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The functional expression of a biologically active fragment of soluble gp130 as an ELP fusion protein in transgenic plants: purification via inverse-transition-cycling

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SYNOPSIS

In murine models of Crohn's disease, rheumatoid arthritis and colon cancer, interleukin (IL)-6 signalling via the soluble IL-6 receptor (sIL-6R, termed IL-6 trans-signalling) has been shown to promote the pathology associated with these conditions. These detrimental activities can however be selectively blocked by soluble forms of gp130, which acts as the universal signalling receptor subunit for all IL-6-related cytokines. Although soluble gp130 (sgp130) therefore represents a viable therapeutic modality for the treatment of these conditions, the mass manufacture of such biologics is often expensive. The advent of molecular farming has however provided an extremely cost effective strategy for the engineering of recombinant proteins. Here we describe the expression and production of a biologically active sgp130 variant which is expressed in transgenic tobacco plants as an elastin-like pentapeptide (ELP) fusion protein (mini-gp130-ELP). Mini-gp130-ELP consists of the first three domains of gp130 (Ig-like domain and cytokine binding module) fused to 100 repeats of ELP. Expression of mini-gp130-ELP did not affect the growth rate or morphology of the transgenic plants, and purification was achieved using inverse transition cycling. This approach led to an overall yield of 141 µg of purified protein per gram of fresh leaf weight. The purified mini-gp130-ELP specifically inhibited sIL-6R-mediated trans-signalling as measured by binding to the IL-6/sIL-6R complex and through its ability to block sIL-6R-mediated activation of STAT3 phosphorylation and proliferation in human hepatoma cells and murine pre-B-cells.

Consequently, this study validates the potential application of molecular farming in transgenic tobacco plants as a strategy for the expression and purification of therapeutically advantageous biologics such as sgp130.

INTRODUCTION

Cytokines are a series of small soluble polypeptides, which control cellular functions like proliferation, differentiation and viability. Dysregulation of cytokine activity is often associated with the progression of chronic disease, and selective targeting of these proteins in various disease states has proven to be clinically beneficial. Such an approach is exemplified by the use of blocking anti-TNF α regimes in rheumatoid arthritis and Crohn's disease.

Cytokines of the gp130-family, including interleukin 6 (IL-6), IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary inhibitory factor (CNTF), cardiotropin-1 (CT-1), cardiotrophin-like related cytokine/neurotrophin-1/B-cell stimulating factor 3 (CLC/NNT-1/BSF-3), neuropoietin (NPN) and IL-27 share the membrane glycoprotein gp130 as a common signal transducer subunit (reviewed in [1] and [2]).

On target cells, IL-6 first binds to the IL-6 alpha receptor (IL-6R). The resulting complex of IL-6 and IL-6R then associates with the signal transducing membrane protein gp130, thereby initiating intracellular signalling [3]. The gp130 subunit is expressed by most, if not all cells in the body whereas IL-6R expression is mainly limited to hepatocytes, neutrophils, monocytes/macrophages and specific lymphocyte subsets. Moreover, a soluble IL-6R (sIL-6R) has been found in various body fluids [4; 5; 6; 7; 8; 9; 10; 11]. Soluble IL-6R forms an agonistic complex with IL-6 that stimulates cells which only express gp130 [12; 13]. A process which we have termed trans-signalling [12; 14].

Interestingly, a naturally occurring soluble gp130 (sgp130) possesses antagonistic properties on IL-6 acting in complex with the soluble IL-6R but not on IL-6 acting via the membrane IL-6R. At 100-fold higher concentrations soluble gp130 also inhibits LIF and OSM but not CNTF and IL-27 [15; 16; 17]. Therefore, we have concluded that sgp130 is the natural inhibitor of IL-6-trans-signalling. Moreover, in mouse models of human diseases, sgp130 has been shown to be a potent candidate for therapy of e.g. Crohn's disease, rheumatoid arthritis and colon cancer [18; 19; 20; 21].

Following the initial report on expression of complete antibodies in transgenic plants [22], several alternative expression strategies have been described for the high yield production of recombinant proteins in transgenic plants [23; 24]. However for many proteins, increased ER retention leads to a trapping of the recombinant protein within the plant [25]. Moreover, we have demonstrated a twofold increase of spider silk protein accumulation in tobacco leaves and a 40-fold increase of single chain antibody accumulation in tobacco seeds after fusion to 100 repeats of an elastin-like pentapeptide (ELP), with one peptide unit made up of Val-Pro-Gly-Xaa-Gly (Xaa is any amino acid except proline; [26; 27, 28]). This amino acid stretch is

derived from the characteristic repeat motif VPGVG found in the native sequence for mammalian elastin. ELPs and ELP-fusion proteins are soluble in water below their transition temperature and become reversibly insoluble when the temperature is raised above the transition temperature (inverse transition cycling) [27]. Since we have shown that inverse transition cycling can be used to purify heat stable spider silk proteins from transgenic tobacco leaves [26], we have now asked whether this principle could be transferred to non-heat stable proteins with therapeutic potential. We therefore generated transgenic tobacco plants, which express a sgp130-ELP (mini-gp130-ELP) fusion protein under the transcriptional control of the plant virus promoter CaMV35S. Such an approach led to the accumulation of large amounts of mini-gp130-ELP in the endoplasmic reticulum, which could be purified as a functionally active variant using inverse transition cycling.

