# Recombinant human interleukin-12 is the second example of a C-mannosylated protein

**Marie-Agnès Doucey, Daniel Hess, Marcel J.J.Blommers[1] and Jan Hofsteenge[2]**

Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland and [1]Novartis Pharma AG, P.O. Box, CH-4002 Basel, Switzerland

*Received on May 16, 1998; revised on September 3, 1998; accepted on September 30, 1998*

[2]To whom correspondence should be addressed

**The β-chain of human interleukin 12 (IL-12) contains at position 319–322, the sequence Trp-x-x-Trp. In human RNase 2 this is the recognition motif for a new, recently discovered posttranslational modification, i.e., the C-glycosidic attachment of a mannosyl residue to the side chain of tryptophan. Analysis of C-terminal peptides of recombinant IL-12 (rHuIL-12) by mass spectrometry and NMR spectroscopy revealed that Trp-319β is (partially) C-mannosylated. This finding was extended by *in vitro* mannosylation experiments, using a synthetic peptide derived from the same region of the protein as an acceptor. Furthermore, human B-lymphoblastoid cells, which secrete IL-12, were found to contain an enzyme that carries out the C-mannosylation reaction. This shows that nonrecombinant IL-12 is potentially C-mannosylated as well. This is only the second report on a C-mannosylated protein. However, the occurrence of the C-mannosyltransferase activity in a variety of cells and tissues, and the presence of the recognition motif in many proteins indicate that more C-mannosylated proteins may be found.**

*Key words:* C-mannosyltransferase/α-mannosyltryptophan/ribonuclease 2

## Introduction

Only one C-mannosylated protein, human RNase 2, has been reported so far (Hofsteenge *et al*., 1994; de Beer *et al*., 1995; Löffler *et al*., 1996). C-Mannosylation was discovered in endogenous RNase 2 purified from human urine and human blood cells (Löffler *et al*., 1996) and was also found in recombinant RNase 2 (Krieg *et al*., 1997). This raises the question as to how general this type of glycosylation is. Although indirect evidence strongly suggests that other C-mannosylated proteins exist (Krieg *et al*., 1997, 1998), no direct proof is available. C-Mannosylation of RNase 2 involves the attachment of an α-mannosyl residue via a C-C link to the indole moiety (Figure 1) of the first tryptophan in the recognition sequence **Trp**-x-x-Trp (Krieg *et al*., 1998). It has been shown that a microsomal transferase which uses Dol-P-Man as the sugar donor is involved (Doucey *et al*., 1998). This C-mannosyltransferase activity is present in a variety of cultured mammalian cells, but not in cells from insects, plants or *E.coli* (Krieg *et al*., 1997). A search of the Swiss-Prot and TrEMBL databases yielded 336 secreted mammalian proteins containing the motif, including human IL-12 (Krieg *et al*., 1998).

