Characterization of a potent human interleukin-11 agonist

Dimitri HARMEGNIES*[1], Xiao-Ming WANG*[1], Paul VANDENBUSSCHE*, Arnaud LEON†, Patricia VUSIO†, Joachim GRÖTZINGER‡, Yannick JACQUES†, Erik GOORMAGHTIGH§, Bart DEVREESE‖ and Jean CONTENT*[2]

*Institut Pasteur de Bruxelles, rue Engeland 642, B-1180 Brussels, Belgium, †Groupe Recherche Cytokines/Récepteurs, Unité INSERM 463, Institut de Biologie, 9 Quai Moncousu, 44035, Nantes Cedex 01, France, ‡Department of Biochemistry, Christian-Albrechts-Universitat zu Kiel, Olshausenstrasse 40, D-24098, Kiel, Germany, §Free University of Brussels, Structure and Function of Biological Membranes, CP206/2, B-1050 Brussels, Belgium, and ‖Ghent University, Laboratory for Protein Biochemistry and Protein Engineering, B-9000 Ghent, Belgium

Human interleukin-11 (hIL-11) is a multi-potential cytokine that is involved in numerous biological activities, such as haematopoiesis, osteoclastogenesis, neurogenesis and female fertility, and also displays anti-inflammatory properties. IL-11 is used clinically to treat chemotherapy-induced thrombocytopenia. Because of its broad spectrum of action, improved IL-11 agonists, as well as IL-11 antagonists, could be of interest for numerous clinical applications. IL-11 signalling is dependent on the formation of a tripartite ligand–receptor complex consisting of IL-11, the IL-11R (IL-11 receptor) α subunit (responsible for the specificity of the interaction) and gp130 (glycoprotein 130) receptor β subunit (responsible for signal transduction). The interaction between IL-11 and IL-11Rα subunit occurs at its recently assigned site I. We have designed an IL-11 mutein whose hydrophobicity at site I has been increased. The mutein has been characterized in terms of structure, affinity, specificity and bioactivity. Electrophoretic analysis, gel filtration, IR spectroscopy and CD indicate that this new protein is more compact than wild-type IL-11. It binds to IL-11Rα with a three-fold-enhanced affinity, and retains the ability to recruit gp130 through site II. However, analysis of its biological activity revealed a complex pattern: although this mutein is 60–400-fold more active than wild-type IL-11 on the proliferation of 7TD1 murine hybridoma cell, it is less active than IL-11 on the proliferation of B9 cells, another murine hybridoma cell line. The results are interpreted on the basis of an IL-11 conformational change induced by the mutations, and the preferential use by the mutein of another unknown transducing receptor chain.

Key words: cytokine, interleukin-11 (IL-11), interleukin-11 agonist, ligand binding, molecular modelling, site-directed mutagenesis.

INTRODUCTION

IL-11 (interleukin-11) was cloned from the primate stromal cell line PU-34 and was initially considered as a haematopoietic cytokine [1]. It was found later to also have effects on non-haematopoietic systems, and to act on many different cell types and tissues [2,3]. Numerous experiments on animal models and clinical trials in patients suffering from acute and chronic inflammatory diseases, including rheumatoid arthritis [4,5], inflammatory bowel disease [6–8], inflammatory liver disease [9,10], mucositis [11] and psoriasis [12], have revealed that IL-11 is an anti-inflammatory and mucosal protective agent, which, by inhibiting nuclear translocation of NF-κB (nuclear factor-κB), can decrease the production of pro-inflammatory cytokines secreted by macrophages such as TNF-α (tumour necrosis factor-α), IL-1β, IL-6 and IL-12 [13–16]. Its radioprotective and septic-shock-protective activities have also been demonstrated in other experiments [15,17–19]. The clinical application of hIL-11 (human IL-11) has been approved by the FDA (Food and Drug Administration) in the U.S.A. for the treatment of chemotherapy-induced thrombocytopenia, owing to the ability of this cytokine to stimulate megakaryocytopoiesis and thrombopoiesis [20–24]. Another potential therapeutic application of IL-11 in the treatment of mild haemophilia A or von Willebrand disease was recently evidenced by the fact that IL-11 is able to increase von Willebrand factor and factor VIII production in a von Willebrand disease mouse model, as well as in healthy mice [25].

Activity of IL-11 requires binding to the α receptor subunit (IL-11Rα) that provides ligand specificity in a functional multimeric signal-transduction complex with gp130 (glycoprotein 130), the common receptor subunit for the cytokine family including IL-6, vIL-6 (viral IL-6), CNTF (ciliary neurotrophic factor), LIF (leukaemia inhibitory factor), OSM (oncostatin M), CT-1 (cardiotrophin) and NNT-1/BSF-3 (novel neurotrophin-1/B-cell-stimulating factor-3) [26–29]. It is believed that IL-11 first interacts with IL-11Rα with a low affinity ($K_d = 10$ nM) and that the IL-11–IL-11Rα complex interacts subsequently with gp130 to form a high-affinity ($K_d = 300$–800 pM) and signal-transducing complex [30]. Three sites responsible for the interaction with the receptor subunits, similar to the situation described for IL-6, have been assigned for IL-11 [31,32]. Site I, constituting amino acids at the end of the AB-loop and the C-terminal part of the D-helix, is implicated in the interaction with IL11Rα. Site II, containing amino acids from the A and C helices and site III, consisting of the N-terminal part of the D-helix and residues from the beginning of the AB-loop, are responsible for gp130 (β-subunit) recruitment (Figure 1). LIF and CNTF were found to have similar receptor-binding topologies, whereas their site III is involved in gp190 [LIFR (LIF receptor)] recruitment [33,34].

The aim of the present study was the rational generation of a potent IL-11 agonist that could be potentially useful in clinical applications. The structure of the complex of the growth hormone (GH) and its receptor (GHR) serves as a paradigm for receptor recruitment by α-helical cytokines [35]. Mutagenesis studies revealed, for GH [36,37] and for IL-6 [28], that the main energy for receptor–ligand binding is provided by hydrophobic interactions

Abbreviations used: ATR, attenuated total reflection; CNTF, ciliary neurotrophic factor; FTIR, Fourier-transform IR; GH, growth hormone; GLM-R, gp130-like receptor; gp130, glycoprotein 130; hIL-11, human IL-11; IL-11, interleukin-11; IL-11Rα, α subunit of IL-11 receptor; LIF, leukaemia inhibitory factor; STAT, signal transduction and activators of transcription.

[1] These authors made an equal contribution to this work.

[2] To whom correspondence should be addressed (e-mail jcontent@pasteur.be).