MATERIAL AND METHODS

Cells and Reagents

HepG2 cells (human hepatoma cells) were obtained from the American Type Culture Collection (Rockville, MD/Manassas, VA, USA). BA/F3 cells (murine pre-B-cells) stably transfected with human gp130 (Ba/F3-gp130 cells) or gp130 and IL-6R cDNAs (Ba/F3-gp130-IL-6R cells) have been previously described [29; 30]. All cells were grown in DMEM High Glucose Culture Medium (PAA Laboratories, Pasching, Austria) supplemented with 10% foetal calf serum, penicillin (60 mg/l) and streptomycin (100 mg/l) at 37°C with 5% CO₂ in a humidified atmosphere. For the cultivation of Ba/F3-gp130-IL-6R cells the standard DMEM medium was supplemented with 10% FCS, penicillin (60 mg/l), streptomycin (100 mg/l) and 10ng/ml IL-6 or 10ng/ml Hyper-IL-6. Hyper-IL-6 is a fusion protein of IL-6 and sIL-6R connected by a flexible polypeptide linker. Soluble gp130Fc is a fusion protein of the extracellular portion of gp130 and the Fc-portion of IgG1 [16; 29]. Both recombinant proteins were expressed and purified as previously described [16; 29]. Hyper-IL-6-Fc is a fusion protein of the Hyper-IL-6 and the Fc-portion of an IgG antibody, which was expressed in transfected EBNA-HEK-293 cells and was directly used as conditioned supernatant. Mouse anti-STAT3 mAb was purchased from Cell signalling technology (Beverly, MA, USA), rabbit anti-phospho-STAT3 mAb (Y705) was from Cell signalling technology (Beverly, MA, USA). Mouse anti human IL-6R mAb M91 was from Immunotech (Marseille, France). Mouse anti c-myc mAb 9E10 was from Abcam (Cambridge, UK). Sheep anti-mouse IgG horseradish peroxidase and goat anti-rabbit IgG horseradish peroxidase were from Amersham Biosciences (Buckinghamshire, UK). All restriction enzymes were obtained from

MBI Fermentas (St. Leon-Rot, Germany). [³H]thymidine was purchased from Amersham Biosciences (Freiburg, Germany).

Construction of the binary vector for mini-gp130-ELP under the control of the CaMV-35S-promoter for the transformation of tobacco plants

The cytokine binding portion (D1-D3) of the cDNA coding for sgp130Fc-opt (see below) was amplified by PCR using the sense strand oligonucleotide primer gp130-Δsignal (5′ GACGTCACAT GTTTAGCAGA GCTGCTGGAT CCTTGC 3′) and the gp130-NaeI (5′ CAGTGCCGGC TCTGTCTCG TAGGTGATGC 3′) anti-sense oligonucleotide primer. The plasmid pCR-Script-sgp130Fc-opt served as template (GeneArt, Munich, Germany). Here, the codon usage of sgp130Fc was adapted to the codon bias for *Cricetulus griseus* (Chinese hamster) genes (for sequence information of the cDNA coding for mini-gp130-ELP and the protein sequence of mini-gp130-ELP see supplementary Figure 1A and B). The purified PCR-product was digested with *Afl*III and *Nae*I and cloned into the plasmid pRTRA7/3-35S-anti-KRES-c-myc-100xELP [27], which was linearised with *Afl*III and *Nae*I. The generation of the 100xELP fusion protein was described previously [26]. The resulting plasmid pRTRA7/3-35S-mini-gp130-ELP was digested with *Hind*III and the fragment containing the expression cassette (35S promoter/LeB4 signal peptide/mini-gp130/c-myc-tag/ELP/KDEL/CaMV 35S terminator) cloned into the binary plasmid pCB301-Kan. The vector pCB301-Kan is based on the vector pCB301 [31] and was produced by the transfer of a *Bgl*III-*Bam*HI-T-DNA-fragment containing the kanamycin resistance gene of the pBIN19 vector [32].

Transformation of tobacco

The mini-gp130-ELP encoding construct was transferred into *Agrobacterium tumefaciens* C58C1 (pGV2260) by electroporation. Tobacco (*Nicotiana tabacum* cv. SNN) leaf discs were transformed as described elsewhere [33]. Regenerated transgenic plants were grown *in vitro* on Murashige–Skoog medium containing 100 mg/l kanamycin. Regenerated plants were grown to maturity in the greenhouse and were screened for high expression by Western blot analysis using the anti c-myc mAB 9E10.

Purification of recombinant mini-gp130-ELP

Green leaves from transgenic tobacco plants were ground in a mortar under liquid nitrogen. 50 ml PBS was added to 10 g ground leaves and the suspension was stirred for 5 min at RT.

