Identification of residues in the putative 5th helical region of human interleukin-6, important for activation of the IL-6 signal transducer, gp130

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Received 3 July 1996; revised version received 23 August 1996

Abstract We have previously shown that L58 in the putative 5th helical region of human interleukin-6 (IL-6) is important for activation of the IL-6 signal transducer gp130 [de Hon et al. (1995) FEBS Lett. 369, 187–191]. To further explore the importance of individual residues in this region for gp130 activation we have now combined Ala substitutions of residues E52, S53, S54, K55, E56, L58 and E60 with other substitutions in IL-6, known to affect gp130 activation (Q160E and T163P). The combination mutant protein with L58A completely lost the capacity to induce the proliferation of XG-1 myeloma cells, and could effectively antagonize wild type IL-6 activity on these cells. Moreover, the data suggest that besides L58, S54 particularly, but also E52, S53, K55 and E56 contribute to gp130 activation.

Key words: Interleukin-6; Structure-function analysis; gp130

1. Introduction

Interleukin-6 is a pleiotropic cytokine which, in concert with other cytokines like IL-1β and TNFα, regulates the host defense reactions in response to infections, trauma or neoplastic growth [1,2]. IL-6 functions through binding to a type I cell surface transmembrane receptor composed of two different chains. A specific 80 kDa α-chain is responsible for low affinity binding of IL-6, whereas the complex of ligand and α-chain subsequently associates with a second non-specific signal transducing (β) chain, gp130 [2]. The end result of IL-6 receptor binding is thought to be a hexameric complex that signals into the cell, composed of dimers of the ligand and each receptor chain [3,4].

Human IL-6 has been implicated in the pathogenesis of a wide variety of human diseases [1]. IL-6 antagonists may therefore have clinical applications. With the aim to develop such molecules, we study the structure-function relationships of human IL-6. Based on homology with granulocyte-colony stimulating factor, the crystal structure of which has been determined, a tertiary structure model of IL-6 has been built [5]. In this model, IL-6 has the characteristic core structure of the long-chain family of cytokines [6,7], consisting of a bundle of 4 α-helices (named A–D), connected with long loops [5,8]. Various regions of IL-6 that are important for α- or β-chain interaction (respectively termed α- and β-sites) have been identified and are dispersed throughout the putative tertiary structure (reviewed in [9]). The α-site is spatially separated from the β-sites (β_1–β_3), and is comprised of residues in the A- and D-helix and in the loop between helices A and B. The β_1-site comprises residues Q153–H165 at the end of the C–D loop and top of helix D, the β_2-site residues K42–A57 covering the loop created by the first cysteine bridge and part of the putative 5th helical region in the A–B loop, and the β_3-site residues Y32, G36, S119 and V122, located in the A- and C-helix [8,10]. The α- and β_3-site of IL-6 correspond to binding sites 1 and 2 of human growth hormone respectively [10,11]. In the hexameric IL-6-receptor complex, regions β_1 and β_2 are thought to form a composite binding site for one gp130 molecule, whereas β_3 is thought to contact the second gp130 [4,12]. Single β_1- but not β_2-site mutant proteins are partial IL-6 receptor antagonists: they antagonized IL-6 activity on the human IL-6 responsive cell lines CESS and HepG2, but not on XG-1 and TF-1 cells [5,13–15]. When β_1 and β_2 substitutions are combined however, a synergistic loss of activity is observed: the resulting combination mutants are completely inactive and antagonize IL-6 activity on the XG-1 and TF-1 cells as well [14,16]. This antagonistic activity can further be improved by introducing two mutations (F171L/S177R, abbreviated 'LR') that enhance the affinity of IL-6 for IL-6Rα [14,16]. Similarly, simultaneous substitution of all β_3 residues results in an IL-6RA on a variety of human cell lines [10].

