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Internal deletions in human interleukin-6: structure-function analysis

(Recombinant DNA; oligodeoxyribonucleotide-directed mutagenesis; *N*-glycosylation; cytokine; conformation-specific immunoprecipitation; mutated proteins)

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SUMMARY

By cDNA mutagenesis, we have constructed internal and C-terminal deletions (Δ^{21-51}, Δ^{52-97}, Δ^{97-104}, $\Delta^{127-174}$, Δ^{97-184} and $\Delta^{134-184}$) in human interleukin-6 (hIL-6). All those deletion-carrying hIL-6 (ΔhIL-6) proteins were then produced in *Xenopus laevis* oocytes and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results show that, at least in frog oocytes, the first potential *N*-glycosylation site (Asn^{45}) is utilized exclusively.

The IL-6 conformation of these deletion-carrying proteins has been studied by immunoprecipitation with two kinds of monoclonal antibodies (mAb's): mAb's that show preference towards denatured hIL-6, or conformation-specific mAb's. The binding pattern of these two series of mAb's indicated that the IL-6 conformation has been largely destroyed for four of our Δ-proteins. Proteins Δ^{21-51} and $\Delta^{127-174}$ have kept a part of the IL-6 tertiary structure since they are still recognized by some conformation-specific mAb's.

All of these ΔhIL-6 proteins were inactive in the IL-6 hybridoma growth factor (HGF) assay and unable to inhibit the HGF activity of the recombinant human wild-type IL-6 (wt hIL-6). Moreover, the oocyte-synthesized ΔhIL-6 (Δ^{21-51}, $\Delta^{127-174}$, Δ^{97-184}, $\Delta^{134-184}$) did not bind to the IL-6 receptor.

Finally, we have produced two proteins with aa 29–33 or 97–104 substituted by corresponding murine IL-6 (mIL-6) sequences. In contrast to mIL-6 that is able to act only on murine cells, these chimeric h/mIL-6 proteins are active toward both murine and human cells. However, drastic reduction in IL-6 activity observed for the 29–33-aa-substituted h/m protein toward both of these kinds of cells suggests that the 29–33-aa region may be in part implicated in modeling the receptor-binding site of IL-6. On the other hand, the 97–104-aa region seems to be involved in the overall structure of IL-6.

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in the regulation of inflammatory and immunological processes. Amongst its major roles, is that it stimulates terminal differentiation of B lymphocytes, activates proliferation and cytotoxic differentiation of T cells, triggers the production of acute phase proteins by hepatocytes and is a growth

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Abbreviations: aa, amino acid(s); BS, BlueScribe vectors (Vector Cloning Systems, La Jolla, CA, USA); BSA, bovine serum albumin; CAT, Cm acetyltransferase; *cat*, gene encoding CAT; Cm, chloramphenicol; *CRP*, human C-reactive protein promoter; Δ, deletion (superscripts indicate the deleted aa); ΔhIL, deletion-carrying hIL protein; h, human; HGF, hybridoma growth factor; IL, interleukin; mAb, monoclonal antibody; m, murine; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium dodecyl sulfate; UT, untranslated region; wt, wild type; *X.*, *Xenopus*.

factor for murine hybridoma and plasmacytoma cell lines (for review see Van Snick, 1990).

The natural mature IL-6 protein, consisting of 184 aa, can be *N*- or *O*-glycosylated and phosphorylated (Gross et al., 1989; May et al., 1988). IL-6 exerts its action via a specific high affinity receptor, which is present on various target cells (Taga et al., 1987; Snyers et al., 1989).

In the absence of the IL-6 three dimensional structure, one way to analyse the interaction between IL-6 and its receptor is to investigate the relation between the IL-6 structure and its biological activity. Furthermore, this might help us to create IL-6 antagonists that would be of clinical use, e.g., for treatment of multiple myeloma, rheumatoid arthritis, psoriasis or renal cells carcinomas (Kawano et al., 1988; Houssiau et al., 1988; Grossman et al., 1989; Miki et al., 1989).

Some structure/function studies have already been initiated by various investigators, who have reported that the N-terminal 27 aa of mature IL-6 are not required for IL-6 activity in contrast to aa 28–33 and the C-terminal aa 180–184; the latter are also recognized by IL-6 neutralizing mAb's (Brakenhoff et al., 1989; 1990; Krüttgen et al., 1990). The aa 153 to 162 are recognized by other IL-6 neutralizing mAb's (Ida et al., 1989).