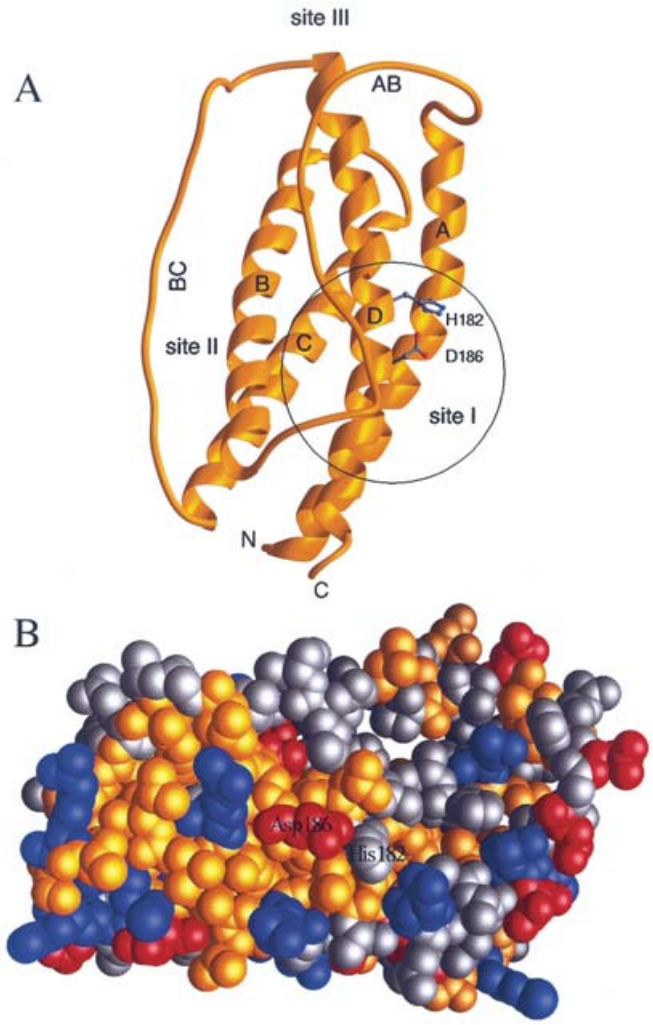

Figure 1 Three-dimensional model of human IL-11

(**A**) Three-dimensional model of the IL-11 based on crystallographic data obtained for CNTF, as described by Tacken et al. [32]. (**B**) Site I view of the IL-11 model. Positively charged amino acids (Arg, Lys) are coloured in blue, negatively charged amino acids (Asp, Glu) are in red, hydrophilic amino acids are in grey and hydrophobic amino acids are in yellow.

of apolar amino acids (a hydrophobic 'hot spot'), surrounded by polar or charged residues that are responsible for the specificity of interaction. Based on the three-dimensional model of IL-11 and previous studies defining three sites responsible for the recruitment of α and β receptors [32,38–40], the hydrophobicity analysis of hIL-11 site I revealed that it is composed of a hydrophobic cluster containing two hydrophilic amino acids, namely His^{182} and Asp^{186} (Figure 1). In an attempt to increase its affinity for IL-11Rα, we therefore decided to increase site I hydrophobicity by replacing His^{182} and Asp^{186} with their most closely related hydrophobic amino acid counterparts, valine and alanine respectively. The human IL-11 mutein thus created has been characterized in terms of structure, affinity, specificity and bioactivity on various IL-11-dependent cell lines.

EXPERIMENTAL

Bacterial strains, enzymes and chemicals

Escherichia coli DH5α was from Invitrogen Life Technologies. BL21 (DE3) and pET-22b(+) were from Novagen. *E. coli* recombinant hIL-11 was from PeproTech Inc. (London, U.K.) and R & D Systems (Wiesbaden-Nordenstadt, Germany). Primers for mutagenesis were from Genset (San Diego, CA, U.S.A.). Anti-hIL-11 monoclonal antibody MAB628 and anti-hIL-11 biotinylated polyclonal antibody BAF218 were from R & D Systems. [γ-^{32}P]ATP with a specific radioactivity of approx. 3000 Ci/mmol was obtained from Amersham Biosciences. Acrylamide and *N*,*N*′-methylenebisacrylamide were from Bio-Rad. RPMI 1640, DMEM (Dulbecco's modified Eagle's medium), glutamine and FCS (foetal calf serum) were from Gibco-BRL. The catalytic subunit of cAMP-independent protein kinase from bovine heart muscle, streptavidin-conjugated alkaline phosphatase, SDS and anti-FLAG M2 monoclonal antibody were obtained from Sigma.

Table 1 Primers used for inverse-PCR mutagenesis of FPΔIL-11

Underlined residues show the sites of mutation. s, sense; as, antisense.

Muteins	Primers
$H^{182} \rightarrow$ V (H/V)	G422 pACACTTGACTGGGCCGTACGGGGAC (s) G412 pCAGAACCAGCCCCCCCAGGATGG (as)
$D^{186} \rightarrow$ V (D/V)	G410 pACACTTGTCTGGGCCGTACGGGGAC (s) G421 pCAGGTGCAGCCCCCCCAGGATGG (as)
$D^{186} \rightarrow$ A (D/A)	G411 pACACTTGCCTGGGCCGTACGGGGAC (s) G421 pCAGGTGCAGCCCCCCCAGGATGG (as)
$H^{182} \rightarrow$ V-$D^{186} \rightarrow$ V (H/V-D/V)	G410 pACACTTGTCTGGGCCGTACGGGGAC (s) G412 pCAGAACCAGCCCCCCCAGGATGG (as)
$H^{182} \rightarrow$ V-$D^{186} \rightarrow$ A (H/V-D/A)	G411 pACACTTGCCTGGGCCGTACGGGGAC (s) G412 pCAGAACCAGCCCCCCCAGGATGG (as)

Mutagenesis

FPΔIL-11 was mutated by inverse-PCR amplification [41] of the plasmid pET-FPΔIL-11, previously described [42], using the primers shown in Table 1, followed by a *Dpn*I digestion to eliminate the parental plasmid. Gel-purified PCR fragments were ligated overnight at 16 °C using T4 DNA ligase and were then used to transform *E. coli* DH5α cells. The corresponding plasmids were amplified in DH5α, sequenced and then used to transform the BL21(DE3) strain of *E. coli*.

Production and purification of parental and mutant FPΔIL-11

BL21 (DE3) cells that were transformed with the plasmid carrying the mutant or parental FPΔIL-11 cDNA were cultured in Luria–Bertani medium containing 100 μg/ml of ampicillin. Expression of the recombinant proteins was induced by 1 mM IPTG (isopropyl β-D-thiogalactoside) for 2 h at 37 °C.

E. coli were then lysed by 30 min incubation at 37 °C in the presence of 0.1 % (v/v) Triton X-100 and 150 μg/ml lysozyme in 50 mM Hepes, pH 7.4, followed by sonication for 5 min at an intensity level of 5 using a microprobe (Vibra Cell, Sonics Materials Inc., Danbury, CT, U.S.A.). Lysates were centrifuged twice at 13 000 ***g*** for 25 min at 4 °C and were then assayed or purified as previously described [42]. Briefly, lysates were precipitated with 60 % $(NH_4)_2SO_4$ in order to concentrate the crude proteins. Salts were eliminated by dialysis against 50 mM Hepes buffer, pH 7.4, before the purification of samples by chromatography on a Mono-S HR5/5 column (Amersham Pharmacia Biotech) using a 50 mM Hepes buffer, pH 7.4, and a 0–1 M NaCl gradient.