Recently we performed alanine scanning mutagenesis of a number of residues in the putative 5th helical region of IL-6 (around the β_2-site), including residues E52, S53, S54, K55, E56, L58 and E60, to identify single amino acids important for gp130 interaction [17]. In that study only the L58A substitution resulted in an IL-6 mutant protein with 5-fold reduced biological activity and gp130 binding [17]. These data were in apparent contradiction with those of Ehlers et al. who showed that simultaneous substitution of residues C51–E56 with the corresponding residues of mouse IL-6 (mutant 2a2) resulted in a 30-fold reduced biological activity and a similar reduction in gp130 binding [16]. Together these data could suggest that residues 51–56 have a minor contribution in gp130 binding, which could only be detected by simultaneous, but not by single substitution of these residues. Because of the strong synergy between β_1 and β_2 substitutions [14,16], we reasoned that another way to detect a contribution of the single residues in this region to biological activity and gp130 binding, might be to combine them with β_1 substitutions

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Abbreviations: wtIL-6, wild type interleukin-6

PII S0014-5793(96)00974-X

[14,16]. In addition, this study was undertaken to determine the minimum number of substitutions required in the β_2 region, to generate an IL-6 receptor antagonist. Combination mutants of the single Ala substitution mutants with IL-6.Q160E/T163P/F171L/S177R (IL-6.β_1/LR) were therefore constructed and tested for biological activity and receptor binding characteristics.

2. Materials and methods

2.1. Cytokines

The wild type (wt)IL-6 preparation used as a standard was mature recombinant human (rh) IL-6 (A1-M185) purified from *E. coli* BL21 (DE3) carrying the pET8c-hIL-6 cDNA expression vector [13]. *E. coli* derived human oncostatin M (rhOM) was from Preprotech (Rocky-hill, USA), human granulocyte macrophage-colony stimulating factor (hGM-CSF) was from Sandoz, Basel, Switzerland.

2.2. Construction of expression vectors

The T7 promoter vector pRSET6D was used for expression of IL-6 mutant proteins [14]. Construction of pRSET6D-IL-6.Q160E/T163P/F171L/S177R (IL-6.β_1/LR) and pRSET6D-IL-6 mutant vectors with Ala substitutions of IL-6 residues E52, S53, S54, K55, E56, L58, and E60 has been described [14,17]. The Ala substitution mutants were combined with the β_1/LR substitutions by ligating *Nco*I-*Xba*I IL-6 cDNA fragments from the Ala substitution plasmids into *Nco*I-*Xba*I digested pRSET6D-IL-6.β_1/LR. Presence of the mutations was verified by restriction enzyme digestion [17] and/or nucleotide sequence analysis on dsDNA with the dideoxy chain termination method using the Sequenase kit (Biochemical Corporation, Cleveland, OH, USA).

2.3. Expression, purification and quantification of IL-6 mutant proteins

For expression of the IL-6 mutant proteins the pRSET6D vectors were transformed to *E.coli* JM109 (DE3) (Promega) and the proteins were purified to ~80% homogeneity from inclusion bodies and quantified by radioimmunoassay as described [13].

2.4. IL-6 bioassays

The human XG-1 myeloma and TF-1 erythroleukemia proliferation assays were performed as described [14,15]. XG-1 cells were kindly provided by Dr. B. Klein (Institute for Molecular Genetics, CNRS, Montpellier, France [18]). TF-1 cells were kindly provided by Dr. R. Kastelein (DNAX, Palo Alto CA, USA).

2.5. Receptor binding assay

To investigate the binding of IL-6 mutant proteins to IL-6Rα an ELISA was used in which the capacity of the IL-6 mutant proteins was measured to compete for binding of biotinylated wtIL-6 to immobilized soluble (s) human IL-6Rα, as described [14,15].