As yet no data have been presented on the effect of deleting internal parts of IL-6 on its biological activity. In the present article we, therefore, investigate the folding, bioactivity and receptor-binding characteristics of a number of internal-deletion mutants of hIL-6.

RESULTS AND DISCUSSION

(a) Construction and biochemical analysis of ΔIL-6 proteins

Natural restriction sites of the *IL-6* cDNA have been used to construct $\Delta^{127-174}$, Δ^{97-184} and $\Delta^{134-184}$. Restriction endonuclease sites created by silent oligo-directed mutagenesis have been used to construct Δ^{21-51}, Δ^{52-97} and Δ^{97-104} (Fig. 1).

Synthetic mRNA's encoding the wt and ΔhIL-6 were first tested in a rabbit reticulocyte lysate translation assay. The decreased M_r values of these proteins in comparison to that of the wt hIL-6 were in agreement with the size of the deletions (Fig. 1). Moreover, these ΔhIL-6 proteins were specifically immunoprecipitated by a polyclonal antibody to hIL-6 (Fig. 3).

In order to produce glycosylated materials, we injected capped mRNA corresponding to the various constructs, into *X. laevis* oocytes in the presence of [^{35}S]methionine. As previously described (Poupart et al., 1987), wt hIL-6 is secreted as multiple glycosylated forms which are seen on SDS-PAGE gel as two major bands of 22 and 27 kDa (Fig. 2). The lower bands are unglycosylated or *O*-glycosylated components whilst the upper bands consist of *N*- and *O*-glycosylated products. In this translation system, we have only succeeded in expressing Δ^{52-97}, $\Delta^{127-174}$, $\Delta^{134-184}$ and Δ^{97-184}. Three of them, $\Delta^{127-174}$, $\Delta^{134-184}$ and Δ^{97-184}, which lacked the second potential *N*-glycosylation site at Asn^{144} are still secreted as two major components in contrast to the one band profile of Δ^{21-51}, which misses the first *N*-glycosylation site (Asn^{45}). We can thus conclude that, at least in oocytes, IL-6 *N*-glycosylation occurs only on Asn^{45}, the first *N*-glycosylation site. Analyzing trypsin-digested IL-6 fragments, the same conclusion was also recently reached for the natural hIL-6 by Dr. Heinrich's group (G. Dufhues, J. Müllberg, S. Rose-John, H.S. Conradt and P.C. Heinrich, manuscript in preparation). Both results are also in agreement with results of Gross et al. (1989), suggesting that 'IL-6 apparently carries only one *N*-linked oligosaccharide chain'. IL-6 glycosylation does not seem to influence its biological activity, since IL-6 produced in *E. coli* or rabbit reticulocyte lysate shows the same specific activity as the natural protein (Geiger et al., 1988; Brakenhoff et al., 1987; Van Damme et al., 1987). In rat, the rate and extent of hIL-6 distribution throughout the fluids and tissues seems independent of its glycosylation profile, since the same clearance and distribution kinetics were observed for oocytes or *E. coli* IL-6 preparation when compared with natural IL-6 (Castell et al., 1990).

(b) Monoclonal antibodies (mAb's) as probes for the IL-6 tertiary structure

First, we probed the structure of the ΔhIL-6 by immunoprecipitation with a mAb that had a higher affinity for denatured than for native IL-6 (Brakenhoff, 1990) (Fig. 3A and Table I). Proteins Δ^{21-51}, Δ^{52-97} and Δ^{97-104} are immunoprecipitated by this mAb in contrast to the wt hIL-6 and $\Delta^{127-174}$ protein. In fact, a part of the epitopes recognized by this mAb corresponds to the deletion in $\Delta^{127-174}$ protein, comprising Thr^{142}-Ala^{145} (Brakenhoff, 1990). These results suggest that proteins Δ^{21-51}, Δ^{52-97} and Δ^{97-104} have lost a part of the native IL-6 conformation.