The extract was cleared by centrifugation at 8,000 g for 30 min. The supernatant was filtered (Syringe Filter 0.22 μ M pore diameter (Roth GmbH, Karlsruhe, Germany)) and sodium chloride added to a final concentration of 2 M. The solution was incubated in a water bath at 40°C for 30 min to allow for aggregation of the ELP-fusion proteins. The aggregates were precipitated by centrifugation at 8,000 g for 30 min at 40°C. The precipitate was dissolved on a vertical shaker at 200 rpm in 50 ml PBS for 15 min at 20°C. Insoluble material was removed by centrifugation at 8,000 g for 15 min at 20°C. The supernatant was supplemented with sodium chloride to a final concentration of 2 M. The solution was again incubated in a water bath at 40°C for 30 min. The precipitate was removed by centrifugation at 8,000 g for 30 min at 40°C. The precipitate was dissolved on a vertical shaker at 200 rpm in 2 ml PBS for 15 min at 20°C. Insoluble material was removed by centrifugation at 8,000 g for 15 min at 20°C.

Size exclusion chromatography

The mini-gp130-ELP protein was further purified on a calibrated HiPrep 26/60 Sephacryl S-300 High Resolution column (Amersham Biosciences, Freiburg, Germany) using PBS as the mobile phase with a constant flow-rate of 1.0 ml/minute. For calibration, the high molecular mass standards (Amersham Biosciences, Freiburg, Germany) were used (see supplementary Figure 4A and B). Fractions of 2.5 ml were collected, analyzed by SDS-PAGE, pooled and concentrated.

Protein concentration

The method of Waxman [34] was used to determine the protein concentration. The extinction coefficient of mini-gp130-ELP at 280 nm was calculated to be 64890 l/mol x cm. The absorption spectra were recorded in the range of 240-320 nm (supplementary Figure 5A).

Edman sequencing

10 μ g of the purified mini-sgp130-ELP dissolved in 10 μ l PBS was sent for N-terminal sequence determination by automated Edman degradation to Sequence laboratories Göttingen GmbH (Seqlab, Göttingen, Germany).

Immunoprecipitation

Purified mini-gp130-ELP protein was incubated with Hyper-IL-6-Fc in DMEM-10% FCS overnight at 4°C followed by addition of 50 μ l of protein A-Sepharose (50% slurry, CL-4B

from Amersham Biosciences, Freiburg, Germany) for at least 1 h at 4°C. Immunoprecipitates were washed twice with washing buffer (10mM Tris pH 7.6, 150mM NaCl, 0.2 % Tween, 2mM EDTA) and once with PBS prior to addition of Laemmli sample buffer (2xLaemmli:100 mM Tris pH 6.8; 4% SDS; 200 mM DTT; 20 % glycerol; 0.1% Bromophenol Blue) and boiling at 95°C for 5 min.

Enzyme-Linked Immunosorbent Assay (ELISA)

Microtitre plates (Greiner Microlon, Solingen, Germany) were coated with purified mini-gp130-ELP (5µg/ml) in PBS. The plate was incubated over night at room temperature. After blocking with 3% (w:v) bovine serum albumin in PBS for 2 h, 100 µl aliquots of Hyper IL-6 (0.1µg/ml, 0.5µg/ml and 1µg/ml) (with or without antagonistic sgp130Fc (5µg/ml)) were added. The plate was incubated for 1 h at room temperature. Hyper IL-6 bound to the plate was detected by the non-neutralizing mAb M91 (working concentration 0.1µg/ml) followed by anti-mouse IgG horseradish peroxidase 1/1000 diluted in PBS/3% BSA. The enzymatic reaction was performed with soluble peroxidase substrate (BM blue POD from Roche, Mannheim, Germany) at 37°C for 30 min and the absorbance was measured at 450 nm on a SLT Rainbow plate reader from Tecan (Maennedorf, Switzerland).

Immunoblotting and enhanced chemiluminescence (ECL) detection

For Western Blotting, proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Hybond-P from Amersham Biosciences, Freiburg, Germany) by a semi-dry electro-blotting procedure. Membranes were blocked in a solution of TBS (10 mM Tris, pH 8, 150 mM NaCl) supplemented with 0.02% Tween-20 and 5% skimmed milk powder, and probed overnight with the indicated antibodies at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected using a chemiluminescence kit (ECL plus Western Blotting Detection System, Amersham Biosciences, Freiburg, Germany) following the manufacturer's instructions. Band intensities were quantified using ImageJ software (National Institutes of Health).

Proliferation assays

Ba/F3-gp130-IL-6R cells were washed 3 times with sterile PBS and re-suspended in DMEM-containing 10% FCS at 5×10^3 cells per well of a 96-well plate. The cells were cultured in a final volume of 100 µl with additional cytokines as indicated in the legend of Figure 4C for

72 h and subsequently pulse labelled with 0.25 μCi [^3H]-thymidine for 4 h. The specific activity of [^3H]thymidine was 25Ci/mmol. Cells were harvested on a glass fibre filter (Filtermat A from Wallac, Turku, Finland) and the filter was microwave-baked for 2 min. Subsequently, the filter was soaked in 4.5 ml liquid scintillation cocktail (Betaplate Scint from Wallac, Turku, Finland). [^3H]-thymidine incorporated into cellular DNA was determined by scintillation counting using the MicroBeta TriLux counter from PerkinElmer (Wellesley, MA, USA). Bioassays were performed with each value being determined in triplicate.

