [PBS, 0.05% (w/v) NaN_3, 5% (v/v) FCS] and incubated for 1 h on ice with weak shaking with 1 : 50 diluted anti-c-myc antibody, 1 nM biotinylated wt IL-22 and different concentrations (0.8–500 nM) of wt IL-22 or its four muteins in a final volume of 50 μl. Afterwards the cells were washed again and incubated with GαM FITC (FITC-labeled goat anti-mouse IgG) and Strep–PE antibodies in a final volume of 50 μl for 1 h on ice at 4°C with weak shaking. Then the cells were washed twice with 1000 μl FACS buffer, once with 1000 μl PBS and resuspended in 250 μl PBS for FACS. A distinct population of yeast cells was detected and blotted using forward scatter and side scatter, while a total of 2.5×10^5 yeast cells were collected and analyzed. Subpopulations expressing both the IL-22BP and the c-myc were detected using anti-c-myc mAb 9e10 and goat anti-mouse antibody conjugated with FITC and biotinylated IL-22 and Strep–PE. Cells were analyzed using the BD FACSCanto system (Becton, Dickinson and Company Franklin Lakes, NJ, USA).

Determination of biological activity in HepG2 cells

HepG2 cells were grown at 37°C in 24-well plates (10^6 cells/ml, 0.5 ml/well) in DMEM + 10% FBS for 24 h and then were serum-deprived for 16 h before stimulation with IL-22. Then the cells were incubated at room temperature for 20 min in 0.5 ml serum-free DMEM in the presence or absence of various concentrations of IL-22 (1, 5, 25, 125 ng/ml, containing 0.5 ml serum-free medium) or with 10, 50, 250 and 1250 ng of PEG-IL-22 per well in a 24-well plate. In the competitive-inhibition experiments, 25 ng/well of wt mIL-22 was added alone or with increasing concentrations (0.1, 1, 10 and 100 μg/well) of mIL-22 muteins and incubated for 20 min. Then the cells were rapidly washed and harvested in 150 μl ice-cold lysis buffer (Sigma, cat #C2978) supplemented with 1 : 1000-diluted protease inhibitor cocktail (Sigma, cat #P8340) and 1 mM sodium orthovanadate (Sigma, cat #S6508) and incubated on ice for 15 min. Lysates were clarified by centrifugation at 12 000 *g* for 10 min and supernatants were kept for western blot analyses. Protein concentrations in the supernatants were determined using the Bradford assay. Cell proteins (30 μg) were resolved by SDS–PAGE (10%) followed by western blotting using anti-p-STAT-3 (Tyr705) (Cell Signaling, cat #9138) and reblotting with anti-STAT-3 (Cell Signaling, cat #9132) antibodies according to the manufacturer's instructions. Then, western blot bands were revealed by enhanced chemiluminescence.

Results and discussion

Purification and physicochemical characterization of wt mIL-22 and four mIL-22 muteins

The refolded and dialyzed wt mIL-22 and four mIL-22 muteins (Y51A, N54A, R55A and E117A) were purified on a Q-Sepharose anion-exchange column equilibrated with 10 mM Tris base buffer pH 10 using a non-continuous NaCl gradient (see Materials and methods). In mIL-22 and all four muteins, the monomeric fractions, as documented by analytical gel-filtration analysis, were eluted by 100 mM NaCl. All five proteins were pure as shown by SDS–PAGE carried out under reducing and non-reducing conditions (Fig. 1A) and consisted of >98% monomers (Fig. 1C and G). As depicted in the supplementary Fig. S3 the mutations did not change