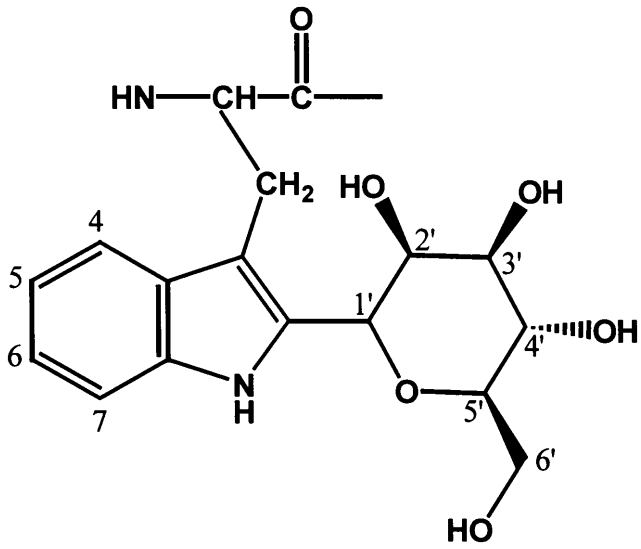


**Fig. 1.** Structure of $C^2$-α-mannosyltryptophan [($C^2$-Man-)Trp].

IL-12 (also called cytotoxic lymphocyte maturation factor) is a cytokine that is structurally unique among the interleukins in that it is a heterodimer composed of two disulfide-linked chains. The α-chain is 253 amino acids long and contains three potential N-glycosylation sites, whereas the β-chain consists of 328 amino acids with five potential N-glycosylation sites in addition to a C-mannosylation site at the C-terminal end (position 319β–322β). IL-12 plays a central role in the immune system by promoting the development of type 1 T-helper cells and by regulating cellular functions of T- and natural killer cells (Trinchieri *et al*., 1994; Hendrzak *et al*., 1995). It has great therapeutic potential because of its potent antitumor activity. This is presently leading to clinical trials, including gene therapy (Tahara *et al*., 1995), for the treatment of human cancer (Zitvogel *et al*., 1995). Furthermore, IL-12 has been demonstrated to be active in several mouse models of infectious diseases caused by viruses, protozoans, fungi, and mycobacteria (Gately *et al*., 1996).

Here we demonstrate by ESIMS analysis and NMR spectroscopy that rHuIL-12 from CHO cells is partially C-mannosylated on Trp-319β. This raises the question as to whether nonrecombinant IL-12 is also modified. Toward this aim, the presence of the C-mannosyltransferase in human B-lymphoblastoid cells (NC-37) which secrete IL-12 was investigated using an *in vitro* assay.