RESULTS

The mini-gp130-ELP fusion protein is expressed in high amounts in leaves of transgenic tobacco plants and can be purified by inverse transition cycling

A cDNA coding for the cytokine binding domains D1-D3 of gp130 was fused to a cDNA encoding 100 repeats of the elastin-like peptide (ELP) and placed under the control of the plant virus promoter CaMV35S (Figure 1A). For sequence information see supplementary Figure 1A and 1B.

We used ELP-units that were made up of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is valine, glycine or alanine [26]. The LeB4 signal peptide and the KDEL signal at the C-terminus caused ER retention. KDEL mediated ER retention was chosen because maintenance of single chain Fv-fragments and spider silk proteins as ELP-fusion proteins in the ER resulted in high level accumulation in transgenic tobacco seeds and leaves, respectively [26; 27]. The expression of mini-gp130-ELP without an ER retention signal in transgenic tobacco plants was not examined. A c-myc tag was used for detection of mini-gp130-ELP in Western blotting. Transgenic tobacco plants were generated by leaf disc transformation [35] and leaves were harvested and tested by Western blotting (data not shown). High-producers were chosen (12 out of 25 for mini-gp130-ELP, supplementary Figure 3A) and proteins were extracted from leaves with PBS. Protein expression of recombinant mini-gp130-ELP could already be detected after SDS-Page-Gel separation and Coomassie-staining of raw leaf extracts (Figure 2A, lane 1 and 2). Recombinant mini-gp130-ELP was purified by two rounds of inverse transition cycling as described in the material and methods section (Figure 1B and Figure 2A, lane 3-8).

Finally, 2 ml of pure mini-gp130-ELP-protein from 10 g of green leaves was further purified by size exclusion chromatography on a calibrated HiPrep 26/60 Sephacryl S-300 High Resolution column (for calibration curve see supplementary Figure 4). We determined the molecular mass of monomeric mini-gp130-ELP by SDS-Page analysis to be 100 kDa. The difference to the calculated molecular mass of mini-gp130-ELP (77.4 kDa) is likely due to glycosylation within the gp130 portion of the mini-gp130-ELP fusion protein. Since the majority of mini-gp130-ELP behaved on the column like a 240 kDa protein as estimated by comparison with a calibration curve of molecular mass standards, it is likely that the majority of the mini-gp130-ELP formed at least dimers (large peak in Figure 2B). A smaller fraction formed even higher ordered oligomers (650 kDa), whereas monomeric mini-gp130-ELP could not be detected. On the other hand, it could not be excluded that the presence of the

ELP-region resulted in an elongated protein that eluted in the position expected for a globular protein of higher molecular mass.

Mini-gp130-ELP from the major peak was collected and the concentration was quantified by UV-spectroscopy using the method of Waxman [34] (see supplementary Figure 5A).

Therefore, the final yield of purified mini-gp130-ELP was 141 μg purified protein per gram leaf fresh weight (supplementary Figure 5B).

The correct cleavage of the LeB4-signal peptide is important since the N-terminus of gp130 is needed for efficient binding to the IL-6/IL-6R complex [36; 37]. The four N-terminal amino acids of the mature mini-gp130-ELP-protein were determined by automated Edman sequencing (performed by Seqlab, Göttingen, Germany) to be ELLD, which corresponds to the wild type sequence of mature gp130. Purified mini-gp130-ELP protein was routinely stored at -20°C but was also stable at 4°C and room temperature without detectable loss of soluble recombinant protein integrity (supplementary Figure 2A and B).

Mini-gp130-ELP is biologically active and inhibits exclusively IL-6 trans-signalling, but not classic IL-6 signalling

4 μg purified mini-gp130-ELP was incubated with a molar excess of Hyper-IL-6-Fc and precipitated by protein A-Sepharose. Hyper-IL-6 is a fusion protein of IL-6 and the soluble IL-6R connected by a flexible linker [29], which can directly bind to gp130. Here, Hyper-IL-6 was additionally fused to the Fc portion of an IgG antibody to allow for direct precipitation with protein A-Sepharose. Hyper-IL-6-Fc was produced in EBNA-HEK-293 cells and was directly used as conditioned supernatant. As depicted in Figure 3A, mini-gp130-ELP was precipitated by Hyper-IL-6-Fc (lane 2), whereas no mini-gp130-ELP remained in the cleared supernatant after immunoprecipitation with Hyper-IL-6-Fc (lane 1). Moreover, mini-gp130-ELP could not be precipitated by protein A-Sepharose alone (lane 3: supernatant; lane 4: immuno-precipitated protein). Since it was very likely that mini-gp130-ELP formed dimers at higher protein concentrations (Figure 2B), we cannot exclude that these dimers also existed at the lower mini-gp130-ELP concentrations used for the immunoprecipitation and for the following experiments. Therefore it is conceivable that the interaction of one out of two sgp130 molecules of the mini-gp130-ELP-dimer with Hyper-IL-6 is necessary for complete immunoprecipitation of mini-gp130-ELP. Since there was no detectable mini-gp130-ELP remaining in the supernatant after immunoprecipitation with Hyper-IL-6-Fc, we conclude that at least 50% of the mini-gp130-ELP molecules are biologically active in terms of binding to the complex of IL-6 and soluble IL-6R.