Quantification of parental and mutant FPΔIL-11 by ELISA

Two antibodies raised against human IL-11, a non-neutralizing monoclonal antibody MAB618 and a biotinylated polyclonal

BAF218, were used to quantify the recombinant human parental and muteins by sandwich ELISA method. Microtitre plates (96-well) were coated overnight at 4 °C with 100 μl of monoclonal antibody MAB618 at a concentration of 2 μg/ml. After blocking with 3 % (w/v) BSA, 100 μl of serially diluted samples was added and incubated for 1 h at 37 °C. After washing with PBST buffer [PBS buffer with 0.1 % (v/v) of Tween 20], plates were incubated for a further 1 h at 37 °C with 100 μl of biotinylated polyclonal antibody BAF218/well at a concentration of 30 ng/ml. Before another incubation at 37 °C for 1 h with streptavidin-conjugated alkaline phosphatase (1/5000), the plates were washed three times with TBS buffer (100 mM Tris/HCl and 150 mM NaCl, pH 7.5). Finally, the test was revealed using an ELISA Amplification System (Gibco-BRL). Commercial recombinant IL-11 was used as a standard and the sensitivity was 2 pg/ml.

Mass spectrometry

The exact molecular mass of the FPΔIL-11 and the mutein was determined using nano-electrospray MS on a hybrid quadrupole time-of-flight Q-TOF mass spectrometer (Micromass, Wythenshawe, U.K.). Before analysis, samples were desalted using Vivaspin microconcentration devices with a cut-off of 10 kDa (Millipore, Bedford, MA, U.S.A.). After washing twice with water, samples were dissolved in a mixture of 50 % acetonitrile and 0.1 % formic acid in water to a concentration of approx. 5 pmol/μl. Samples (4 μl) were loaded in a nano-electrospray capillary (MDS Proteomics, Odense, Denmark) that was then placed in the special holder delivered with the instrument. Spray was initiated by slightly breaking the needle tip and supplementing a small back-pressure of nitrogen. The capillary voltage was set at 1250 V. Spectra were accumulated for about 5 min, collecting data from *m*/*z* 1000–2500 at 1 s/scan. Data processing was performed using the Masslynx and MaxEnt software delivered with the instrument.

IR spectrometry

ATR–FTIR (attenuated total reflection–Fourier-transform IR) spectrometry spectra were recorded at room temperature (20 °C) on a Bruker IFS55 FTIR spectrophotometer equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector at a nominal resolution of 2 cm^{-1} and encoded every 1 cm^{-1}. The internal reflection element (IRE) was a germanium plate (50 mm × 20 mm × 2 mm) with an aperture angle of 45°, yielding 25 internal reflections. The spectrophotometer was continuously purged with air dried on a FTIR purge gas generator 75-62 Balston (Maidstone, U.K.) at a flow rate of 10–20 l/min in the sample compartment and 5 l/min in the optic compartment. Thin films were obtained by slowly evaporating a sample under a stream of nitrogen on one side of the ATR plate [43]. The ATR plate was then sealed in a liquid sample holder. The sample on the ATR plate was rehydrated by flushing ^2H_2O-saturated N_2, at room temperature. For each measurement a mean of 256 scans was calculated. Secondary-structure determination was based on the shape of the amide I band (1600–1700 cm^{-1}), which is sensitive to the secondary structure [44].

For hydrogen/deuterium exchange kinetics, nitrogen was saturated with ^2H_2O by bubbling in a series of three vials containing ^2H_2O. Before starting the deuteration, ten spectra of the sample were recorded to test the stability of the measurements. At zero time, the ^2H_2O-saturated N_2 flux, at a flow rate of 100 ml/min (controlled by a Brooks flow meter), was connected to the sample. For each kinetic time point, 24 scans were recorded and averaged at a resolution of 4 cm^{-1}. All the spectra of the kinetics were corrected for atmospheric-water absorption and side-chain contribution. The subtraction of atmospheric water was done automatically by home-written software that computed the subtraction coefficient as the ratio of the atmospheric-water band between 1579 and 1572 cm^{-1} on the sample spectrum, and on a reference atmospheric-water spectrum [45–49]. The area of amide II, characteristic of the δ(N-H) vibration, was obtained by integration between 1596 and 1502 cm^{-1}. For each spectrum, the area of amide II was divided by the corresponding amide I ν(C=O) area. This ratio, expressed as a percentage, was plotted against deuteration time. The 100 % value is defined by the amide II/amide I ratio obtained before deuteration. The 0 % value corresponds to a zero absorption in the amide II region, observed for a full deuteration of the protein.

Circular dichroism

CD measurements were carried out on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) equipped with a temperature control unit and calibrated according to Chen and Yang [50]. The spectral bandwidth was 2 nm (< 250 nm) and 1 nm (> 250 nm) respectively. The measurements were carried out at a temperature of 20 °C, and the solvent was PBS throughout. The time constant ranged between 1 and 4 s and the cell path length was between 0.1 and 10 mm.

Labelling of FPΔIL-11 and of its mutein

FPΔIL-11 and the H/V-D/A mutant were labelled through protein phosphorylation with [γ-^{32}P]ATP or [γ-^{33}P]ATP in the presence of bovine heart kinase, and phosphorylation was checked by autoradiography as previously described [42].

SDS/PAGE and Western blot

SDS/PAGE was carried out as previously described [51]. Muteins and parental FPΔIL-11 were transferred from gels on to a nitrocellulose membrane and were detected by incubation with biotinylated goat polyclonal antibody BAF218 (R & D Systems), then with streptavidin-conjugated alkaline phosphatase, and finally revealed with the NBT (Nitro Blue Tetrazolium)/BCIP (5-bromo-4-chloroindol-3-yl phosphate) system (Sigma). Alternatively, proteins were detected using biotinylated anti-FLAG antibody M2.

Binding of [^{32}P]H/V-D/A to cells

Binding of [^{32}P]H/V-D/A on 7TD1 cells was carried out as described for the parental [^{32}P]FPΔIL-11 on B13Rα1 cells by Wang et al. [42]. 7TD1 cells (5×10^5) were pre-incubated in culture medium lacking growth factor for 2 h and were washed three times with PBS, pH 7.4. For binding studies, radiolabelled H/V-D/A was added to cells at the indicated concentration in PBS containing 0.5 % (w/v) BSA. The mixture was incubated at 4 °C for the appropriate time, and bound radiolabelled H/V-D/A was separated from the free radioactivity by centrifugation at 3000 ***g*** for 1 min through a 0.2 ml layer of a mixture of dioctyl phthalate and dibutyl phthalate (2:3, v/v) (Janssen Chemica, Beerse, Belgium). After quick freezing, the tip of each tube containing the cell pellet was cut off, and radioactivity was counted in a Beckman β-counter. Non-specific binding was determined by incubating cells with radiolabelled H/V-D/A in the presence of a 200-fold molar excess of unlabelled H/V-D/A.

Surface-plasmon-resonance studies

These experiments were performed with a BiaCore 2000 optical biosensor (BiaCore, Uppsala, Sweden). A fusion protein of human IL-11R and IL-2 (IL-11R–IL-2) [52] was coupled through primary amino groups to a carboxymethyl-dextran flow cell (CM5) at a low immobilization level (about 500 resonance units per flow cell) compatible with kinetic binding studies. Subsequent binding of parental FPΔIL-11 or mutein was carried out in Hepes buffer (pH 7.4) at a flow rate of 10 μl/min at 24 °C.