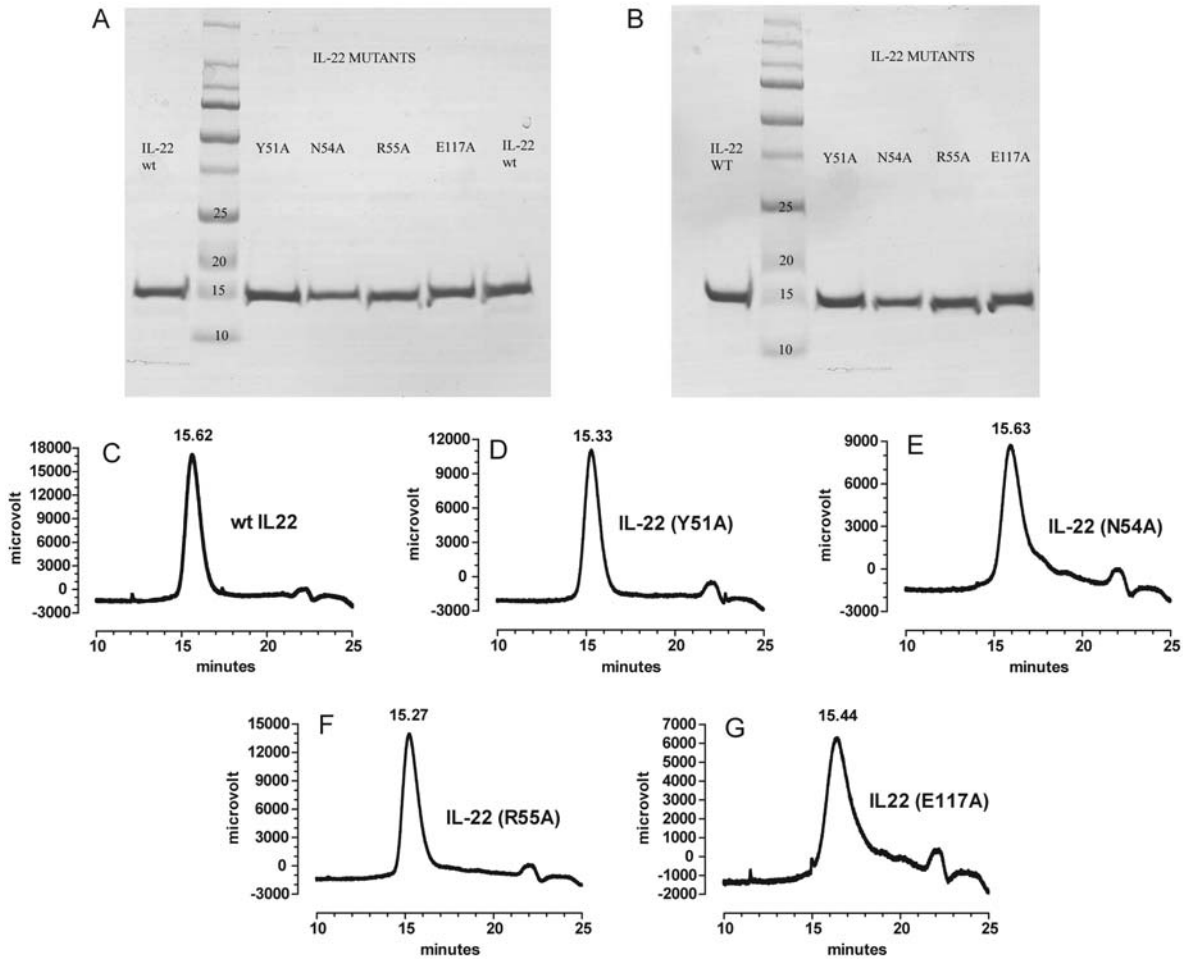

Fig. 1. SDS–PAGE (15%) of IL-22 and its four muteins in the absence (**A**) or presence (**B**) of reducing agent and gel filtration on analytical Superdex 75 column developed with TN buffer pH 8 at room temperature (**C–G**). The column was developed at 0.8 ml/min and calibrated with BSA (66 kDa, RT = 11.52), ovine placental lactogen (23 kDa, RT = 14.48) and lysozyme (14 kDa, RT = 15.02). Protein concentration of the eluate was monitored by absorbance at 280 nm and is presented as microvolts. RT is shown as a number above the peak. A color version of this figure is available as supplementary data at *PEDS* online.

the CD spectra which were almost identical to that of the non-mutated IL-22 and quite characteristic to cytokines having the 4-bundle α-helical structure.