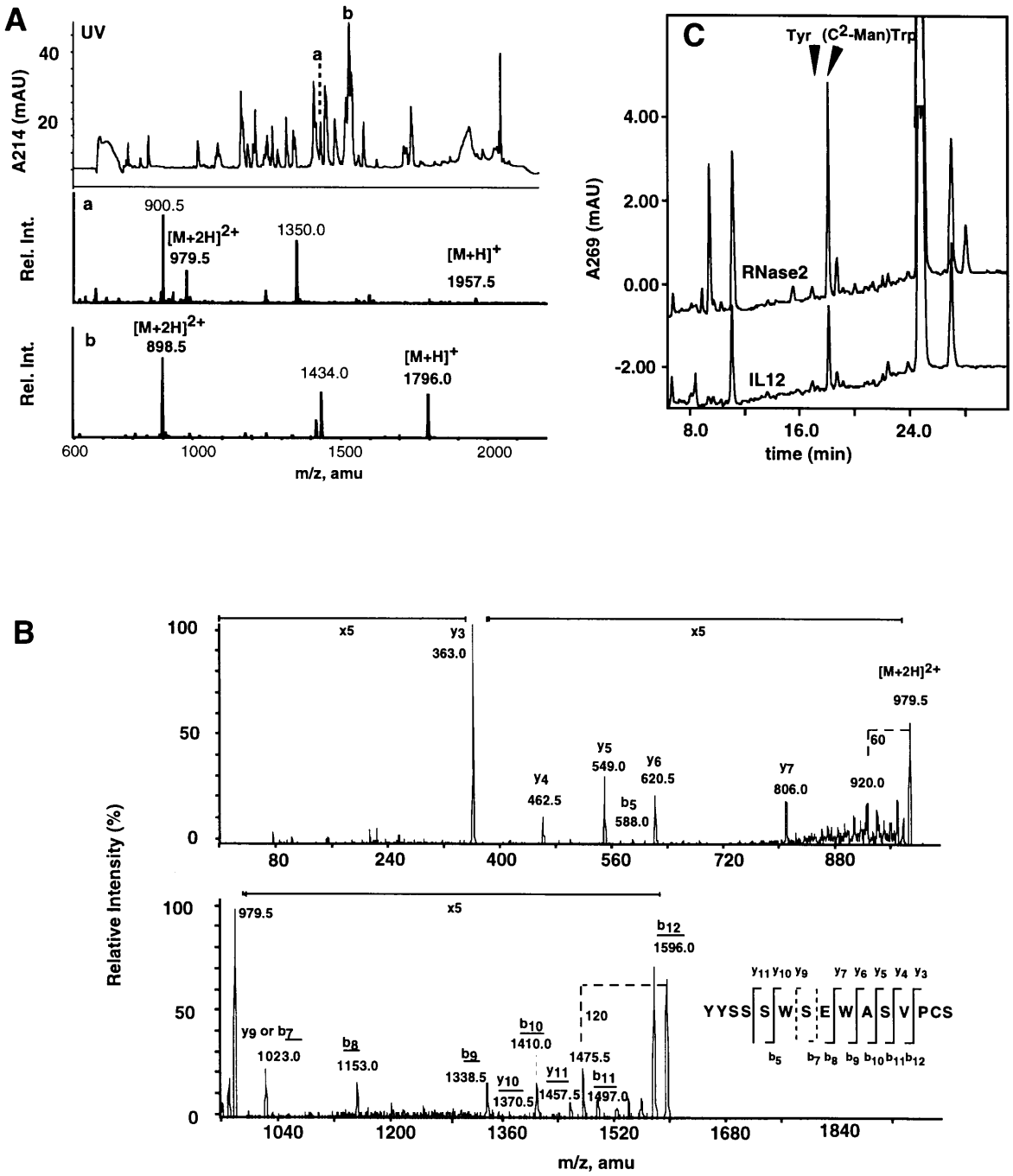


**Fig. 2.** Characterization of the modified C-terminal peptide from rHuIL-12. (**A**) Reduced and carboxamidomethylated rHuIL-12 was digested with trypsin, and the two C-terminal peptides (a and b) were isolated by LC-ESIMS (upper panel) and characterized by nanospray ESIMSMS. The spectra of peptide a and b are shown in the lower panels. The numbers indicate the *m/z*. (**B**) The ESIMSMS spectrum of the modified peptide ("a") is shown. Ions with *m/z* 162 amu larger than in the unmodified peptide have been underlined. The loss of 120 Da from the $b_{12}$- and the $[M+2H]^{2+}$ ion has been indicated with "120" an "60," respectively. (**C**) Edman degradation of peptide "a" of rHuIL-12. The phenylthiohydantoin amino acid observed at cycle 6 (lower trace) coelutes with the ($C^2$-Man-)Trp derivative obtained from position 7 of RNase 2 (upper trace).

## Results

### *rHuIL-12 from CHO cells is C-mannosylated*

To examine whether Trp-319 of the β-chain of rHuIL-12 is C-mannosylated, tryptic peptides were isolated from the reduced and carboxamidomethylated protein. The C-terminal tryptic fragment of the rHuIL-12 β-chain was isolated by reversed phase HPLC interfaced with LC-ESIMS. Two peptides from this region of the molecule were isolated, peak "a," eluting at 39.6 min, and peak "b," eluting at 44.1 min, with a mass of 1957 and 1795 Da, respectively (Figure 2A). The 162 Da higher mass and shorter elution time of peptide "a" strongly suggested it to be mono-hexosylated (Hofsteenge *et al.*, 1996). The ion count of the modified peptide was 20% of that of the unmodified peptide suggesting that the ratio of the modified to unmodified peptide was approximately 1:5. Both peptides were purified to homo-