An enzyme-linked immunosorbent assay (ELISA) was developed to detect the binding of mini-gp130-ELP to the IL-6/sIL-6R-complex. Mini-gp130-ELP protein was surface adsorbed and increasing amounts of Hyper IL-6 were added followed by detection via an anti-IL-6R antibody M91 (Figure 3B). The monoclonal antibody M91 directed against the human IL-6R was chosen for the immunochemical detection of Hyper IL-6. M91 recognizes the Ig-like domain of IL-6R and therefore does not affect binding of Hyper-IL-6 to mini-gp130-ELP. Mini-gp130-ELP alone did not cross react with the detection antibodies. Binding of Hyper-IL-6 to mini-gp130-ELP could be competed by a molar excess of sgp130Fc. sgp130Fc consists of the complete extracellular part of gp130 (domains 1-6) fused to a dimerization domain (Fc-part of a human IgG-antibody) [16]. These experiments clearly show that mini-gp130-ELP binds to Hyper-IL-6, which can be competed with the sgp130Fc protein.

Next, we asked whether mini-gp130-ELP could inhibit IL-6 or Hyper-IL-6 induced STAT3-phosphorylation. Human hepatoma cells (HepG2) were stimulated with 50 ng/ml IL-6 or 50 ng/ml Hyper-IL-6. We have shown previously that sgp130Fc exclusively inhibits the IL-6/sIL-6R complex without affecting the activity of IL-6 [16]. As shown in Figure 3C, increasing amounts of mini-gp130-ELP led to a dose-dependent inhibition of Hyper-IL-6 induced STAT3-phosphorylation. On the other hand, mini-gp130-ELP was not able to inhibit STAT-3 phosphorylation when the cells were stimulated with IL-6 alone. These results indicate that mini-gp130-ELP specifically inhibits IL-6 signals that were mediated by the soluble IL-6 receptor but not by the membrane bound IL-6 receptor.

The IL-3 dependent murine pro B-cell line Ba/F3 is the only mammalian cell line known, which completely lacks gp130 expression [38]. However, if these cells are transfected with gp130 (Ba/F3-gp130 cells) their reliance on IL-3 can be circumvented by stimulation with Hyper-IL-6 [39; 40; 41; 29]. Ba/F3-gp130 cells were stimulated with a fixed concentration of Hyper-IL-6 in the presence of increasing amounts of mini-gp130-ELP. Mini-gp130-ELP inhibited Hyper-IL-6 induced STAT3-phosphorylation of Ba/F3-gp130 cells (Figure 4A). To prove that mini-gp130-ELP selectively blocked sIL-6R-mediated responses, an identical experiment was performed in Ba/F3 cells transfected with cDNA for both gp130 and the IL-6R (Ba/F3-gp130-IL-6R cells). These cells respond to either IL-6 or Hyper-IL-6, as shown in Figure 4B mini-gp130-ELP selectively blocked Hyper-IL-6 induced activation of STAT3, but not the IL-6 mediated phosphorylation of STAT3 in these cells. The activity of sgp130Fc

was used as a comparative control protein. Moreover, the proliferation of Ba/F3-gp130-IL-6R cells stimulated by Hyper-IL-6 could be blocked by mini-gp130-ELP in a dose dependent manner (Figure 4C). As expected, mini-gp130-ELP had no inhibitory effect on the proliferation of Ba/F3-gp130-IL-6R cells treated with IL-6 alone. Thus, mini-gp130-ELP specifically inhibits IL-6 trans-signalling, but not classical IL-6-signalling.

DISCUSSION

Plant expression systems have the potential to be used for the production of safe and biologically active recombinant proteins. It has been estimated that proteins can be produced 10-50 times cheaper in plants than in *E. coli* [42]. Therefore, the combination of safe and low cost protein production with a protein-intrinsic purification strategy would be very desirable. So far, there have been examples of high yield production of recombinant proteins in transgenic plants, mainly in the field of molecular medicine such as antibodies, enzymes and vaccines [42; 44; 45]. Moreover, health risks that may arise from contamination of recombinant proteins with potential human pathogens or toxins are minimized by molecular farming [46]. Recently, an improvement of the production of recombinant single chain antibodies and spider silk proteins, respectively, in seeds and leaves has been achieved by ER-retention of ELP-fusion constructs [26; 27]. Furthermore, inverse transition cycling of a spider silk ELP-fusion protein has been used for purification from leaf extracts [26]. Spider silk proteins are heat stable and therefore represent an ideal model for inverse transition cycling since the temporary heat treatment did not lead to protein denaturation. Initially, we generated transgenic tobacco plants accumulating sgp130Fc or sgp130Fc-ELP in the ER of leaves. It turned out that these proteins of around 800 and 1300 amino acids respectively were produced very poorly (< 0.01 % and 0.05 % of total soluble protein, respectively; supplementary Figure 3A). Therefore, we decided to generate transgenic plants, which accumulate a minimal mini-gp130-ELP-fusion protein in the ER of leaves. Mini-gp130-ELP only contains the cytokine-binding portion of gp130 (domains 1 - 3, 303 amino acids) fused to 100 repeats of ELP and was found to be produced in high amounts in transgenic tobacco leaves by ER-retention (compare supplementary Figure 3A and B). So far, it has not been examined whether ELP-fusion proteins, which are expressed in the cytoplasm or in plastids, will also result in increased protein accumulation compared to the non-fusion proteins. Inverse transition cycling was applied to purify the mini-gp130-ELP to homogeneity. It has previously been shown that for sgp130Fc, a 2-5 fold molar excess was sufficient to inhibit IL-6/sIL-6R mediated BA/F3-gp130 cell proliferation by 50%. In sgp130Fc the complete