IL-11 bioassay

IL-11 activity was measured using the 7TD1 cells. Cells, at a concentration of 2×10^3 cells/well, were cultured in flat-bottom 96-well microtitre plates for 7 days in the presence of serial dilutions of the purified mutein or parental FPΔIL-11, or *E. coli* crude lysates containing different muteins previously adjusted to the same protein concentration. The cell number in each well was then determined by a colorimetric assay for hexosaminidase [53]. Bioactivity was assayed similarly on 1×10^4 B9 cells/well for approx. 3 days and was revealed by XTT {sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzenesulphonic acid hydrate} colorimetric assay. Each sample was tested in triplicate using a commercial recombinant human IL-11 as a standard.

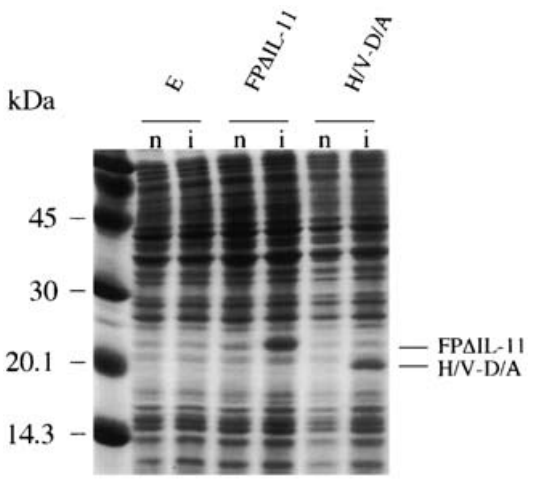

Figure 2 Expression of FPΔIL-11 and H/V-D/A mutein analysed by SDS/PAGE

BL21 *E. coli* were transformed with pET-22b(+) vector encoding FPΔIL-11 and H/V-D/A mutein or empty vector (E). After induction (i, induced; n, not induced) of protein production, bacteria were lysed as described in the Experimental section. Supernatants (100 μg of total protein per lane) were then analysed by SDS/PAGE and stained with Coomassie Blue. Molecular mass indications are given in kDa.

RESULTS

Expression, purification and initial characterization of the H/V-D/A mutein

FPΔIL-11 was used as the human IL-11 parental molecule to generate IL-11 muteins by mutagenesis, because (i) it has the same biological activity as the wild-type human recombinant IL-11 and (ii) the presence of the FLAG-tag (F), the phosphorylation site (P) and the absence of the first ten amino acids of IL-11 (Δ) allow a strong expression, a simple purification and an easy radio-labelling of IL-11 [42].

To evaluate the involvement of His^{182} and Asp^{186} with respect to biological activity and receptor binding, these residues were altered by site-directed mutagenesis using an inverse-PCR method [41]. These two residues were replaced by a valine residue ($H^{182} \rightarrow V$) and an alanine residue ($D^{186} \rightarrow A$) respectively to generate a mutein named H/V-D/A.

The expression of these parental and mutant FPΔIL-11 in *E. coli* was analysed by SDS/PAGE (Figure 2). The parental molecule had an apparent molecular mass of about 24 kDa, a value higher than its theoretically expected one (20.05 kDa). This difference could be due to the introduction of numerous charged residues present in the FLAG-tag and the phosphorylation site at the N-terminus of FPΔIL-11 (one glutamic, five aspartic, two arginine and two lysine residues). Indeed, when the two charged residues His^{182} and Asp^{186} of FPΔIL-11 were replaced by two hydrophobic amino acids, the resulting mutein moved faster in gels than its parent, so that its apparent molecular mass (19 kDa) was close to its calculated one (19.9 kDa). This observation reinforced the hypothesis that the charged residues could influence the molecular mobility in SDS/PAGE. However, to rule out the possibility that the reduced mobility of the H/V-D/A mutein could be linked to a truncation of the protein, purified parental and mutant FPΔIL-11 were submitted to mass spectrometric analysis. FPΔIL-11 and H/V-D/A were found to have masses of 20.016 kDa and 19.934 kDa respectively, in perfect agreement with their predicted molecular masses (results not shown).

Even though the increased electrophoretic mobility of the H/V-D/A mutein on SDS/PAGE is probably due to charge modifications, we cannot rule out the possibility that it could be partially due to a structural and/or conformational change of the molecule induced by mutagenesis. Such changes could render the mutein more compact than the parental molecule, therefore making it more resistant to heat denaturation and enabling it to move faster in polyacrylamide gels.

Structural analysis by IR spectrometry and CD

In order to evaluate further a potential conformational change induced by mutagenesis, the parent and mutant proteins, purified to homogeneity, were characterized by ATR–FTIR spectrometry. This technique has been used successfully to investigate the structure of soluble and membrane proteins [44]. The method is based on the analysis of the vibration bands of protein and particularly the amide I band, ν(C=O), whose absorption frequency is dependent upon the secondary structure. Figure 3 represents the ATR–FTIR deuterated spectra of those two proteins recorded at pH 7.4. Their similar spectra suggest that the replacement of two amino acids (His^{182} and Asp^{186}) by a valine and an alanine residue respectively does not have a detectable effect upon the protein secondary structure. The main absorption peak within the amide I is located in a region associated with the α-helical structure, confirming that this structure is predominant in both IL-11 (parent and mutein).

Parental and mutant IL-11 were also submitted to CD analysis, because this technique is more sensitive to α-helical structures. Figure 4 shows their CD spectra. Both spectra have the same shape, but their intensity is different. Secondary-structure analysis [28] of the far-UV CD spectra of both proteins reflects the α-helical character of the proteins (parental IL-11: α-helix 44.8%, β-sheet 14.0%, turn 15%, remainder 26.2%; mutant IL-11: α-helix 38.8%, β-sheet 17.0%, turn 15.7%, remainder 28.5%), which are typical for a four-helix bundle cytokine. The somewhat lower helical content of the IL-11 mutein compared with the

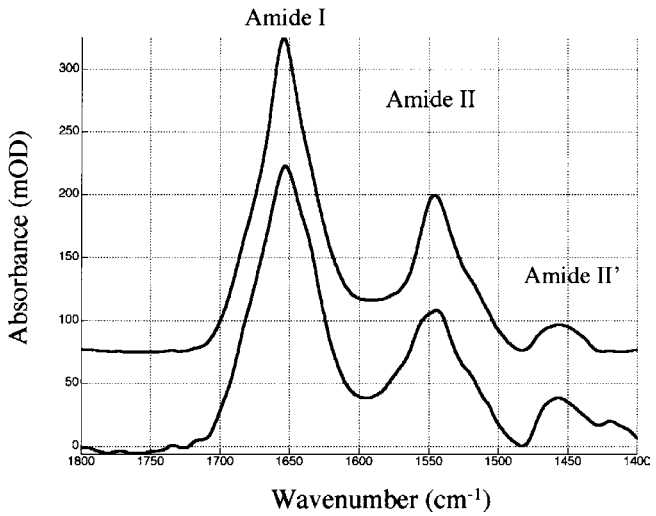

Figure 3 IR spectra of the FPΔIL-11 (upper spectrum) and H/V-D/A (lower spectrum) in the 1800–1400 cm^{-1} frequency range

The absorbance is reported in mOD. The absorbance scale is given for the bottom spectrum. The upper spectrum has been offset for clarity.