2.6. ELISA protocols

The reactivity of the IL-6 mutant proteins with conformation specific monoclonal antibodies (mAbs) CLB.IL-6/8, CLB.IL-6/12 and CLB.IL-6/14 was measured in sandwich ELISAs with the mAbs as coating antibody, serial dilutions of the mutant protein preparations and affinity purified biotinylated sheep polyclonal anti-rhIL-6 as detecting antibodies as described [19]. mAb 8 and 12 are neutralizing mAbs. mAb 8 recognizes the carboxyl-terminus of IL-6 and mAb 12 recognizes the amino-terminus around residue I25. mAb 14 is a non-neutralizing mAb, with an epitope that partially overlaps with that of mAb 8 [19].

3. Results and discussion

3.1. Synergistic loss of IL-6 biological activity by combining substitutions in the IL-6.β_1 site with Ala substitutions of single residues in the putative 5th helical region of IL-6

Expression vectors encoding IL-6.β_1/LR substitution mutant proteins combined with either E52A, S53A, S54A, K55A, E56A, L58A or E60A were constructed and the proteins were expressed in *E. coli*. Following partial purification of the IL-6 mutant proteins, their capacity to stimulate the proliferation of human XG-1 myeloma cells was tested. In combination with the β_1/LR substitutions, the L58A substitution resulted in a complete loss of activity. Also for the combination of IL-6.β_1/LR with either E52A, S53A, S54A, K55A, or E56A a significant loss of activity was observed (Fig. 1, Table 1). This reduction in activity was characterized both by an increase in EC_{50} value of the mutants and by a reduction in the maximum induced response (Table 1). The maximum response induced by the IL-6.S54A/β_1/LR mutant was consistently lower than that induced by the IL-6.E52A/β_1/LR, IL-6.S53A/β_1/LR, IL-6.K55A/β_1/LR, and IL-6.E56A/β_1/LR mutant proteins, which displayed similar residual activity. Similar results were found when the capacity of the mutant proteins was measured to inhibit the proliferation of the human melanoma cell line A375 (data not shown). To explain the observed reduction in biological activity of the mutant proteins, receptor binding characteristics and reactivity with IL-6 conformation specific mAbs were determined. Compared to IL-

Table 1
Characteristics of IL-6.Ala substitution/β_1/LR mutant proteins[a]

	XG-1[b]		shIL-6Rα[c]	mAb binding (%)[d]		
	Activity (%)	Max. (%)	binding (%)	8	12	14
wtIL-6	100	100	100	100	100	100
β_1/LR	1.18 ± 0.36	86 ± 26	17 ± 4	70 ± 10	50 ± 22	74 ± 14
E52A/β_1/LR	0.14 ± 0.11	46 ± 13	9 ± 4	40 ± 3	44 ± 8	68 ± 0.5
S53A/β_1/LR	0.21 ± 0.06	55 ± 16	14 ± 6	39 ± 0.5	36 ± 13	65 ± 5
S54A/β_1/LR	0.09 ± 0.06	24 ± 6	17 ± 8	28 ± 4	31 ± 11	47 ± 12
K55A/β_1/LR	0.50 ± 0.32	38 ± 7	12 ± 2	49 ± 6	38 ± 16	54 ± 16
E56A/β_1/LR	0.16 ± 0.12	42 ± 4	13 ± 5	40 ± 3	31 ± 17	45 ± 5
L58A/β_1/LR	< 0.0002	nd[e]	13 ± 6	48 ± 2	43 ± 26	50 ± 12
E60A/β_1/LR	0.84 ± 0.46	68 ± 19	21 ± 7	45 ± 2	60 ± 11	55 ± 5