We then examined whether the same ΔhIL-6 proteins are still immunoprecipitated by nine IL-6-conformation-specific mAb's recognizing various epitopes of the wt hIL-6 (Fig. 3B and Table I). It was observed that Δ^{52-97} and Δ^{97-104} are not immunoprecipitated by any of these mAb's (this was also observed for $\Delta^{134-184}$, data not shown), which suggests that the native IL-6 tertiary structure has been largely destroyed. In contrast, Δ^{21-51} and $\Delta^{127-174}$ proteins can be still recognized by some of these mAb's. In fact, the remaining non-reactive mAb's have their epitopes mapped in the deleted regions of the Δ^{21-51} and $\Delta^{127-174}$ proteins (Brakenhoff et al., 1990). These deletion mutants, therefore, seem to have lost some but not all of the IL-6

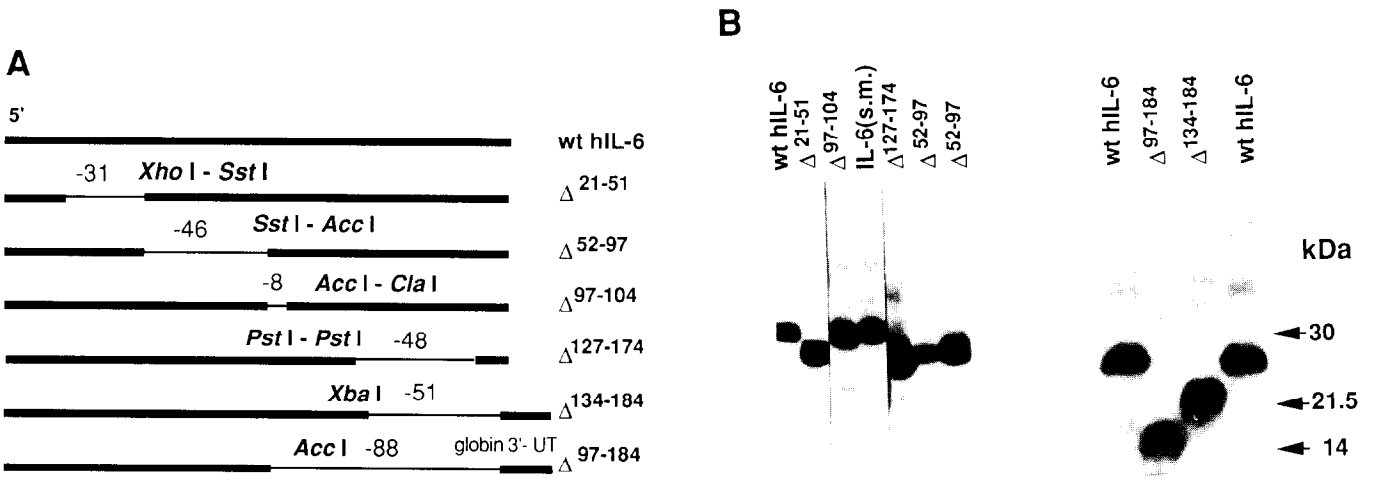

Fig. 1. Deletion mutants of *hIL-6* cDNA and their expression in rabbit reticulocyte lysate. (Panel **A**) Construction of *IL-6* cDNA deletions. All of these deletion mutants were derived from the pBS-IL-6$^-$ plasmid. To construct pBS-IL-6$^-$, the pSP64-T26K plasmid (Poupart et al., 1987) was digested with *Hin*dIII + *Sal*I. The *Hin*dIII-*Sal*I fragment consisting of the *IL-6* cDNA flanked by 5′ and 3′ untranslated regions of β-globin, which allows efficient translation in *X. laevis* oocytes, was subcloned in the *Hin*dIII + *Sal*I-digested BlueScribe M13$^+$ vector (Vector Cloning Systems, La Jolla, CA, USA) (pBS-M13$^+$). In order to simplify future digestions, the resulting plasmid (pBS-IL-6) was then cleaved with *Sst*I + *Sal*I to remove polylinker sites (pBS-IL-6). We took advantage of BlueScribe M13 phagemid, which can be packaged by a M13 helper phage and thereafter obtained as single-stranded DNA for oligo-directed mutagenesis (Nakamaye and Eckstein, 1986) using the mutagenesis kit of Amersham International (Amersham, U.K.). We used the computer database of Dr. G. Volckaert (Katholieke Universiteit, Leuven, Belgium) to identify positions where new restriction sites could be created by silent changes in cDNA. Amongst those we selected unique sites, which are mutually in frame after filling in with the Klenow fragment of *E. coli* DNA polymerase I (Pharmacia, Uppsala, Sweden) or digestion with the mung bean nuclease (New England Biolabs, Beverly, MA) and ligation with T4 DNA ligase (Amersham). Plasmid pSP64-T 26K $\Delta^{127-174}$ was constructed using *Pst*I natural restriction sites of the *IL-6* cDNA. Plasmid pSP64-T 26K Δ^{97-184} and $\Delta^{134-184}$ were created by subcloning *Bgl*II-*Acc*I or *Bgl*II-*Xba*I fragments (ligated to a *Bgl*II linker) of the *IL-6* cDNA into the *Bgl*II-digested pSP64T vector described by Krieg and Melton (1984). Because the first stop codon is 17 aa further in the 3′-untranslated region of β-globin these two last constructions express an additional C-terminal region (represented as 'globin 3′-UT') with the sequence Ser-Asp-Leu-Val-Thr-Thr-Lys-Pro-Ala-Ser-Arg-Thr-Pro-Glu-Trp-Ser-Leu for Δ^{97-184}, and Ala-Asp-Leu-Val-Thr-Thr-Lys-Pro-Ala-Ser-Arg-Thr-Pro-Glu-Trp-Ser-Leu for $\Delta^{134-184}$. *Xho*I, *Sst*I and *Cla*I restriction sites were created by mutagenesis in order to construct pBS-IL-6$^-$ Δ^{21-51}, pBS-IL-6$^-$ Δ^{52-97} and pBS-IL-6$^-$ Δ^{97-104}. All constructions have been verified by sequencing (Sanger et al., 1977). Thin lines represent the deletion in the IL-6 proteins and the numbers above them indicate the number of aa deleted, by excision with indicated enzymes. (Panel **B**) Synthesis of ΔIL-6 proteins in reticulocyte lysate. Intact or deleted *IL-6* cDNA cloned in the pSP64T plasmid were transcribed as previously described (Poupart et al., 1987). *Eco*RI-linearized pBS-IL-6 plasmid or its derivates (1 μg) were transcribed using the T3 RNA polymerase (GIBCO BRL, Gent, Belgium) using all four NTPs and 500 μM of the methylated m7G(5′)ppp(5′)G as cap (Pharmacia). Following RNA synthesis, the DNA template was digested with 20 μg/ml of RNase-free DNase I (GIBCO BRL) in the presence of 10 mM vanadylribonucleosides (GIBCO BRL), inhibitors of eventual RNase. Free nt were removed by filtration on 'Nick column' (Pharmacia). The translation of the mRNA in reticulocyte lysate was carried for 45 min at 30°C in the presence of [^{35}S]methionine, as described by Poupart et al. (1987). The products of translation (1 μl) were purified by 0.1% SDS-17% PAGE. Gels were fixed, treated with 'Amplify' (Amersham International), dried and autoradiographed, as described by Poupart et al. (1987). IL-6(s.m.) represents IL-6 protein expressed from the IL-6 cDNA containing silent mutations coding for *Xho*I, *Sst*I and *Cla*I restriction sites that have been described for panel **A**.

conformation. The loss of one disulfide-bridge in Δ^{21-51} and Δ^{52-97} proteins may partly explain the loss of the IL-6 tertiary structure of these proteins and their inactivity. Indeed, we know that hIL-6 treated for 4 h at 37°C with 5 mM dithiothreitol in 'physiological' conditions (10 mM Tris · HCl pH 7.5/0.14 M NaCl) loses 98% of its hybridoma growth factor (HGF) activity (unpublished results).

(c) Biological activities of the ΔhIL-6 proteins

The ΔhIL-6 proteins produced either in reticulocyte lysate or in oocytes were further tested for IL-6 activity by the HGF-bioassay using the B murine hybridoma 7TD1 cells, as described by Van Snick et al. (1986). The results show that they are at least 10000-fold less active than the wt hIL-6. In addition, they are also unable to inhibit the HGF activity of the wt hIL-6 even if they are present in a 10000-fold molar excess. This was confirmed for Δ^{21-51}, $\Delta^{127-174}$, Δ^{97-184} and $\Delta^{134-184}$ proteins which cannot bind to the IL-6 receptor present on UAC cells or on CESS cells. This was done as described by Snyers et al. (1990), using [^{35}S]IL-6 or Δ^{21-51} or $\Delta^{127-174}$ preparations or, as described by Coulie et al. (1987), for competition experiments for the IL-6 receptor, using cold oocyte-mutants preparations in molar excess of 15-, 30-, 344- and 1376-fold, respectively. Therefore, none of these ΔhIL-6 proteins are either agonists or eventual antagonists of IL-6.