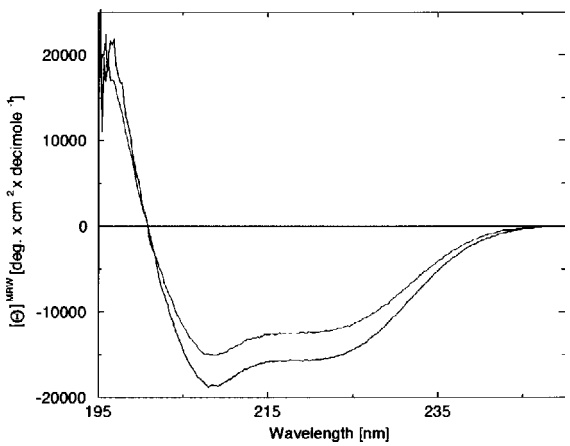

Figure 4 CD spectra of the FPΔIL-11 (lower spectrum) and H/V-D/A (upper spectrum)

parental IL-11 might reflect a conformational change introduced by the mutated amino acids.

To characterize further the conformational changes that take place upon mutagenesis of FPΔIL-11, deuteration kinetics of the mutein and its parental protein were measured. In a soluble protein, the rate of hydrogen/deuterium exchange is essentially related to protein-structure stability (local unfolding dynamics in secondary structures govern the exchange). The hydrogen-exchange rate of the proteins was followed by monitoring the amide II absorbance peak decrease [δ(N-H) maximum in the 1596–1502 cm^{-1} region], because of its shift to the 1460 cm^{-1} region [amide II′, δ(N-D)] upon deuteration (results not shown). The variations with time of the percentages of non-exchanged residues, calculated from the ratio of amide II/amide I as described in the Experimental section, are shown in Figure 5. It appears that the FPΔIL-11 is undergoing a fast exchange, whereas the H/V-D/A mutein is more resistant to hydrogen/deuterium exchange, suggesting that the mutein might form oligomers and/or have a more compact structure than parental FPΔIL-11.

By gel filtration on a Superdex-75 column, parental and mutant proteins were both eluted at a similar position corresponding to a monomeric form (Figure 6), indicating that the increased hydrophobicity due to mutagenesis at site I did not lead to the formation of dimers or oligomers.

Interaction with soluble IL-11Rα

In order to find out if mutagenesis and the associated conformational change of H/V-D/A have an effect on its interaction with IL-11Rα, the association (k_{on}) and dissociation (k_{off}) kinetic constants describing parent IL-11 and H/V-D/A mutein binding to human IL-11Rα were determined by surface plasmon resonance biosensor analysis using dextran-immobilized purified human IL-11Rα–IL-2 [52] fusion protein as a matrix. As depicted in Table 2, the k_{on} and k_{off} kinetic constants of H/V-D/A were both much higher (35- and 14-fold respectively) than those of parental FPΔIL-11, leading to an equilibrium dissociation constant (K_d) for the mutein that was 3-fold lower than for FPΔIL-11. If one translates the K_d in terms of free energies of interaction [$\Delta G = -RT\ln(1/K_d)$], binding of FPΔIL-11 or H/V-D/A to IL-11R–IL-2 is accompanied by free-energy changes of 9.2 or 9.8 kcal/mol respectively (1 kcal ≡ 4.184 kJ), indicating that the mutagenesis and its induced conformational change favour the interaction of IL-11 with the IL-11Rα receptor.

Interaction with cell-surface IL-11 receptors

B13Rα1 and 7TD1 cells were used to test H/V-D/A binding to human and murine IL-11 receptors. B13Rα1 are Ba/F3 cells stably transfected with human gp130 and hIL-11Rα [54]. The non-specific binding component, determined by adding a 200-fold molar excess of unlabelled H/V-D/A, was low (< 5 % of the total association). Analysis of the specific-binding data by the method of Scatchard indicated the existence of a single class of binding sites (Table 3). We could only detect high-affinity receptors on these cells, probably because of an excess of gp130 expression on the surface of the transfected cells. The dissociation constant for the mutein ($K_d = 0.7$ nM) was higher than that for its parent ($K_d = 0.4$ nM). Binding of [^{32}P]H/V-D/A could be completely inhibited by an excess of FPΔIL-11, and the reverse was also found, showing that the two molecules compete with each other for this binding.

7TD1 is a murine hybridoma cell line resulting from the fusion of the mouse myeloma cell line Sp2/0-Ag14 with spleen cells from a C57BL/6 mouse. This cell line is well known to respond to picogram amounts of IL-6 [53], but also has a proliferating response to nanogram amounts of IL-11 [42].

When 7TD1 cells were used for ^{32}P-labelled H/V-D/A or FPΔIL-11 receptor-binding assays, two classes of binding sites were observed (Table 3): low-affinity receptors with a K_d in the nanomolar range, which probably correspond to the binding of IL-11 or mutein to isolated IL-11Rα chains, and high-affinity receptors with a K_d in the picomolar range, which probably correspond to the association of IL-11–IL-11Rα with gp130-transducing subunits. Similar numbers of either type of receptors were detected with labelled FPΔIL-11 and H/V-D/A, in agreement with the above observation that the two molecules compete for common receptors. In the context of low-affinity binding to isolated IL-11Rα chains, the affinity of H/V-D/A ($K_d = 2.7$ nM) was found to be approx. 3-fold higher than that determined for FPΔIL-11 ($K_d = 7.2$ nM), in agreement with the biosensor experiments (Table 2). In the context of the high-affinity receptor complex, however, no differences were found between H/V-D/A and FPΔIL-11 binding ($K_d = 0.60$ nM compared with 0.65 nM).

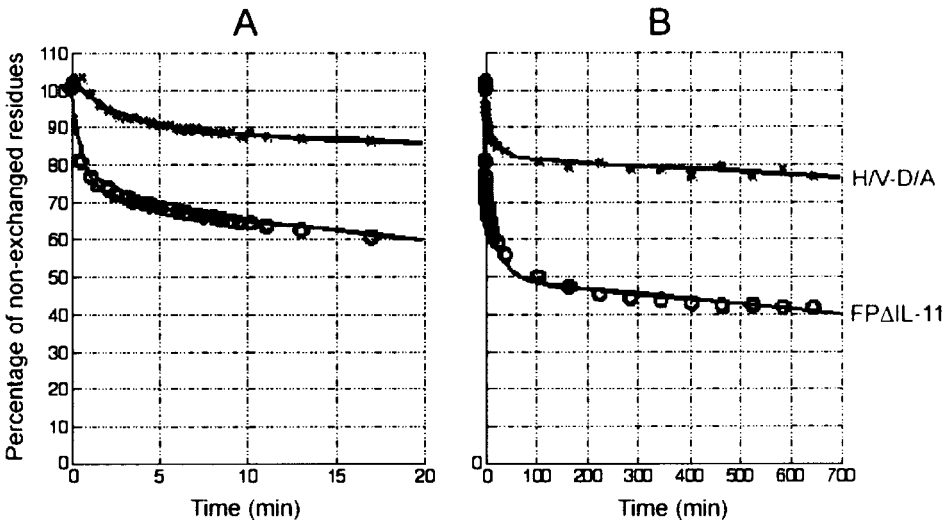

Figure 5 Evolution of the integrated intensity of the amide II band as a function of the time of exposure to ^2H_2O for the FPΔIL-11 (○) and for the H/V-D/A (×)

Fitting was carried out with a three exponential decay. (**A**) Over 0–20 min. (**B**) Over 0–700 min.