[a]Data represent (EC_{50} wtIL-6/EC_{50} IL-6 mutant protein) × 100% (mean ± S.E.) for XG-1 and mAb binding experiments (EC_{50}, the effective concentration giving a half maximal response). For the shIL6Rα assay, data represent (IC_{50} wtIL-6/IC_{50} IL-6 mutant protein) × 100% (mean ± S.E.; IC_{50}, concentration leading to half maximal competition).
[b]Data are derived from 3 independent experiments. EC_{50} wtIL-6: 19 ± 2 pg/ml. Max. indicates the maximum proliferation induced by a mutant protein expressed as a percentage of the maximum of wtIL-6.
[c]$n = 2$; EC_{50} wtIL-6: 43 ± 13 ng/ml.
[d]$n = 2$ for mAb 8 and 14 ELISAs, $n = 3$ for mAb 12 ELISA. EC_{50} wtIL-6 mAb 8, 0.9 ± 0.3 ng/ml; mAb 12, 0.9 ± 0.3 ng/ml; mAb 14, 0.7 ± 0.1 ng/ml.
[e]The maximum induced response of the L58A/β_1/LR protein was < 5% of that of wtIL-6, which is within the S.E. of the background proliferation.

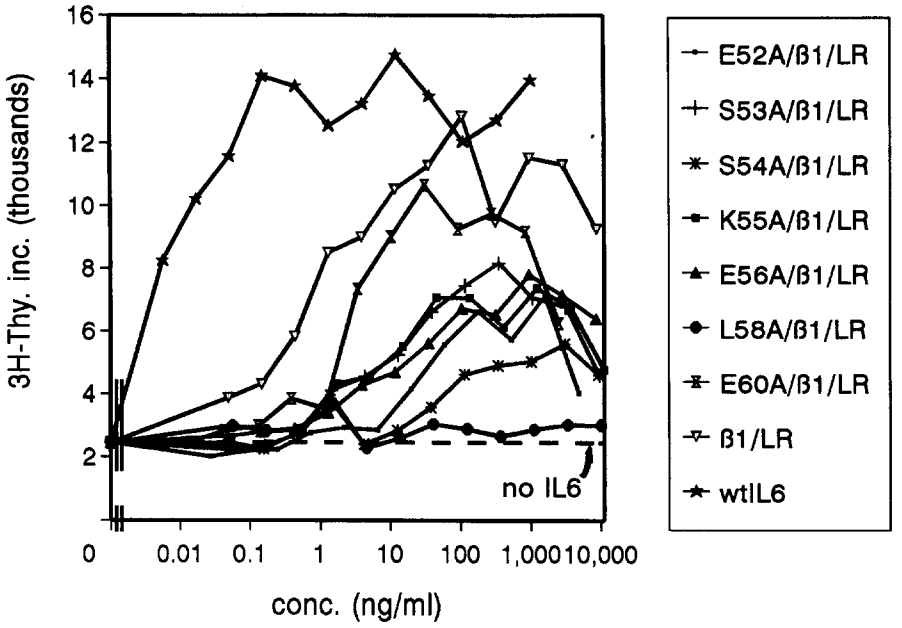

Fig. 1. Capacity of IL-6 Ala substitution/β_1/LR mutant proteins to induce proliferation of XG-1 myeloma cells. XG-1 cells were cultured for 3 days in the presence of the indicated concentrations of the IL-6 mutant proteins and the induced proliferation was measured by incorporation of tritiated thymidine. Background proliferation (mean ± S.E.), $2.5 \pm 0.6 \times 10^3$ cpm. Data points represent the mean of triplicate measurements. One representative experiment out of three is shown.