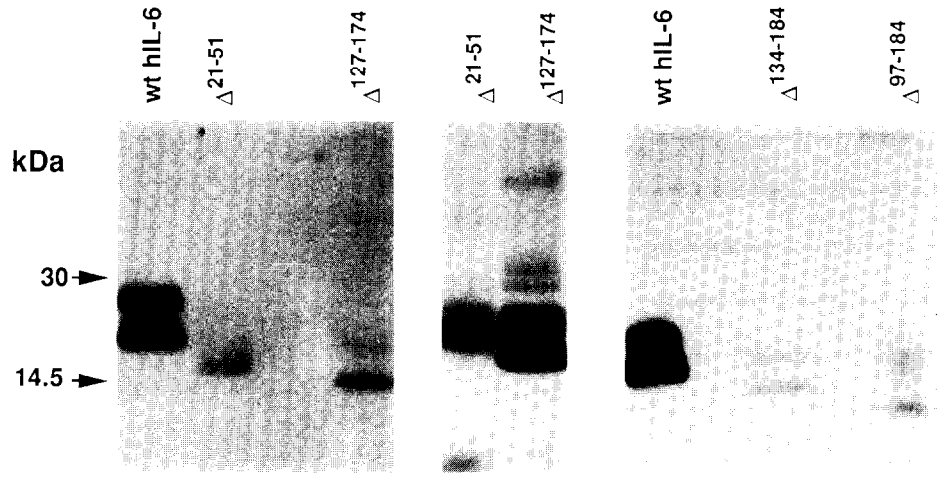

Fig. 2. Expression of *IL-6* deletion mutants in *X. laevis* oocytes. Groups of five oocytes were injected with wt h*IL-6* or *Δ*h*IL-6* mRNAs (2 ng/oocyte) and incubated for 20 h in Barth's medium containing (per ml) 0.1 mg BSA/10 units Trasylol/900 μCi [^{35}S]methionine [Trasylol (Bayer, Leverkusen, Germany) is the trade name for aprotinin]. Incubation media were analysed by PAGE as described in Fig. 1. Proteins $\Delta^{134-184}$ and Δ^{97-184} were immunoprecipitated with a monospecific rabbit anti-recombinant hIL-6 polyclonal antiserum in presence of *Staphylococcus aureus* Cowan I-protein-A, as described by Kessler (1981).

TABLE I

Structural conformation studies of hIL-6 using mAb's

The IL-6 proteins[a]	Antibodies[b]											
	mAb7	8	9	12	14	15	16	B1	B2	B4	Co	Poly
	Immunoprecipitation[c]											
wt hIL-6	−	+	+	+	+	+	+	+	+	+	−	+
Δ^{21-51}	+	+	+	−	+	+	+	−	−	−	−	+
Δ^{52-97}	+	−	−	−	−	−	−	−	−	−	−	+
Δ^{97-104}	+	−	−	−	−	−	−	−	−	−	−	+
$\Delta^{127-174}$	−	±	−	−	−	−	−	±	−	−	−	+

[a] Deletion mutants have been constructed at the cDNA level as described in Fig. 1 (panel **A**). The proteins were produced in a reticulocyte lysate as described in Fig. 1 (panel **B**).

[b] mAb7 recognizes with a higher affinity the unfolded wt hIL-6 than the native IL-6 and binds hexapeptides having aa Thr^{142} → Ala^{145} of hIL-6 in common (Brakenhoff, 1990). The nine hIL-6-conformation-specific mAb's (numbers 8, 9, 12, 14, 15, 16, B1, B2, B4) are described in Brakenhoff et al. (1990). Neutralizing mAb's 8, 12, B1 and B2 recognize partially overlapping epitopes corresponding to aa of both the N- and the C-ends of IL-6 (site I). mAb's 12 and B2 recognize N-terminal aa around Ile^{25}, whereas mAb 8 binds at the C terminus and B1 binds to both regions. mAb's 9 and 14 bind close to site I but they do not inhibit the biological activity. mAb's 15 and 16 recognize a second site which has not yet been mapped. mAb B4 behaves similarly to mAb's 12 and B2 in mapping experiments. We used a monospecific rabbit anti-recombinant hIL-6 polyclonal antiserum (lane Poly) as a positive control and an IgG_1 mAb as a negative control (lane Co).

[c] Immunoprecipitations of proteins have been done as previously described by Brakenhoff et al. (1990).

+, immunoprecipitated; ±, weakly immunoprecipitated; −, non-immunoprecipitated.