extracellular part of gp130 (domains 1-6) was fused to a dimerisation domain (Fc-part of a human IgG-antibody). Thereby sgp130Fc mimics the receptor signal complex on the membrane, which also exists as a preformed dimer [47]. Interestingly, the preformed sgp130Fc-dimer has a ten-fold higher efficiency to inhibit IL-6/sIL-6R mediated responses than the sgp130 monomer [16]. For mini-gp130-ELP an 80-fold molar excess inhibited IL-6/sIL-6R mediated BA/F3-gp130 cell proliferation by 50%. Therefore, we conclude that plant produced mini-gp130-ELP antagonized the activity of Hyper-IL-6 less efficiently than the preformed sgp130Fc dimer but with comparable efficiency as a sgp130-monomer purified from mammalian cell culture supernatants [16]. In this respect, mini-gp130-ELP resembles the functional properties of the highly truncated spliced variant of gp130 known as gp130-RAPS, which consists of D1-D3 of gp130 plus a unique COOH-terminal amino acid sequence [48; 49]. Interestingly, mini-gp130-ELP very likely formed dimers, which in contrast to the preformed sgp130Fc-dimer are non-directional, since mini-gp130-ELP exhibited antagonistic properties comparable to the monomeric sgp130 and not to dimeric sgp130Fc.

In summary, we have generated transgenic tobacco plants accumulating high amounts of biologically active mini-gp130-ELP in the endoplasmic reticulum, which could be purified to homogeneity by inverse transition cycling. The results reported in this manuscript are encouraging and will stimulate the generation of constructs with a cleavable ELP-portion, which can then be dimerised artificially and in a directed fashion, e.g. by free accessible C-terminal cysteines. This strategy might lead to plant produced mini-gp130-cytokine antagonists with even higher activity.

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FIGURE LEGENDS

Figure 1 Expression and purification of mini-gp130-ELP.

(A) Schematic representation of the plant expression cassette and (B) illustration of the purification of ELP-fusion proteins from plant leaves by two rounds of 'inverse transition cycling'.

Figure 2 Purification of mini-gp130-ELP by 'inverse transition cycling'.

(A) Lane 1: raw extract of leaf material ground in a mortar under liquid nitrogen and extracted in PBS; lane 2: centrifuged and filtered (0.2 μ M pore size) raw extract; lane 3: supernatant after 1st round of inverse transition cycling; lane 4: soluble proteins after 1st round of inverse transition cycling; lane 5: soluble proteins after 1st round of inverse transition cycling cleared by centrifugation; ; lane 6: supernatant after 2nd round of inverse transition cycling; lane 7: soluble proteins after 2nd round of inverse transition cycling (concentrated by a factor of 25 when compared to raw extract); lane 8: soluble proteins after 2nd round of inverse transition cycling cleared by centrifugation (concentrated by a factor of 25 when compared to raw extract). Proteins were separated on a 10% SDS polyacrylamide gel and stained with Coomassie. M: molecular mass marker (kDa).

(B) Size exclusion chromatography of pure mini-gp130-ELP (2 ml) after 2 rounds of transition cycling on a calibrated HiPrep 26/60 Sephacryl S-300 High Resolution column using PBS as the mobile phase with a constant flow-rate of 1.0 ml/minute. Fractions of 2.5 ml were collected. Proteins were separated on a 10 % SDS polyacrylamide gel and silver stained.

Figure 3 Plant produced mini-gp130-ELP is biologically active.

(A) Immunoprecipitation of mini-gp130-ELP by Hyper-IL-6-Fc. 4 μ g mini-gp130-ELP was coincubated with conditioned culture supernatant containing Hyper-IL-6-Fc or with culture supernatant without Hyper-IL-6-Fc. Precipitated proteins and supernatants were separated by SDS/PAGE and blotted onto a PVDF membrane. Proteins were detected with the c-myc specific antibody 9E10 and visualized by ECL detection. Lane 1: supernatant after precipitation of mini-gp130-ELP with Hyper-IL-6-Fc. Lane 2: Pellet after precipitation of mini-gp130-ELP with Hyper-IL-6-Fc. Lane 3: Supernatant after precipitation of mini-gp130-ELP without Hyper-IL-6-Fc. Lane 4: Pellet after precipitation of mini-gp130-ELP without Hyper-IL-6-Fc.