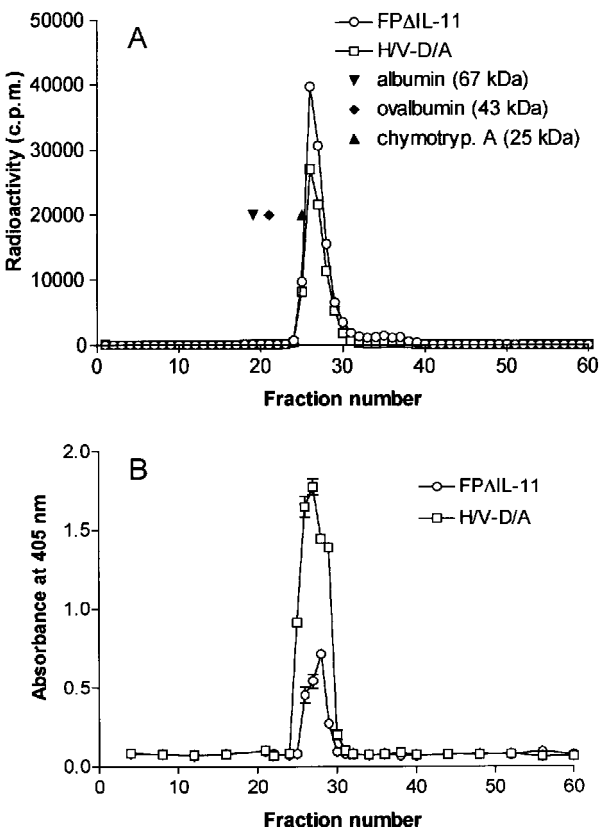

Figure 6 Gel-filtration chromatography of parental FPΔIL-11 and mutant H/V-D/A and their bioactivity tested from fractions collected during the chromatography

(**A**) Superdex-75 column (K16; Amersham Pharmacia Biotech) was used and calibrated with three proteins: albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (chymotryp. A; 25 kDa), before loading 30 μg of each analysed unlabelled protein in the presence of 50 ng of the same ^{33}P-labelled one as a tracer. Each collected fraction (50 μl) was submitted to radio-counting. (**B**) IL-11 activity was measured using the mouse hybridoma cell line 7TD1. Cells were cultivated in flat-bottom 96-well plates (2×10^3 of 7TD1 cells/well) in the presence of 0.2 μl of each eluted fraction. After 7 days of culture, the number of surviving cells was determined by colorimetric assays for hexosaminidase. Results are means ± S.D. ($n = 3$).

Table 2 Kinetic association (k_{on}) and dissociation (k_{off}) and equilibrium dissociation (K_d) constants for the binding of FPΔIL-11 and H/V-D/A to the recombinant human IL-11Rα–IL-2, determined by surface plasmon resonance

IL-11	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
FPΔIL-11	$5.90(\pm 0.90) \times 10^3$	$9.75(\pm 0.05) \times 10^{-4}$	165(± 25)
H/V-D/A	$2.30(\pm 0.74) \times 10^5$	$1.34(\pm 0.46) \times 10^{-2}$	58(± 1.5)

Table 3 Dissociation constants and numbers of sites per cell of FPΔIL-11 and H/V-D/A binding in B13Rα1 and 7TD1 cells

			7TD1			
	B13Rα1		Class 1 sites		Class 2 sites	
Ligands	K_d (nM)	Sites/cell	K_d (nM)	Sites/cell	K_d (nM)	Sites/cell
[^{32}P]FPΔIL-11						
Competed with FPΔIL-11	0.44	3079	7.20	391	0.65	16
Competed with H/V-D/A	0.40	2900	ND*	ND	ND	ND
[^{32}P]H/V-D/A						
Competed with H/V-D/A	0.71	3462	2.70	486	0.60	16
Competed with FPΔIL-11	0.72	3531	ND	ND	ND	ND

* ND, not determined

Induction of cell proliferation

To investigate to what extent the increased affinity of the mutein for the IL-11Rα could impact on its bioactivity, cell proliferation assays were conducted on different cell lines.

As shown in Figure 7(A), H/V-D/A mutein, like IL-11, supports 7TD1 cell proliferation dose-dependently. However, the EC_{50} of the mutein was much lower (400-fold) than that required for the wild-type IL-11 ($EC_{50} = 0.03$ ng/ml for H/V-D/A compared with 15 ng/ml for FPΔIL-11 and rhIL-11). This increased activity of the mutein was found consistently in several experiments, with a H/V-D/A/FPΔIL-11 activity ratio ranging from 60 to 400. Gel-filtration experiments (Figure 6) showed that parental and mutant

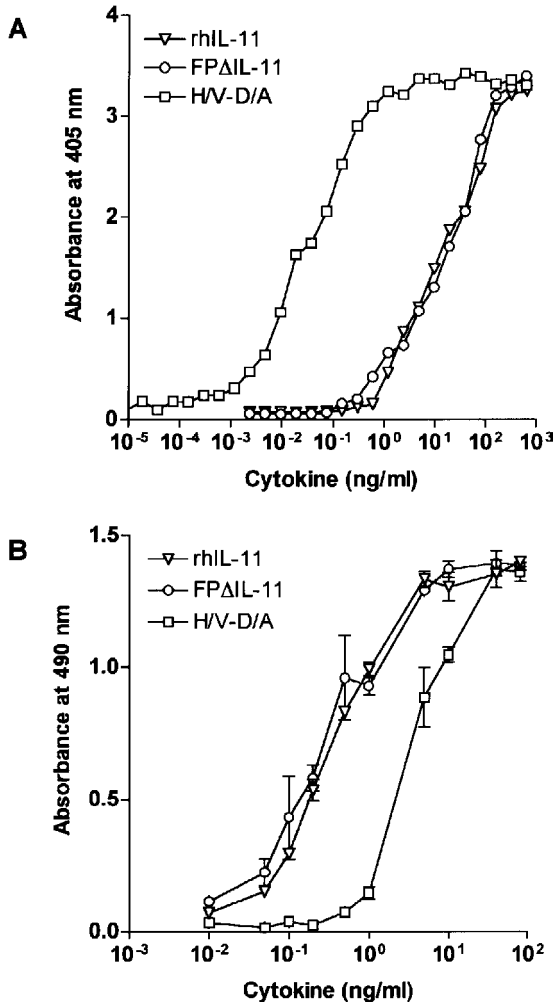

Figure 7 Bioactivity of parental and mutant FPΔIL-11 tested on 7TD1 (A) and B9 (B) cells

Cells were cultivated in flat-bottom 96-well plates (2×10^3 7TD1 cells/well; 1×10^4 B9 cells/well) in the presence of serial dilutions of parental FPΔIL-11, mutein H/V-D/A or commercial rhIL-11 (R & D Systems). After 7 days for 7TD1 and 3 days for B9 cells culture, the number of surviving cells was determined by colorimetric assays for hexosaminidase (7TD1 cells) and for XTT {sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzenesulphonic acid hydrate} (B9 cells). Results are means ± S.D. ($n = 3$).