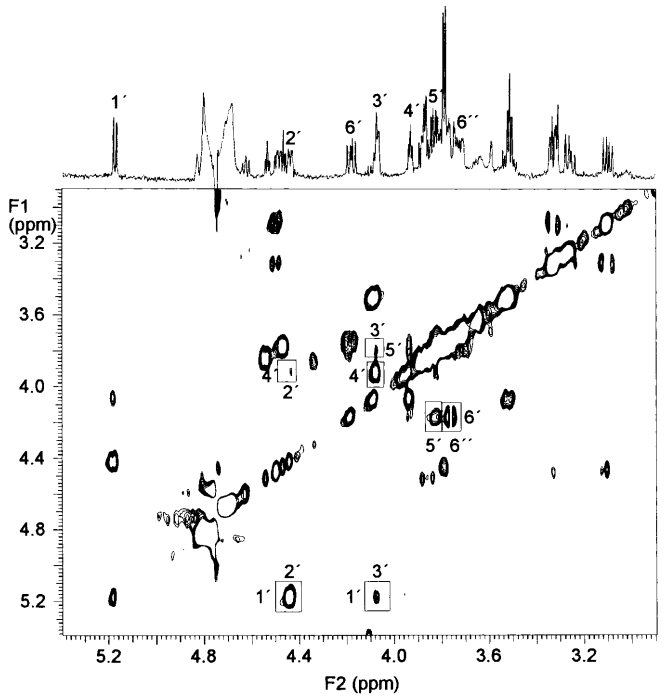


**Fig. 3.** Part of the 600 MHz TOCSY spectrum at 300 K of the peptide comprising residues 316β-322β of rHuIL-12, recorded with a mixing time of 80 ms. The resonance assignment of the modification is indicated in the 1-dimensional spectrum placed on top. Crucial cross peaks are boxed and annotated. The peptide is identified as S-S-S-($C^2$-Man-)W-S-E-W. Non-annotated peaks are α-β connectivities of Ser and Trp.

geneity and analyzed by ESIMSMS. The spectrum of peptide "a" (Figure 2B) showed that the ions $b_5$ and $y_3$ to $y_7$ had the same m/z values as in the unmodified peptide "b," whereas those of $b_8$ to $b_{12}$ and $y_{10}$ to $y_{11}$ were 162 atomic mass units (amu) higher. This demonstrated that the modification was located either on Trp-319β, Ser-320β, or Glu-321β. The ion at 1023.0 amu could either be the unmodified $y_9$- or the modified $b_7$ ion, which restricted the localization of the modification to Trp-319β or Ser-320β. Previously, a characteristic 120 Da loss has been observed in MSMS experiments of ($C^2$-Man-)Trp-containing peptides from RNase 2 (Hofsteenge *et al*., 1996). The same was observed here (Figure 2B). To determine the position of the modified amino acid, peptide "a" was subjected to Edman degradation, which yielded the sequence Y-Y-S-S-S-W*-S-E-W-A-S-V-P-C-S. The PTH-derivative of the residue at position 6 of the peptide (W*) comigrated exactly with that of ($C^2$-Man-)Trp in RNase 2 (Figure 2C).

In order to obtain unequivocal proof for the identity of the hexosyl residue, the chymotryptic peptide comprising residues 316β-322β was analyzed by NMR spectroscopy. Figure 3 shows the part of the TOCSY spectrum where resonances of the non-amino-acid substituent are observed. The assignment of its resonances followed readily from the spectrum. The chemical shifts (Table I) were virtually identical to those reported earlier for the α-mannopyranosyl moiety linked to C2 of Trp-7 in human RNase 2 (Hofsteenge *et al*., 1994; de Beer *et al*., 1995). Also the aromatic resonances of the Trp residue were in good agreement with the published values. The 1-dimensional spectrum was well-resolved, and the observed coupling constants were in close agreement with those found for ($C^2$-Man-)Trp (Table I). It should be noted that the chemical shifts differed significantly from the ones published for O-mannosylated Ser, present in a small protein (Bergwerff *et al*., 1998). This further confirms that Ser-320 is not modified.

**Table I.** Chemical shifts and J-couplings of the C-glycosylated amino acid residue, obtained at 300 K

| Chemical shift (ppm) | | | $^3$J-Coupling (Hz) | | |
|---|---|---|---|---|---|
| IL-12 residue 319β | | RNase 2[a] residue 7 | IL-12 residue 319β | | RNase 2[a] residue 7 |
| H1′ | 5.18 | 5.22 | $J_{1'2'}$ | 7.5 | 7.8 |
| H2′ | 4.44 | 4.42 | $J_{2'3'}$ | 3.0 | 3.2 |
| H3′ | 4.07 | 4.09 | $J_{3'4'}$+ $J_{4'5'}$ | 9.1 | 9.3 |
| H4′ | 3.93 | 3.96 | $J_{5'6'}$ | 8.3 | 8.3 |
| H5′ | 3.83 | 3.87 | $J_{5'6''}$ | 3.4 | 3.3 |
| H6′ | 4.18 | 4.21 | $J_{6'6''}$ | -12.5 | — |
| H4 | 7.65 | 7.67 | | | |
| H5 | 7.14 | 7.14 | | | |
| H6 | 7.20 | 7.20 | | | |
| H7 | 7.42 | 7.41 | | | |