The average yields of wt IL-22, IL-22 (Y51A), IL-22 (N54A), IL-22 (R54A) and IL-22 (E117A) muteins were, respectively, 130, 270, 50, 280 and 40 mg of pure protein from 1 l of fermentation culture. Specific extinction coefficients at 280 nm for a 0.1% (w/v) solution assuming an extra Ala at the N terminus was calculated according to Pace *et al*. (Pace *et al.*, 1995), yielding value of 0.26 for wt IL-22 and its N54A, R55A and E117A muteins and 0.18 for the Y51A mutein.

Preparation and physicochemical characterization of wt PEG-IL-22 and PEG-IL-22 (Y51A)

Pegylation of IL-22 and IL-22 (Y51A), carried out as described in Methods, resulted in an average yield of 33–45 mg and 33–70 mg of PEG-IL-22 and PEG-IL-22 (Y51A), respectively, obtained from 150 mg of non-pegylated protein. The purity and homogeneity of the PEG-IL-22 and PEG-IL-22 (Y51A) were documented by SDS–PAGE (Fig. 2A). The main band appeared at ∼55 kDa, instead of the theoretical value of 37 kDa, as also evidenced by comparison to mono-pegylated superactive mouse leptin antagonist whose theoretical calculated is also 36 kDa. This discrepancy, which was also seen for pegylated leptin antagonists (Elinav *et al.*, 2009; Shpilman *et al.*, 2011), likely results from the increased hydrodynamic volume of the protein due to pegylation. A minor band with apparent molecular mass of ∼100 kDa, likely double-pegylated proteins, was also detected. The gel-filtration profiles of PEG-IL-22 and PEG-IL-22 (Y51A) (Fig. 2B and C, respectively) exhibited 75–80% mono-pegylated IL-22 or IL-22 (Y51A), ∼20–25% double-pegylated protein (detected as a smaller peak with retention time in minutes (RT) = 14.2 min preceding the main peak), and no detectable non-pegylated protein. As pegylation increases the hydrodynamic volume, resulting in slower migration in SDS–PAGE (see above), we concluded that the ∼20–25% peak detected in the gel-filtration profile at a retention time of 14.2 min (Fig. 2B and C) is likely double-pegylated PEG-IL-22 and PEG-IL-22 (Y51A), and not a single pegylated dimer. As no signal was detected in the N-terminal amino acid analysis and as we used mPEG-propionyl-ALD-20 kDa under conditions that according to the manufacturer, favor pegylation of the N-terminal amino group, we concluded that the N terminus is blocked, suggesting that pegylation occurred mainly at the N terminus. Pegylated IL-22 and IL-22 Y51A conjugates were also