6.β_1/LR all mutant proteins retained a similar capacity to bind to sIL-6Rα (Table 1). The observed differences in bioactivity cannot be explained therefore by variability in IL-6Rα binding. Also, the reactivity of the mutant proteins with IL-6 conformation specific mAbs that recognize different epitopes on IL-6 was similar to that of IL-6.β_1/LR (Table 1), suggesting that compared to IL-6.β_1/LR gross structural perturbations do not occur in the combination mutants. Paonessa et al. and Ciaponni et al. recently demonstrated that IL-6.β_1-site mutant proteins (IL-6.T163D, or IL-6.W158R/D161R), but also IL-6.β_2 and IL-6.$\beta_{1,2}$ combination mutant proteins, retained the capacity to bind to sIL-6Rα and a single sgp130 molecule [4,12]. This binding was proposed to occur through the β_3-region which is still intact in these mutant proteins [4,12]. All these mutant proteins were impaired in their capacity to induce dimerization of sgp130 [4]. The same phenotype was recently confirmed for the IL-6.β_1 mutant protein Q160E/T163P used in this study, by Hammacher et al. [20]. Taken together, these observations and our results suggest that the reduced biological activity of the IL-6.β_1/LR Ala substitution mutants described here, as compared to that of the IL-6.β_1/LR protein itself, is most likely due to a further decreased capacity to induce gp130 dimerization. The dose-response curves of wtIL-6 and the IL-6 mutant proteins in the XG-1 assay suggest that the defects in gp130 dimerization capacity of the mutant proteins can be completely (IL-6.β_1/LR) or partially (IL-6.E52A/β_1/LR, IL-6.S53A/β_1/LR, IL-6.S54A/β_1/LR, IL-6.E56A/β_1/LR) compensated for by the occupancy of spare receptors: the EC_{50} of the IL-6.β_1/LR protein is strongly increased, but there is no or little reduction in maximum response (Fig. 1, Table 1). For the above mentioned IL-6.β_1/LR Ala substitution mutants the increase in EC_{50} is larger than the decrease in maximal response (Fig. 1, Table 1). Such a partial or complete compensation for receptor activation defects by occupancy of spare receptors has also been described for mouse IL-2 mutant proteins [21].

As is clear from Fig. 1, Table 1, and ref. [17], combining the single Ala substitutions with the β_1/LR substitutions resulted in a synergistic, rather than an additive loss of IL-6 bioactivity. How can this synergism between the single Ala substitutions and β_1-(Q160E/T163P) substitutions be explained at the structural level? (The 'LR' substitutions are not considered here because they do not play a role in gp130 dimerization, but affect IL-6Rα chain binding ([14] and references therein).) According to Wells, synergism between mutations in a protein is observed when the mutated residues interact with each other, by direct contact or indirectly through electrostatic interaction or structural perturbations, so that they no longer behave independently [22]. Alternatively the mutation causes a change in mechanism or rate-limiting step of the reaction [22]. We favor the first possibility to explain the observed synergy of the mutations in IL-6. In the 3D model of IL-6 Q160 is a surface exposed residue (Fig. 2). Substitution with Glu may therefore disturb a contact in the hexameric complex. The contribution of Q160 to gp130 dimerization likely is a small one based on the following considerations: the T163P substitution alone results in a 250-fold reduced bioactivity on XG-1 cells. Combination of T163P with Q160E further results in a minor four-fold reduction in bioactivity [15]. Also, no loss of bioactivity was detected by simultaneous Ala substitution of Q160 and D161 [15]. In contrast to the minor effects of Q160 substitutions, T163 substitutions can have large effects [15]. T163 is buried in the structure however, suggesting that the substitutions indirectly affect (a) surface exposed residue(s) (Fig. 2). In the model, the distances from the α- and γ-carbon of T163 to the β-carbon of A59 in the putative 5th helix are 4.8 and 3.6 Å respectively. Taking the hydrogen atoms into account, this suggests that these residues are in contact. Re-