Chemical shifts were referenced against internal acetone (δ = 2.225; Vliegenthart *et al*., 1983). The notation for the atoms is as depicted in Figure 1.
[a]These values were taken from Hofsteenge *et al*. (1994).

### *The C-terminal peptide of IL-12-β is a substrate for the C-mannosyltransferase*

The results described above predict that the C-terminal peptide of the IL-12-β chain should be an acceptor for the C-mannosyltransferase. Incubation of the peptide Ac-RYYSSSWSEWAS-$NH_2$ with Dol-P-[2–$^3$H]Man and rat liver microsomes (55 μg of protein) for 30 min at 37°C resulted in the incorporation of $4.2 \times 10^4$ c.p.m., compared to $1.37 \times 10^3$ c.p.m. in a control without peptide. To obtain sufficient product for a detailed structural characterization, the peptide was incubated for 20 h at 26°C, at which time 83% of the [2–$^3$H]Man had been transfered from Dol-P-[2–$^3$H]Man to the peptide. The peptide was treated with trypsin to remove the N-terminal acetylated Arg and purified. The purification yielded a single radioactive peptide with a molecular mass of 1514 Da, corresponding to that of a monomannosylated peptide (Figure 4A).

The purified radiolabeled peptide was subjected to solid-phase Edman degradation to determine the site of mannosylation. A burst of radioactivity appeared with the Trp at position 7 (Figure 4B). Due to its C-terminal blocking group, the peptide was coupled to the solid-phase through the side chain carboxylate of the Glu preceding the Trp at position 10. As a result, no information about this Trp residue was obtained. C-mannosylation of this residue was excluded by MSMS analysis of the peptide. All ions containing Trp-7 were 162 Da heavier than those of the unmodified peptide. Furthermore, no ions with a modified Trp 10 were identified (data not shown). The presence of a C-C linkage between the tryptophan and the mannosyl residue was concluded from the typical 120 Da loss from ions containing Trp 7, as was also found in the peptide from rHuIL-12 (Figure 2B).