IL-11 behaved as monomeric molecules (at approx. 20 kDa) with no sign of aggregation, and biological activity was associated fully with these monomers.

In sharp contrast with what was found on 7TD1 cells, the H/V-D/A mutein was about 10-fold less active on B9 cells (Figure 7B), another murine hybridoma cell line, indicating that the mode of action of the IL-11 mutein was more complex than expected.

In order to check if the H/V-D/A activity was mediated through gp130 transduction, we used an anti-IL-11 monoclonal antibody (H2) that has been demonstrated to react with an epitope localized in site II of IL-11 [52]. By interfering with gp130 recruitment, this antibody inhibits the binding of FPΔIL-11 to its receptors, and consequently inhibits IL-11-dependent cell proliferation [42]. Figure 8 shows that this neutralizing antibody is able to inhibit 7TD1 cell proliferation induced by both the parental and mutant FPΔIL-11, indicating that the epitope recognized by the antibody H2 (site II) is conserved in the H/V-D/A mutein, and that H/V-D/A, like parental IL-11, requires the gp130 subunit for exerting

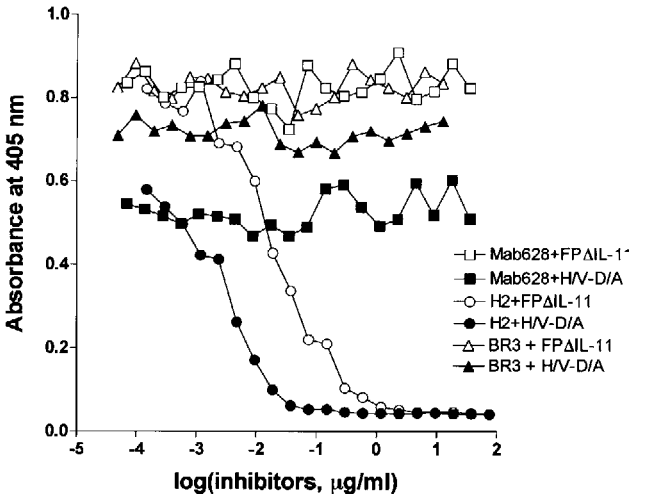

Figure 8 Inhibition of 7TD1 cell proliferation stimulated by FPΔIL-11 or H/V-D/A by anti-hIL-11 and anti-(human gp130) neutralizing antibodies

Cells were incubated with the indicated concentrations of anti-hIL-11 monoclonal antibodies H2 (circles) and anti-(human gp130) monoclonal antibodies MAB628 (squares) and B-R3 (triangles). Results are means of triplicate determinations.

its bioactivity. The anti-(human gp130) antibodies MAB628 and B-R3 did not affect parental or mutant IL-11 proliferation of the murine 7TD1 cells, and served as controls. As far as these two antibodies have been shown to inhibit cell proliferation on human cells [55], these results also indicate that the epitopes recognized by these antibodies on human gp130 are not shared by murine gp130.

When analysing the dose–response curves depicting the inhibitory effect of H2 antibody (Figure 8), it appeared that the IC_{50} of H2 was approx. 10-fold lower in the case of the H/V-D/A mutein than in the case of parental IL-11. This indicates that the H/V-D/A mutations at site I induce a conformational change at site II that results in an increased affinity for the H2 antibody. Other experiments showed that H/V-D/A, like IL-11, was able to stimulate the proliferation of Ba/F3 cells co-transfected with human IL-11Rα and human gp130 (results not shown), whereas Ba/F3 cells only transfected with human gp130 were insensitive to either molecule. Therefore H/V-D/A, like parental IL-11, cannot activate gp130 in the absence of IL-11Rα.

Relative roles of His^{182} and Asp^{186} in the properties of H/V-D/A

In order to investigate the relative importance of His^{182} and Asp^{186}, these residues were mutated separately or in combination, generating H182/V, D186/V, D186/A and H182/V-D186/V (H/V-D/V) muteins, in addition to H/V-D/A. As shown in Figure 9, SDS/PAGE and Western blot analysis indicate a good expression of all recombinant proteins. As observed before for H/V-D/A, all muteins showed systematic differences between their apparent molecular mass on SDS/PAGE and their predicted one. Muteins D186/V and D186/A moved faster than the mutein H182/V, suggesting that the negatively charged residue (Asp) had more impact on the molecular mobility in gels than the positively charged one (His). The difference of mobility between D186/V and D182/A also indicated that the charge is not the only factor involved in the mobility change. This reinforces our previous hypothesis that, beyond the charge, an SDS-resistant conformational change of the molecules resulting from mutagenesis could also contribute to the mobility change. The two double muteins H/V-D/V and

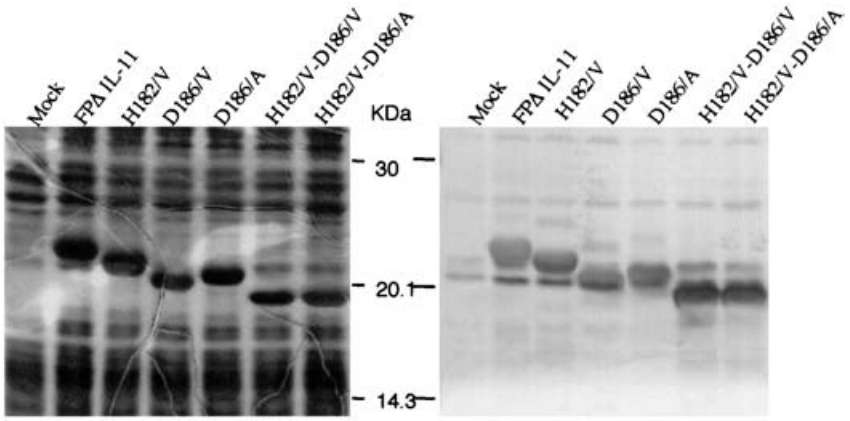

Figure 9 Expression of FPΔIL-11 and its muteins analysed by SDS/PAGE and immunoblotting

BL21 *E. coli* were transformed with empty vector (Mock) or expression vector encoding parental FPΔIL-11 or mutated proteins as indicated. After induced expression of the proteins, cells were lysed by sonication, and lysates (100 μg of total protein/lane) were analysed by SDS/PAGE (left-hand panel) and immunoblotting (right-hand panel) using a polyclonal antibody raised against IL-11 (BAF 218).

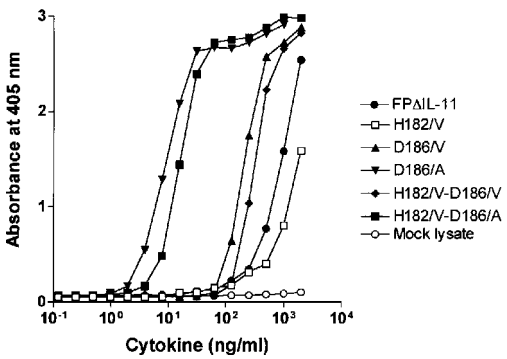

Figure 10 Proliferation of 7TD1 cells in response to FPΔIL-11 and its muteins

7TD1 cells were incubated in the presence of serial dilutions of *E. coli* lysate containing mock, FPΔIL-11 or muteins, which were previously adjusted to 2 μg/ml. After 7 days of culture, the number of cells was determined by a colorimetric assay for hexosaminidase.