(B) Binding of Hyper-IL-6 to mini-gp130-ELP and competition with sgp130Fc as measured by enzyme-linked immunosorbent assay – Microtiter plates were coated with mini-gp130-ELP (5 µg/ml) in PBS. After blocking with 3% BSA in PBS, 100 µl aliquots of Hyper IL-6 (0.1 µg/ml, 0.5 µg/ml and 1 µg/ml) (with or without antagonistic sgp130Fc (5 µg/ml)) were added. Hyper IL-6 bound to the plate was detected by the non-neutralizing anti-IL-6R mAb M91 followed by anti mouse immunoglobuline-peroxidase conjugate. The enzymatic reaction was performed with soluble Peroxidase substrate at 37°C for 30 min and the absorbance was measured at 450 nm.

(C) Inhibition of Hyper-IL-6-induced STAT3-phosphorylation by mini-gp130-ELP in human hepatoma cells (HepG2). HepG2 cells were stimulated with 50 ng/ml IL-6 or Hyper-IL-6 for 5 min in the presence or absence of mini-gp130-ELP. Unstimulated cells were used as controls. STAT3 phosphorylation was detected by Western Blot using anti-phospho-STAT3 specific antibodies. Western Blotting against STAT3 served as loading control.

Figure 4 Plant produced mini-gp130-ELP leads to an inhibition of Hyper-IL-6-induced Ba/F3-gp130/IL-6R proliferation

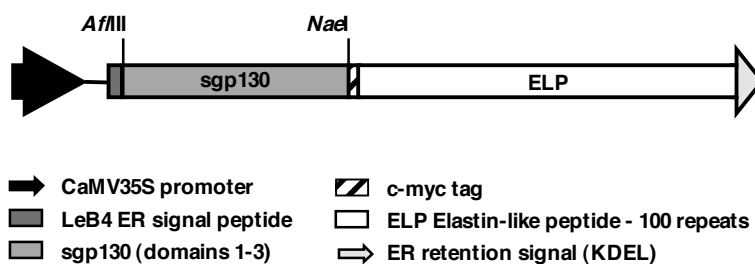
(A) Inhibition of Hyper-IL-6-induced STAT3-phosphorylation by mini-gp130-ELP in Ba/F3-gp130 cells. Ba/F3-gp130 cells were stimulated with 100 ng/ml of IL-6 or Hyper-IL-6 for 5 minutes in the presence or absence of mini-gp130-ELP (0.2 µg/ml, 2 µg/ml, 20 µg/ml). Unstimulated cells were used as controls. STAT3 phosphorylation was detected by Western Blot using anti-phospho-STAT3 specific antibodies. Western Blotting against STAT3 served as loading control.

(B) Inhibition of Hyper-IL-6-induced STAT3-phosphorylation by mini-gp130-ELP in Ba/F3-gp130/IL-6R cells. Ba/F3-gp130/IL-6R cells were stimulated with 10 ng/ml of IL-6 or Hyper-IL-6 for 5 minutes in the presence or absence of mini-gp130-ELP. Unstimulated cells were used as controls. STAT3 phosphorylation was detected by Western Blot using anti-phospho-STAT3 specific antibodies. Western Blotting against STAT3 served as loading control.

(C) Equal amounts of stably transfected Ba/F3-gp130-IL-6R-cells were cultured for 3 days in the presence of IL-6 or Hyper-IL-6 in combination with increasing amounts of mini-gp130-ELP. Proliferation was measured by pulse labelling the cells after 72 h with [³H]-thymidine for 4 h. Cells were harvested and incorporated radioactivity was measured by scintillation counting. Bioassays were performed with each value being determined in triplicate.