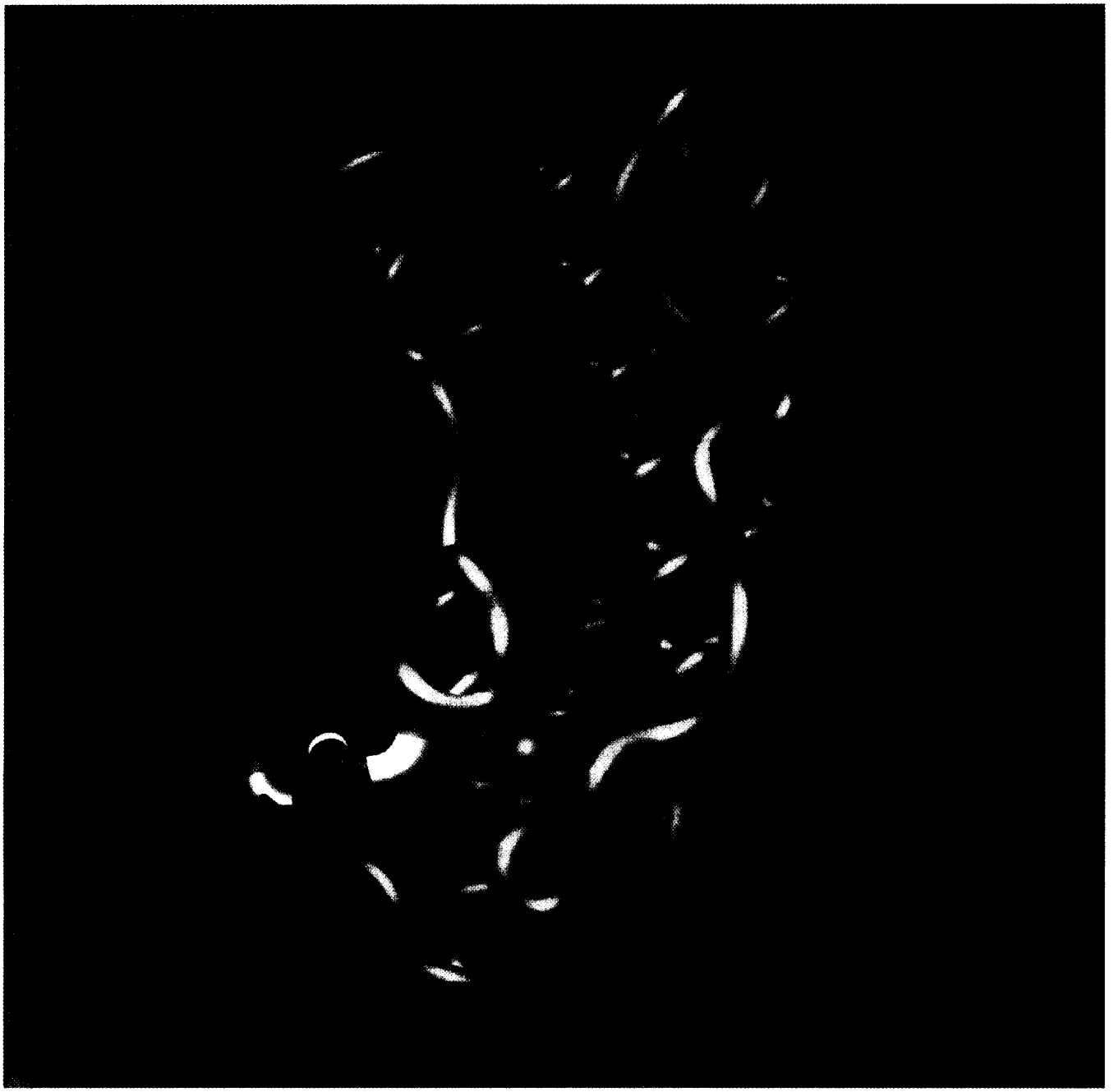

Fig. 2. Location of T163 and residues in the 5th helical region in the tertiary structure model of hIL-6. Capitals indicate the positions of helices A–D. T163 is shown in blue spheres to emphasize close proximity to A59 (white); Q160 in blue sticks. L58 and S54 in the 5th helical region are shown in sticks. Color coding of the residues in the 5th helical region corresponds to their importance for IL-6 biological activity (EC_{50} values IL-6.Ala substitution/β_1/LR mutant proteins in XG-1 assay (Table 1): L58 in dark red). The position of the 'LR' (F171L and S177R) substitutions, affecting IL6Rα binding, is also indicated.