(d) Effects of aa substitutions in critical regions of hIL-6; human/murine hybrid IL-6

In the previous studies, we have found that deleting parts of the IL-6 aa sequence results in large changes in the tertiary structure, which makes it difficult to draw conclusions on the localisation of the receptor-binding site(s). Human and murine IL-6 are expected to have a similar tertiary structure; their aa sequences are 42% homologous (Van Snick et al., 1988), the disulfide-bridges are identical (Simpson et al., 1988; Clogston et al., 1989) and both hIL-6 and mIL-6 bind to the mIL-6 receptor (for review see Van Snick, 1990). However, mIL-6 is not active on human cells (Coulie et al., 1989). The construction of chimeric molecules would thus be expected to allow dissection of

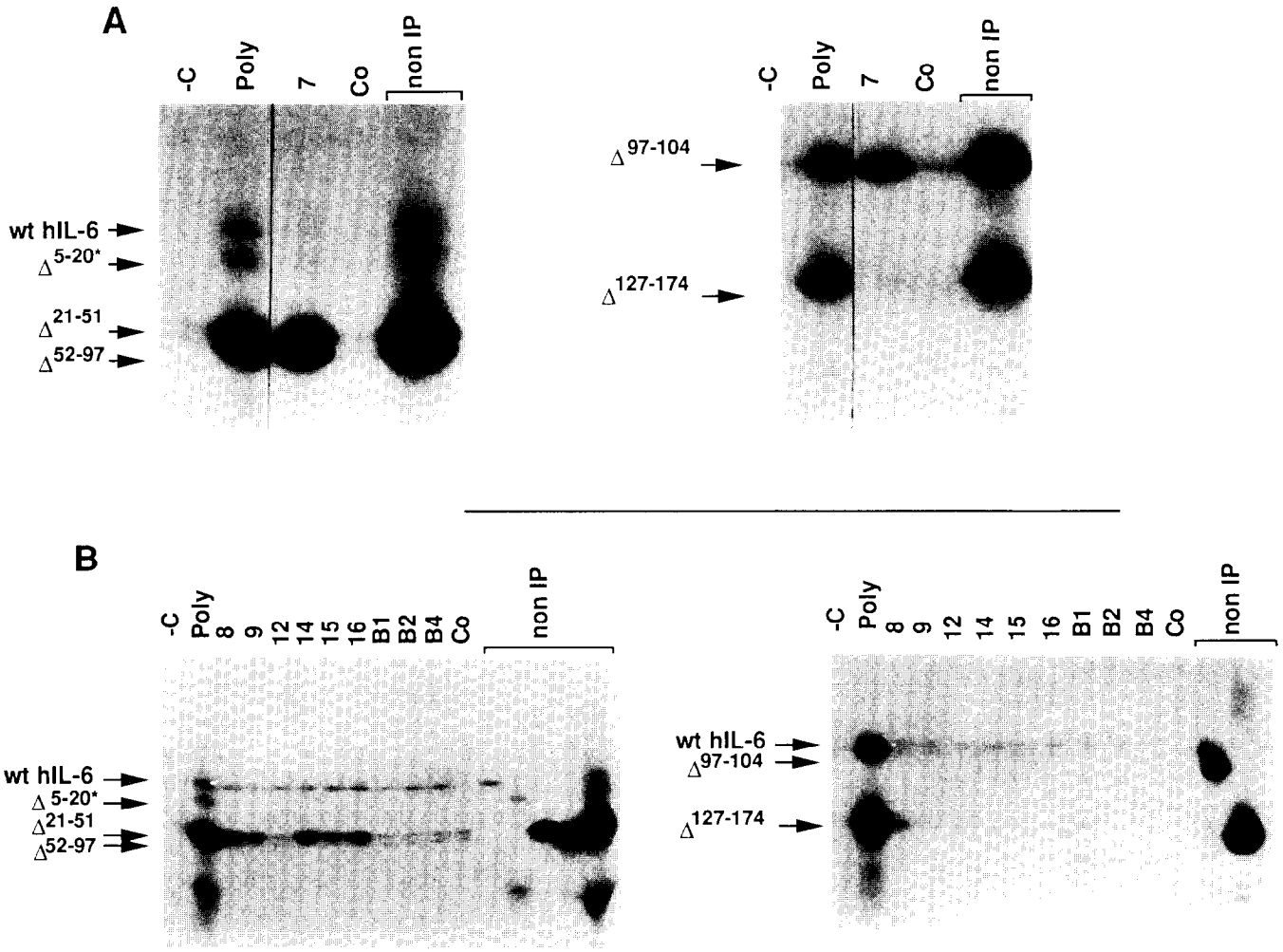

Fig. 3. Structural integrity analysis of the ΔhIL-6 proteins: immunoprecipitation with anti-IL-6 mAb's. Proteins were synthesized as described in Fig. 1. Goat anti-mouse antibodies coupled to Sepharose were incubated overnight with 500 ng mAb's/ml, together with mixtures of the ΔhIL-6 proteins (± 50000 cpm of each protein), as described by Brakenhoff et al. (1990). After the overnight incubation, the Sepharose-bound proteins were washed, dissolved in 20 μl sample mix and subjected to 0.1% SDS-17.5% PAGE under reducing condition, followed by autoradiography as described by Brakenhoff et al. (1990). (Panel **A**) Immunoprecipitation with a mAb No. 7 (lane 7), recognizing mainly the unfolded wt hIL-6 (Brakenhoff, 1990). (Panel **B**) Immunoprecipitation with nine IL-6 conformation-specific mAb's, numbers 8, 9, 12, 14, 15, 16, B1, B2 and B4 (the lane numbers correspond to the number of the mAb's), has been described in Brakenhoff et al. (1990). Control lanes for **A** and **B** are as follow: - C, goat anti-mouse-Sepharose, negative control; Poly, polyclonal anti-IL-6-Sepharose, positive control; Co, negative control IgG_1; non IP, input of non-immunoprecipitated materials (mixtures of 2 or 4 different labelled proteins in the case of panel **A**).

All ΔhIL-6 proteins used in this study are indicated on the left margins. Results concerning Δ^{5-21}* protein are not discussed in the context of this work.

functional sites without disruptive changes in the general shape of the hybrid molecules compared to native IL-6. We therefore decided to investigate in more detail the short 97–104 aa region and to study if this region is only structurally involved in hIL-6 (as shown in section **b**) or also functionally. We were also interested in investigating the 29–33 aa region since we know that this region may be in part involved in the IL-6 conformation (as shown in section **b**) and that neutralizing mAb's bind to IL-6 in close