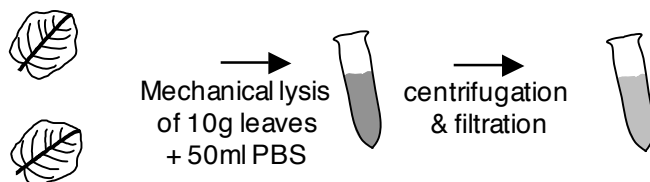
Figure 1

A

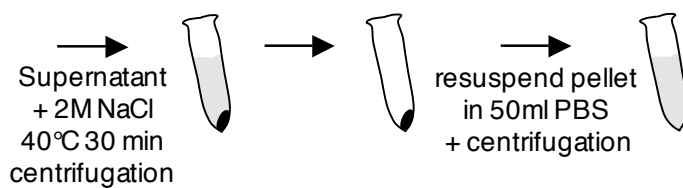


B

Preparation of leaf raw extract



1st round of inverse transition cycling



2nd round of inverse transition cycling

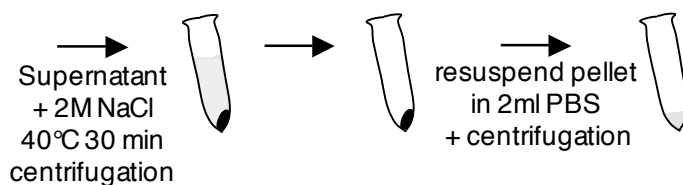


Figure 2

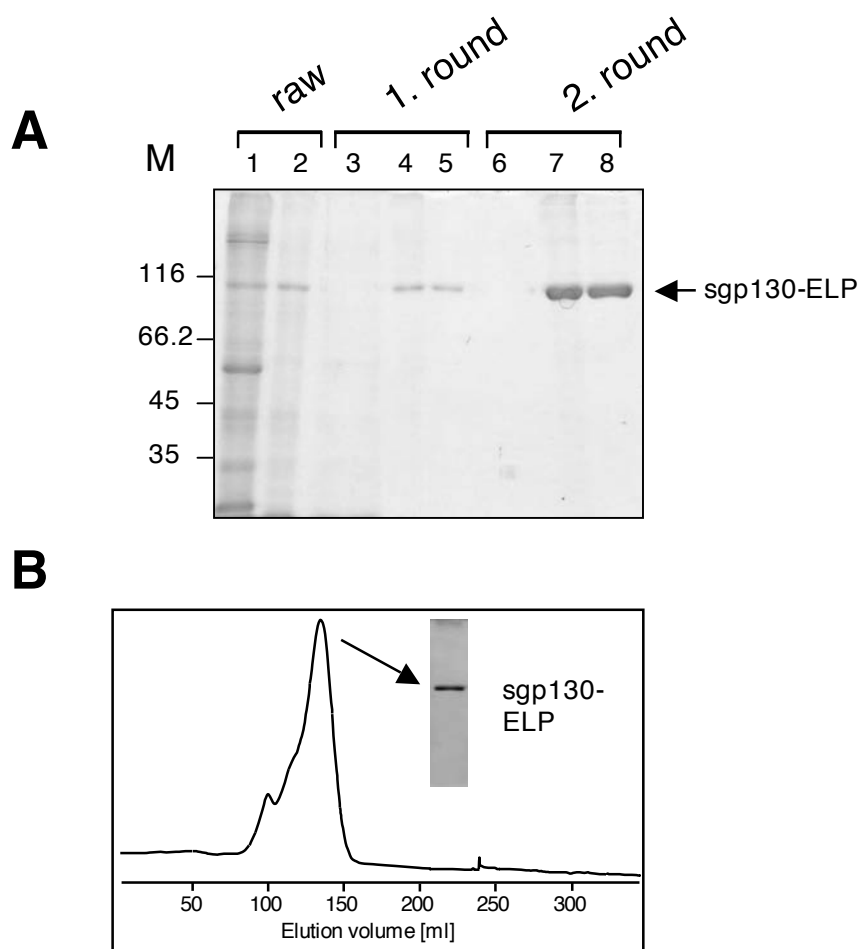
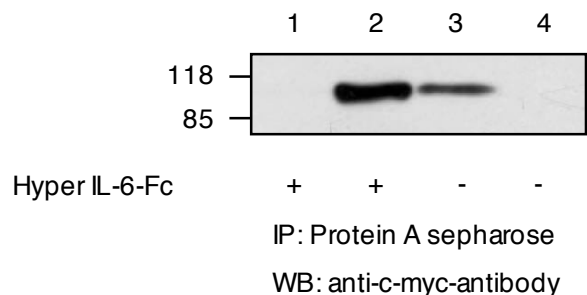
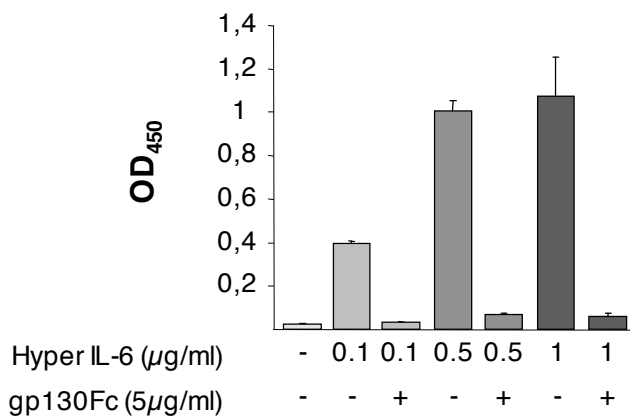


Figure 3

A



B



C

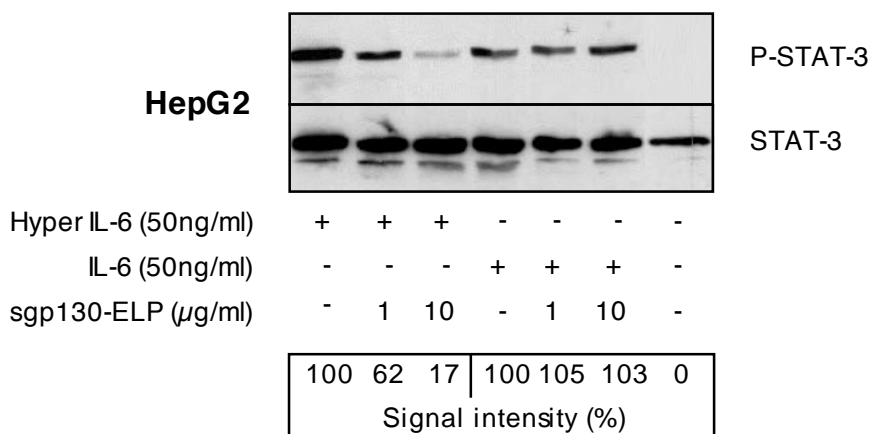


Figure 4