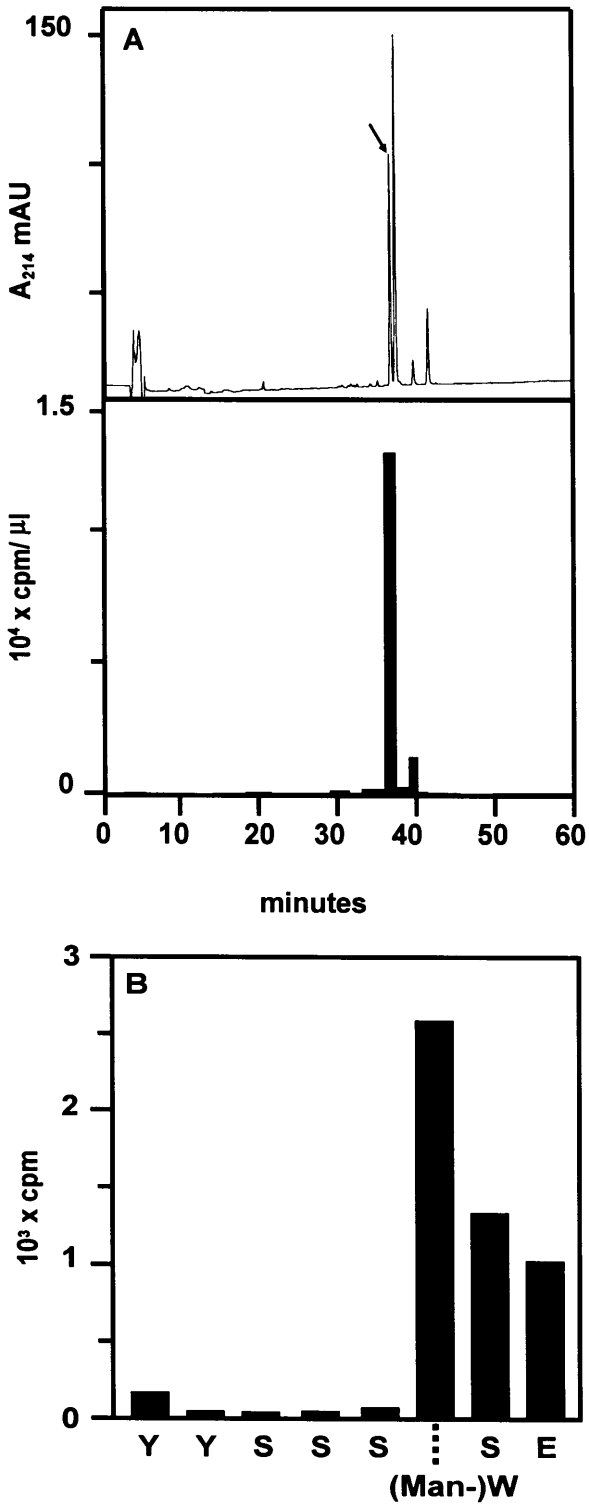


**Fig. 4.** *In vitro* C-mannosylation of synthetic IL-12 peptide. (**A**) The radiolabeled peptide was digested with trypsin and fractionated by C8 reversed phase HPLC. The elution of the column was monitored at 214 nm (upper panel). The lower panel shows the radioactivity; the width of the bar indicates the fraction size. (**B**) the purified IL-12 radiolabeled peptide was subjected to solid-phase Edman degradation and the radioactivity of the anilinothiazolinone amino acid released at each cycle was measured.

*IL-12 secreting B-lymphoblastoid cells contain the C-mannosyltransferase*

The question remains whether nonrecombinant IL-12 is also C-mannosylated. The direct analysis of endogenous IL-12 is not practical due to its extremely low abundance. Stern *et al.* (1990) obtained only 10 µg of purified IL-12 from 60 l of conditioned medium from EBV-transformed lymphoblastoid cells. This can be compared with the 9.6 mg used in the complete analysis of the C-mannosylation of the recombinant protein by MS and NMR. As an alternative, we examined C-mannosyltransferase activity in IL-12 secreting NC-37 cells. Incubation of NC-37 cell microsomes with Dol-P-[2–$^3$H]Man and the IL-12 derived peptide (Ac-RYYSSSWSEWAS-$NH_2$), or the general acceptor tetrapeptide Ac-WAKW-$NH_2$, resulted in the transfer of radioactivity from the Dol-P-[2–$^3$H]Man to the peptides (Figure 5A). The reaction was linear with time (Figure 5B) and its rate increased with the microsomal protein concentration (Figure 5C). Previously, rat liver has been shown to contain an enzyme that catalyzes protein C-mannosylation (Doucey *et al.*, 1998). Heat-treatment of microsomes from NC-37 cells abolished C-mannosyltransferase activity (Figure 5A). Furthermore, the reaction displayed a rectangular hyperbolic dependence on the concentration of the peptide substrate (Figure 5B, inset). Treatment of the microsomes with chymotrypsin strongly decreased the rate of the reaction (data not shown). In RNase 2 the sequence **Trp**-x-x-Trp is the recognition signal for C-mannosylation (Krieg *et al.*, 1998). Therefore, a peptide lacking this motif, Ac-WAK**A**-$NH_2$, was examined as a further control. The amount of radioactivity transferred in this case was the same as the background observed in the absence of any peptide (Figure 5A). The small amount of radioactivity found in these controls must be due to incorporation of mannose into endogenous microsomal acceptors. These results demonstrate that C-mannosylation is also enzyme-catalyzed in NC-37 cells.