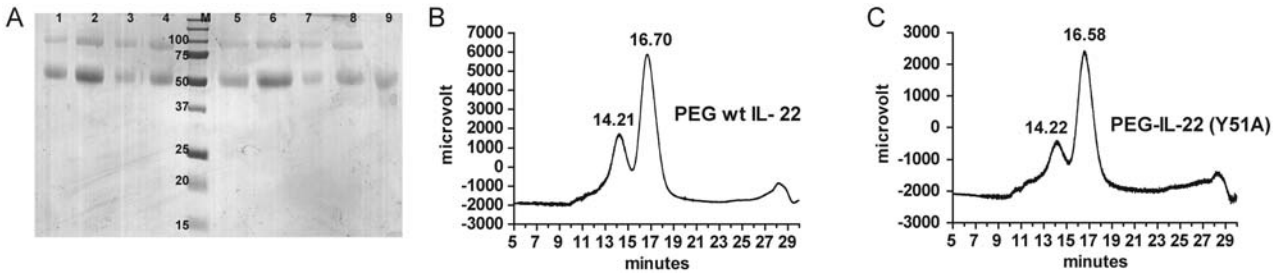

Fig. 2. SDS–PAGE (12%) of final purified pegylated (PEG)-IL-22 and PEG-IL-22 (Y51A) in the presence or absence of reducing agent (**A**): lanes 1, 5, and 2, 6—PEG-IL-22, 2.5 and 5 μg/lane, respectively; lanes 3, 7, and 4, 8—PEG-IL-22 (Y51A), 2.5 and 5 μg/lane, respectively; lane 9—mono-pegylated superactive leptin antagonist; M—molecular mass markers in kDa from the top: 200, 150, 100, 75, 50, 37, 25, 20 and 15; lanes 1–4 and lanes 5–9, without and with reducing agent, respectively. Gel-filtration on analytical Superdex 200 column developed with TN buffer pH 8 at room temperature (**B** and **C**). The column was developed at 0.7 ml/min and calibrated with bovine IgG (150 kDa, RT = 17.6), BSA (66 kDa, RT = 20.5), ovine placental lactogen (23 kDa, RT = 23.5) and lysozyme (14 kDa, RT = 25.3). Protein concentration of the eluate was monitored by absorbance at 280 nm and is presented as microvolts. RT is shown as a number above the peak. A color version of this figure is available as supplementary data at *PEDS* online.

analyzed by matrix-assisted laser desorption ionization-time of flight (Bruker Reflex III^{TM}), indicating that the mono-pegylated species of 37985 Da for pegylated IL-22 and 37617 Da for pegylated IL-22 (Y51A) as shown in Supplementary Fig. 4A and B, are the dominant one, consisting of ~ 90% slightly higher than the corresponding to the values obtained be gel-filtration (Fig. 2B and C). The nature of the double-pegylated PEG-IL-22 and PEG-IL-22 (Y51A) was not tested, but it likely exists as an N-terminus-pegylated species with a single or a few partially pegylated lysines. It should be noted that the molecular mass of PEG-IL-22 and PEG-IL-22 (Y51A), as calculated from gel filtration, was ~220 kDa, most likely because of the larger hydrodynamic volume due to pegylation as previously shown for mono-pegylated superactive mouse leptin antagonist (Shpilman *et al.*, 2011). The stability of both pegylated and non-pegylated IL-22 and its muteins in solution was tested at 4°C. Both proteins could be stored as sterile (1 mg/ml) solutions for at least 30 days at pH 8 without undergoing any changes in their monomeric content.

Receptor-binding assays

The binding activity of mIL-22 was compared with that of its four muteins (Y51A, N54A, R55A and E117A) in a competitive non-radioactive binding assay using biotinylated mIL-22 as a ligand and mIL-22 R1-ECD as a receptor. To ensure the validity of this assay, we first documented that the biological activities of the biotinylated and non-biotinylated IL-22 tested in HepG2 cells are similar, if not identical (Fig. 4A). Two experiments were performed, and one of them is shown in Fig. 3A and B. The IC_{50} value for mIL-22 calculated from six experiments (three of them shown in Fig. 4A, B and C) was 6.81 ± 0.52 μM (mean ± SEM). The four IL-22 muteins competed effectively with biotinylated IL-22 for binding and the results are summarized in Table II. Statistical analysis of each experiment revealed no significant differences compared with the corresponding values for wt IL-22. Two experiments comparing the binding of PEG-IL-22 and PEG-IL-22 (Y51A) to wt IL-22 were performed. One of them is presented in Fig. 3C. Pegylation lowered the affinity of PEG-IL-22 and PEG-IL-22 (Y51A) as summarized in Table II.