placement of T163 with Pro could therefore induce a change in the position of A59 and surrounding residues including the surface exposed L58. Apparently this conformational change is subtle and local, because we do not detect large changes in binding affinity for sIL-6Rα and conformation specific mAb in binding experiments with a T163P (or Q160E/T163P) mutant protein [15]. As suggested by the residual bioactivity of T163P and Q160E/T163P mutant proteins, this conformational change is not sufficient to completely abolish gp130 dimerization [13–15]. Apparently the presence of the side-chains of residues E52, S53, K55, E56 and more importantly S54 and L58 also contributes to the full gp130 dimerization capacity of IL-6. Thus, together with replacement of in particular the L58 side chain, the conformational change induced by T163P is sufficient to destroy the capacity of this region to induce gp130 dimerization.

3.2. IL-6.L58A/β_1/LR is a specific IL-6 antagonist

Because no biological activity of the IL-6.L58A/β_1/LR mutant protein could be detected, we next determined whether it could antagonize the activity of wtIL-6 on XG-1 cells. Addition of 10 μg/ml of the IL-6.L58A/β_1/LR mutant protein to

increasing concentrations of wtIL-6 reversibly inhibited the biological activity of wtIL-6 on XG-1 cells (Fig. 3A). Specificity of inhibition by the IL-6.L58A/β_1/LR mutant protein was further suggested by the observation that this protein specifically inhibited wtIL-6 activity, but not that of hOM, or hGM-CSF on the human erythroleukemia cell line TF-1 (Fig. 3B). We have previously shown that an IL-6.$\beta_{1,2}$/LR mutant protein could antagonize wtIL-6 activity on XG-1 cells [14]. In this protein K42–A57 of human IL-6 had been replaced with the corresponding mouse residues, resulting in 10 amino acid substitutions, in addition to the 4 β_1/LR substitutions. When we compared the antagonistic capacity of the IL-6.L58A/β_1/LR mutant protein with that of IL-6.$\beta_{1,2}$/LR, both proteins were found equally potent in inhibiting wtIL-6 activity on XG-1 cells (Fig. 4).