vicinity to these aa (Brakenhoff et al., 1990). In addition, deletion of aa 1–28 has no effect, and further removal of aa 29–30 and 29–34 results in a 50- and 10000-fold reduction in specific activity, respectively (Brakenhoff et al., 1989). We have therefore substituted the non-homologous aa of these regions, by oligo-directed mutagenesis of pBS-IL-6 (as defined in Fig. 1 legend) construction, and converted them into their murine counterparts. Protein 9 was substituted in the 97–104 aa region where the Tyr-Leu-Glu-Tyr-*Leu-Gln*-Asn-*Arg* hIL-6 region was modified into Tyr-Leu-Glu-Tyr-*Met-Lys*-Asn-*Asn*. Protein 11 is an $Arg^{104} \rightarrow Asn^{104}$ derivative of protein 9. Protein 4 was substituted in the aa 29–33 region, where the human sequence Ile-*Arg-Tyr-Ile*-Leu was modified into Ile-*Thr-His-Val*-Leu.

We observed that replacement of aa 97–104 with murine

sequence (m-97–104 substitution) induces a higher decrease in IL-6 activity toward m cells than toward h cells (Table II). This suggests that this m substitution in a hIL-6 environment seems to have partly destroyed the IL-6 conformation resulting in the decrease of the IL-6 activity. This is also true for the $Arg^{104} \rightarrow Asn^{104}$ modified protein. A similar but more drastic reduction in specific biological activity toward m cells was observed for m-29–33 substitution. Nevertheless, the same biological activity ratio is observed both toward h and m cells (Table II). We also observed by immunoprecipitation experiments that these reticulocyte lysate-synthesized ΔhIL-6 proteins, though recognized by mAb7 (recognizing mainly the unfolded wt hIL-6), are still immunoprecipitated by conformation-specific mAb8. Therefore, they must have lost only a part of the IL-6 conformation. Because m-29–33 substitution affects much more the activity of IL-6 in comparison to the m-97–104 substitution, it seems that the 29–33 aa region may be more directly implicated in the structural conformation of the active site of IL-6.

TABLE II

Relative activities of the h/m hybrid IL-6

Proteins[a]	HGF activity[b]	*CRP-cat* expression[c]
wt hIL-6	100%	100%
Protein 4	5– 7%	5–10%
Protein 9	19–35%	50–60%
Protein 11	26–32%	40–70%

[a] The non-homologous aa of the aa 29–33, 97–104 or 104 region have been substituted in their murine counterparts in the proteins 4, 9 and 11, respectively, as described in section **d**. All proteins were produced in reticulocyte lysate as described in Fig. 1. Various activities are adjusted for the same amount of protein as calculated by densitometry analysis of SDS-PAGE autoradiograms. For the four proteins, the determinations of both kinds of biological activities have been repeated six times (i.e., twice for three independent protein preparations).