To test whether the mutations affect also the affinity toward IL-22BP in an additional binding experiment we used yeast cells constructed to express IL-22BP on their surface, and used biotinylated mIL-22 as the ligand and non-biotinylated mIL-22 and its four IL-22 muteins as competitors. As shown in Fig. 3D, both wt IL-22 and the four IL-22 muteins competed effectively with biotinylated IL-22 for the binding. The respective IC_{50} values for the wt IL-22, IL-22 (Y51A), IL-22 (N54A), IL-22 (R55A) and IL-22 (E117A), given in nM, were: 78, 134, 133, 97 and 182. Statistical analysis revealed that the decrease in the affinity of IL-22 (Y51A), IL-22 (R55A) and IL-22 (E117A) was significant ($P < 0.05$, using a 95% confidence interval).

Biological activity

The biological activity of wt IL-22, its four muteins, and PEG-IL-22 was tested in HepG2 cells using phosphorylation of STAT-3 as the readout (Fig. 4). To verify that the total amount of STAT-3 in the treated cells was similar, each blot was also reacted with antibody against STAT-3. Though the assay is semi-quantitative in nature, the results clearly showed that: (i) biotinylated IL-22 was almost equally active in HepG2 bioassay as compared with the non-biotinylated IL-22 (Fig. 4A); (ii) the activity of PEG-IL-22 is 5- to 10-fold lower than that of IL-22 (Fig. 4B); (iii) muteins E117A, N54A and R55A exhibits very low agonistic activity, whereas mutein Y51A, has no agonistic activity at all; and (iv) all four muteins act in a competitive manner as potent antagonists (Fig. 4C).

Conclusions

Our results clearly demonstrate that recombinant muteins Y51A, N54A, R55A and E117E of mIL-22 prepared in this work were refolded properly as determined by binding experiments and CD spectra. Those mutations not only lead to loss of activity as reported previously (Yoon *et al.*, 2010), but also confer potent antagonistic activity to IL-22 signaling in HepG cells without changing the affinity toward immobilized ECD of IL-22 R1 and with only small changes in IL-22 (Y51A), IL-22 (R55A) and IL-22 (E117A), toward binding IL-22BP expressed on the surface of yeast cells. Pegylation of mIL-22 and IL-22 (Y51A) that yielded mainly the mono-pegylated species decreased their overall affinity for immobilized ECD of IL-22R1 by 3- to 4-fold and the biological activity of pegylated mIL-22 by 5- to 10-fold. As shown previously for many other pegylated proteins