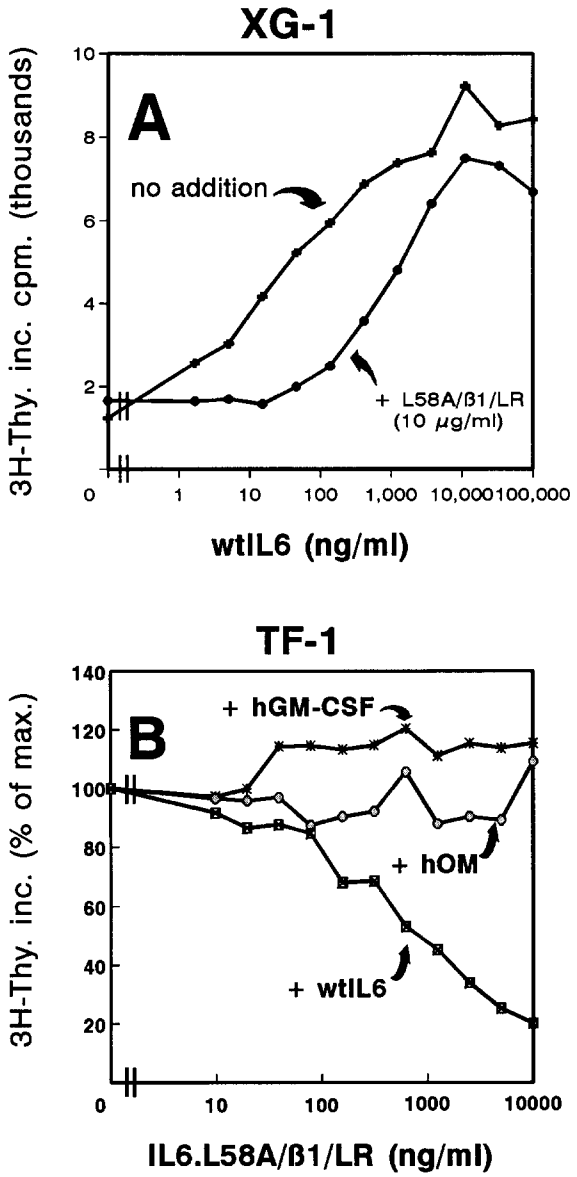

Fig. 3. IL-6.L58A/β_1/LR is a specific IL-6 antagonist. A: IL-6.L58A/β_1/LR reversibly inhibits wtIL-6 induced XG-1 myeloma cell proliferation. Proliferation of XG-1 cells was measured following incubation with wtIL-6 at the indicated concentrations, in the absence or presence of 10 µg/ml of the IL-6.L58A/β_1/LR mutant protein. B: IL-6.L58A/β_1/LR inhibits hIL-6, but not hGM-CSF or hOM activity on TF-1 erythroleukemia cells. Proliferation of TF-1 cells was measured after 2 days of culture with fixed amounts of wtIL-6 (1 ng/ml), hGM-CSF (1 ng/ml) or hOM (5 ng/ml) in the presence of increasing concentrations of the IL-6.L58A/β_1/LR mutant protein. Data are expressed as a percentage of the maximal proliferation induced by each cytokine in the absence of IL-6.L58A/β_1/LR. Background proliferation (1.3×10^3 cpm) was subtracted. Maximal cpm (mean ± S.E.) wtIL-6, $12.0\pm0.7\times10^3$; hGM-CSF, $63.5\pm1.4\times10^3$; hOM, $4.7\pm0.5\times10^3$. Data points of A and B represent the mean of triplicate measurements of one experiment out of two.

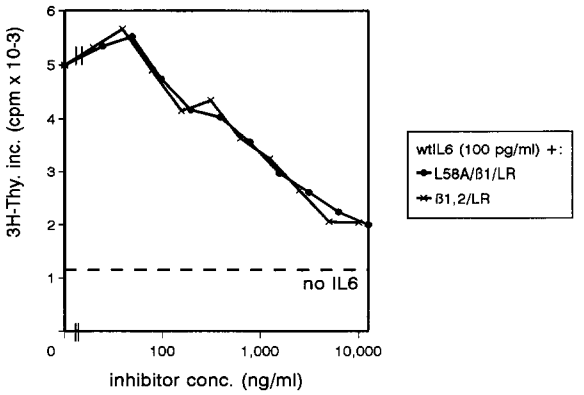

Fig. 4. IL-6.L58A/β_1/LR has a similar capacity to antagonize wtIL-6 activity as IL-6.$\beta_{1,2}$/LR. XG-1 cells were cultured with 100 pg/ml of wtIL-6 in the presence of increasing concentrations of IL-6.L58A/β_1/LR or IL-6.$\beta_{1,2}$/LR. Proliferation (mean ± S.E.) induced by 100 pg/ml wtIL-6, $5.0\pm0.4\times10^3$ cpm; background proliferation 1.1×10^3 cpm. The mean of triplicate measurements of one experiment out of two is shown.

In conclusion we have shown that besides L58, residue S54, and to a lesser extent residues E52, S53, K55 and E56 in the putative 5th helical region in the A–B loop of IL-6 are important for bioactivity of IL-6. These residues likely contribute to the IL-6Rα-chain-dependent dimerization of the IL-6 signal transducer gp130. In addition we have shown that a mutant protein with only five substitutions (IL-6.L58A/β_1/LR) has a similar capacity to antagonize wtIL-6 as the previously described IL-6.$\beta_{1,2}$/LR mutant protein, in which 14 residues of hIL-6 were replaced. When considering therapeutical application of IL-6 receptor antagonists, minimization of the number of amino acid substitutions in these molecules, may reduce their potential immunogenicity.

Acknowledgements: The research of J.P.J.B. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

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