H/V-D/A had similar and higher mobilities than the single muteins, indicating cumulative effects of the two mutations.

7TD1 cells were used to measure the bioactivity of the various FPΔIL-11 muteins (Figure 10). It appeared that the D186/A mutation alone resulted in a strong increase in activity, even stronger than the H/V-D/A combination. The D186/V mutation also resulted in an increase in activity, but to a far lower extent than the D186/A mutation. In contrast, the H182/V mutation always resulted in a slight reduction of bioactivity: H182/V, H/V-D/V and H/V-D/A were less active than wild-type, D186/V and D186/A respectively.

These results suggest that Asp[186] is a key amino acid in site I and plays an essential role in the activity of IL-11. Of note, replacement of Asp[186] by valine instead of alanine resulted in a much lower increase of activity, suggesting that, in addition to the hydrophobic nature, the size of the side chain at position 186 is crucial for this enhancement of activity. The His[182] residue also appears to be involved in the interaction at site I, but with a minor role.

DISCUSSION

The aim of the present study was to create potent agonists of human IL-11 by changing amino acids located in the area (site I) responsible for binding to the specific receptor chain (IL-11Rα). A model of IL-11 was built by homology considerations based on the known receptor-interaction sites of the related cytokines IL-6, CNTF and LIF [29]. Supported by mutagenesis experiments, the model predicts that the main energy for receptor–ligand binding is provided by hydrophobic interactions of a few apolar side chains shielded by a surrounding scaffold of polar or charged residues that guarantee the specificity of the interaction by the formation of hydrogen bonds and salt bridges [28]. Therefore, in order to enhance the interaction of IL-11 with its α-receptor subunit, we replaced two charged amino acid residues His[182] and Asp[186] located in the middle of the site I hydrophobic cluster by two hydrophobic ones. We anticipated that increasing the local hydrophobicity on the surface of site I could influence the quaternary structure of the molecule: a putative large hydrophobic-interaction zone generated by mutagenesis might favour H/V-D/A to form oligomers. Superdex-75 chromatography has evidenced that H/V-D/A is in fact expressed as a soluble functional monomeric protein. However, IR hydrogen/deuterium-exchange kinetics showed that the H/V-D/A mutein is more resistant to hydrogen/deuterium exchange, suggesting that the mutein might have a more compact structure than parental FPΔIL-11. IR hydrogen/deuterium kinetic studies were indeed recorded at a higher protein concentration, since the proteins were concentrated in a film for that experiment. It is then conceivable that additional interactions are present in the IR experiment. Yet, such local interactions encompassing the new, more hydrophobic, domain found in the H/V-D/A mutant could not explain the large effect reported in Figure 5, where almost 40 % of the residues experience a slower exchange, nor can such a difference be explained in view of the minor differences in the secondary structures. On the other hand, a more compact structure is deduced from the mutant's faster mobility on SDS/PAGE, in good agreement with the slower IR hydrogen/deuterium exchange and CD data.

Analysis of the binding characteristics of the H/V-D/A mutein confirmed our earlier studies that residues at the end of the D-helix are implicated in recognition for and interaction with IL-11Rα [32,38,40]. Indeed, biosensor studies showed that the H/V-D/A mutations were associated with modifications in the parameters of binding to the isolated IL-11Rα chain. Both the association and dissociation constants were markedly increased, indicating that the nature of the molecular bonds involved in the cytokine–receptor interaction at site I was strongly modified. Despite these

changes, the binding affinity of the mutein for IL-11Rα was only 3-fold higher than that of parental IL-11. Equilibrium studies on cell-surface receptors confirmed this 3-fold increase in affinity, and showed further that, in the context of the high affinity IL-11Rα–gp130 complex, the mutein and wild-type IL-11 displayed similar affinities.

The relative bioactivity of the H/V-D/A mutein as compared with wild-type IL-11 was not correlated with the difference in affinity between the two molecules. Indeed, on the 7TD1 murine hybridoma cell line, the H/V-D/A had a considerably (> 400-fold) increased activity, whereas on another murine hybridoma cell line (B9), its bioactivity was reduced by about 10-fold. Such variations are in line with a previous study showing that, on another murine plasmacytoma cell line (T10), the substitution of alanine for Asp^{186} (D186/A mutein) rendered the cytokine 500-fold less active than the wild-type [40].

What makes the H/V-D/A more active on 7TD1 cells? Since 7TD1 cells are highly responsive to IL-6, such a high H/V-D/A bioactivity could result from their stimulation via IL-6Rα-mediated signal transduction. As parental FPΔIL-11 was found in the present study to compete fully with ^{32}P-labelled H/V-D/A for its high-affinity binding to 7TD1 cells, and since the binding of this radiolabelled protein to IL-6Rα was not detectable in a RIA (results not shown), this hypothesis can be refuted. The induction by H/V-D/A of murine IL-6 can also be excluded, since we found that H/V-D/A bioactivity on 7TD1 was not modified in the presence of an IL-6-neutralizing antibody (results not shown). One has therefore to hypothesize that another factor, whose expression is cell-line-dependent, is responsible for the enhanced activity of the H/V-D/A mutein. Such a factor could be another unknown receptor chain participating in the structure of the functional IL-11 receptors. The stoichiometry of the IL-11 ligand–receptor complex is still an open question, and the possibility that a transducing subunit different from gp130 might participate in IL-11-mediated signal transduction has been raised earlier by several groups, including ours [31,56–58]. A possible candidate for this unknown subunit is the gp130-like receptor (GLM-R) that has been recently identified and found to be expressed predominantly on activated monocytes [59]. This receptor is able to transduce a proliferation signal and induce activation of the transcription factors STAT-3 (signal transduction and activators of transcription) and STAT-5. Even though its ligand has not yet been identified, GLM-R was not found to be a receptor for IL-11 itself.

In the frame of such a hypothesis (heterocomplex of gp130 with GLM-R), one could postulate that the conformational change induced by mutagenesis could render the mutein H/V-D/A more prone than wild-type IL-11 to recruit and/or activate this unknown gp130-like factor. As far as our studies on 7TD1 cells showed that H/V-D/A and wild-type IL-11 displayed similar high-affinity binding, the higher activity of H/V-D/A would be related to a higher signal-transduction efficiency. Therefore, on cells that would express gp130-like factors in excess of gp130 (such as 7TD1), the mutein would be more active, and conversely on cells (such as B9 or T10) that would express gp130 in excess of gp130-like factors, the mutein would be less active. Neutralizing-antibodies inhibition experiments showed that site II of H/V-D/A remained functional, although its conformation was modified with respect to antibody H2 binding. Such a modification at site II could lead to the recruitment by H/V-D/A of a gp130-like molecule instead of gp130. Alternatively, site II of H/V-D/A would still be involved in recruitment of gp130, and site III involved in recruitment of gp130-like factors.

In conclusion, we have generated a novel hIL-11 mutein with enhanced affinity for IL-11Rα and strongly enhanced activity in 7TD1 cells. This mutein therefore constitutes the basis for the engineering of agonist molecules that are potentially useful in pathologies in which IL-11 has been shown to be beneficial. In addition, it should be a valuable molecule in further studies aimed at precisely defining the structure and function of the IL-11 receptors.

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