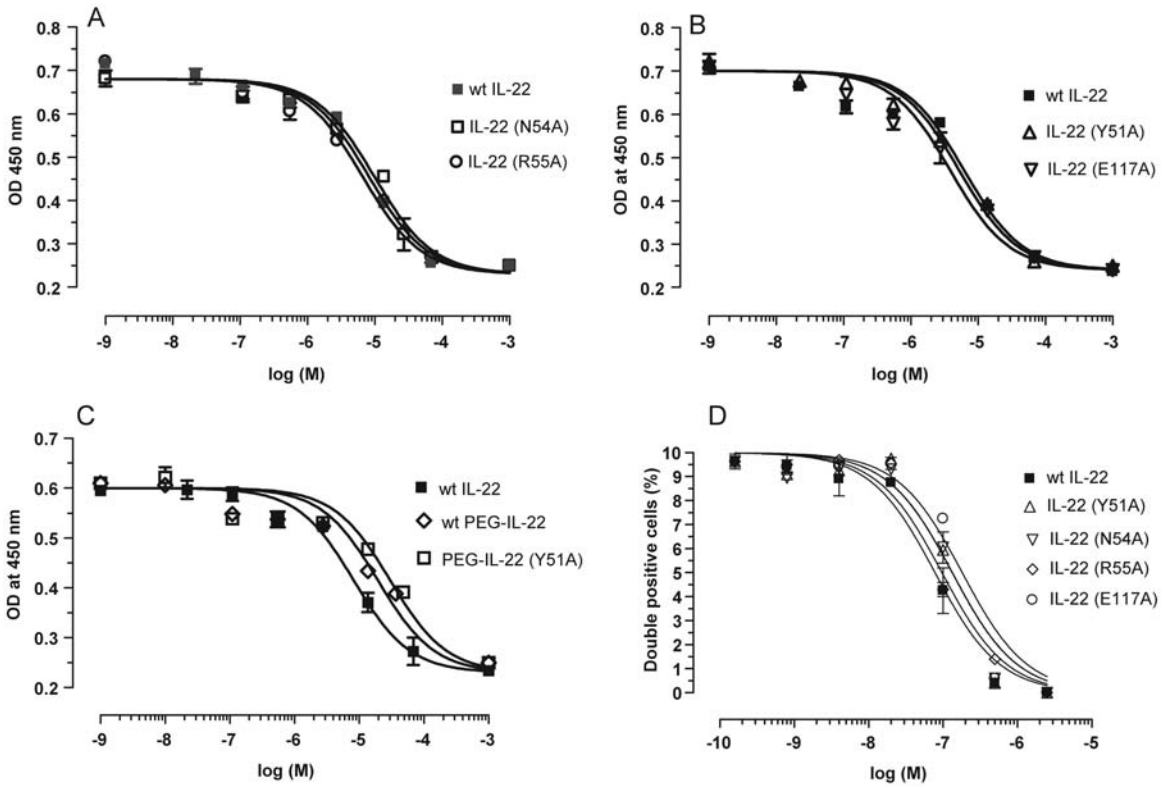

Fig. 3. Competitive non-radioactive receptor-binding assay of wt mIL-22 and its mutants N54A and R55A (**A**), and Y51A and E117A (**B**), and of wt pegylated (PEG)-IL-22 and PEG-IL-22 (Y51A) (**C**). Binding of biotinylated mIL-22 to immobilized mIL-22 Fc-R1-ECD (IL-22 receptor extracellular domain) was performed in the presence of the indicated concentrations of non-biotinylated mIL-22 or its non-pegylated (A, B) or pegylated (C) muteins. The experiment was carried out in triplicate and the results are presented as mean ± SEM. As the variations in this assay were very small, most of the error bars cannot be seen. Binding of wt IL-22 and its four muteins to IL-22 binding protein expressed on the surface of yeast cells was also tested (**D**). The experiment was carried out in duplicate and the results are presented as mean ± SD. Other details of this procedure are given in the text.