[b] The HGF assays were performed with mouse B hybridoma 7TD1 cells as described by Van Snick et al. (1986).

[c] IL-6 assays in human hepatoma cells were realized according to Ganter et al. (1989). Briefly, a plasmid (*CRP-cat* construct 3′Δ-121/-46) containing a fusion between a DNA segment (*CRP*) of the human C-reactive protein promoter linked to the SV40 early promoter and to the bacterial *cat* gene was transfected into Hep3B cells with the calcium phosphate precipitation technique (Graham and Van der Eb, 1973; Arcone et al., 1988). This construct is highly responsive to recombinant IL-6 and not to IL-1 as shown in Ganter et al. (1989). After 15 h, transfected cells were washed with culture medium and wt hIL-6 or h/m hybrid IL-6 were added to the culture medium. Induction was extended for 24 h. In each case, equivalent amounts of wt or hybrid IL-6 were added to the cells. Cell extracts and CAT assays were performed as described (Gorman, 1985). CAT assays were performed with 20 μl of cell extracts and incubated at 37°C for 15 min. After autoradiography individual spots were cut, eluted and the percentage of acetylated versus total [^{14}C]Cm was determined (% of Cm conversion to acetylated forms).

(e) Conclusions

Our first approach in investigating the IL-6 structure/function relationship was to examine the biological activity of six ΔhIL-6 proteins carrying deletions Δ^{21-51}, Δ^{52-97}, Δ^{97-104}, $\Delta^{127-174}$, Δ^{97-184} and $\Delta^{134-184}$. Their expression in *X. laevis* oocytes gave us information about *N*-glycosylation of the wt hIL-6 since we observed that proteins $\Delta^{127-174}$, Δ^{97-184} and $\Delta^{134-184}$, which lack the second *N*-glycosylation site on Asn^{144} of the mature protein, are still *N*-glycosylated, in contrast to the Δ^{21-51} protein, which lacks the first *N*-glycosylation site on Asn^{45}. We therefore concluded that in *X. laevis* oocytes hIL-6 *N*-glycosylation occurs only on the Asn^{45} of the mature protein. The deletion mutants produced in oocytes as well as in a reticulocyte lysate were tested by the IL-6 HGF assay, where only the full-length IL-6 molecule displayed an IL-6 activity; all the ΔhIL-6 proteins were at least 10000-fold less active. Binding studies demonstrated that all of these deletion mutants were also unable to bind to the IL-6 receptor.

Using mAb's as probes for the IL-6 tertiary structure, we demonstrated that all the ΔhIL-6 proteins were recognized by an mAb which recognizes mainly the unfolded wt hIL-6. However, Δ^{21-51} and $\Delta^{127-174}$ proteins were found to have lost only part of the native IL-6 conformation, since they were still recognized by some IL-6 conformation-specific mAb's; the remaining deletion mutants were found to have largely lost the IL-6 tertiary structure, since they were not recognized by those conformation-specific mAb's. This partial or complete disruption of the IL-6 tertiary structure may then explain the inactivity of these proteins.

The aa 29–33 region of the mature IL-6 deleted in our Δ^{21-51} protein has already been demonstrated to be a critical region, since N-terminal ΔhIL-6 proteins lose their activity if these aa are removed (Brakenhoff et al., 1989). Moreover, this region is recognized by some neutralizing mAb's (Brakenhoff et al., 1990). However, we have shown by murine substitutions that this region is most probably only important for the modeling of the receptor-binding site of hIL-6. In contrast, the aa 97–104 region appeared to be important for the overall IL-6 conformation. Thus, none of these regions were found to be involved in the binding of IL-6 to its receptor.

The Δ^{97-184} and $\Delta^{134-184}$ proteins, which were shown to be inactive and unrecognized by any of the mAb probes for the IL-6 conformation, have lost the specific C-terminal region recognized by all neutralizing monoclonal antibodies which could consist of a 7 aa α-helix as shown by secondary structure prediction data (Gilbrat et al., 1987; Brakenhoff et al., 1990). These rather large α-helix regions must then be structurally and functionally involved in the development of the IL-6 activity.

The aa region removed in the $\Delta^{127-174}$ protein is also recognized by an IL-6 neutralizing mAb (Ida et al., 1989).

This region may therefore be of interest for further studies, since we expect that the inactivity of the $\Delta^{127-174}$ protein, still recognized by some IL-6 conformation-specific mAb's, could be explained by more than the partial loss of the IL-6 tertiary structure.

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