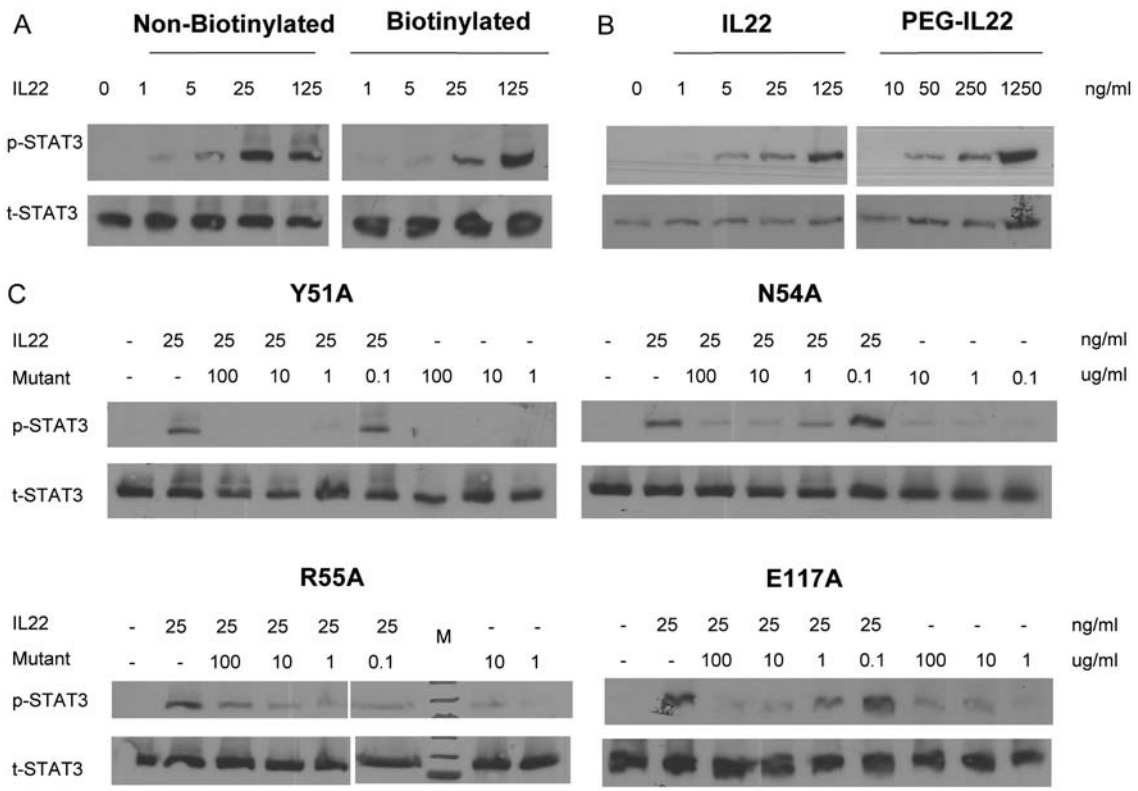

Fig. 4. Biological activity (STAT-3 phosphorylation) of mIL-22 in HepG cells. Comparison of activities of biotinylated and non-biotinylated mIL-22 (**A**), pegylated and non-pegylated (PEG) mIL-22 (**B**), and inhibition of mIL-22-induced phosphorylation of STAT-3 by mIL-22 muteins Y51A, N54A, R55A and E117A (**C**). For other details see text. The lane marked M in the blot of R55A shows (from the top) three molecular mass markers of 150, 100 and 75 kDa.

Table II. The absolute IC_{50} values for mIL-22, its four mutants and pegylated IL-22 and IL-22 (Y51A) mutant in binding assay using immobilized ECD of IL-22R1

Protein tested	No. of experiments	IC_{50} (μM)[a]
IL-22	6	6.81 ± 0.52
IL-22 (Y51A)	2	5.93 ± 0.71
IL-22 (N54A)	2	9.98 ± 2.69
IL-22 (R55A)	2	5.31 ± 0.31
IL-22 (E117A)	2	6.88 ± 0.71
Pegylated IL-22	2	19.50 ± 2.60
Pegylated IL-22 (Y51A)	2	30.30 ± 3.40

[a]The values of IL-22 are given as mean ± SEM and the values of other proteins as mean ± SD.

(Bailon and Won, 2009), pegylation prolonged half-life in the circulation and despite lower intrinsic activity is expected to result in higher activity *in vivo* as also proven recently in our lab comparing pegylated and non-pegylated leptin antagonists (Shpilman *et al.*, 2011). In view of the potential pharmaceutical uses of recombinant IL-22 antagonists the general question of how the biopotency of recombinant proteins can be enhanced *in vivo* needs to be explored. One possible approach which is currently performed in our lab is to increase the antagonist's affinity for the receptor by increasing k_{on}, or mainly by decreasing k_{off}, and thus prolonging receptor occupation as recently reported by our labs for creating superactive leptin antagonists (Shpilman *et al.*, 2011). Theoretical thermodynamic considerations show that if antagonist and agonist exhibit the same affinity, at a 100-fold molar excess of antagonist, 99% of all occupied receptors will be occupied by the latter. A 100-fold increase in antagonist affinity will lead to similar results at an ~1 : 1 molar antagonist : agonist ratio. Pegylation of such high-affinity antagonists combines both approaches, resulting in a potent and effective, long-acting competitive antagonistic activity *in vivo*.

Supplementary data

Supplementary data are available at *PEDS* online.

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