## Discussion

The results presented here show that rHuIL-12 contains ($C^2$-Man-)Trp at position 319 of the β-chain. Initial evidence was obtained by ESIMS and Edman degradation of the C-terminal tryptic peptide. Although these techniques allow the analysis of picomolar amounts of material, they do not provide unequivocal identification of the hexosyl moiety. For example, due to the lack of synthetic standards of the various ($C^2$-hexosyl-)tryptophans, it cannot be excluded that their PTH-derivatives comigrate on HPLC following Edman degradation. Unambiguous proof has been obtained by NMR spectroscopy (Figure 3, Table I). This showed that the chemical structure of residue 319β is identical to that of ($C^2$-Man-)Trp present in human RNase 2, which was previously characterized in detail by the same technique (Hofsteenge *et al.*, 1994; de Beer *et al.*, 1995). The chemical shifts of the ($C^2$-Man-)Trp residue in the peptide from IL-12 [S-S-S-($C^2$-Man-)W-S-E-W] are remarkably similar to those found in the peptide from human RNase 2, which has a different primary structure [F-T-($C^2$-Man-)W-A-Q-W]. Both peptides are presumably disordered. The J-couplings of the mannosyl residue in both peptides differ substantially from those expected for the regular chair conformation. Previously, it has been concluded that the sugar moiety, at least in the peptide, does not adopt one, but several conformations (de Beer *et al.*, 1995). Thus, the present data also show that the conformational dynamics of the mannosyl residue is the same in the two peptides.

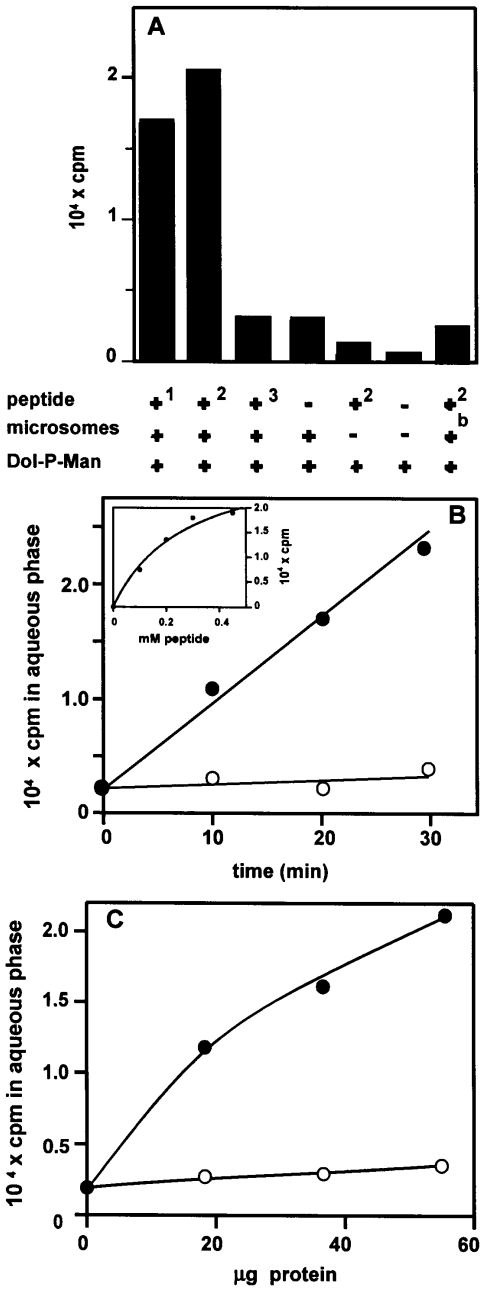


**Fig. 5.** C-Mannosyltransferase activity in IL-12 producing cells. (**A**) The presence of C-mannosyltransferase activity in microsomes from NC-37 cells was determined by incubation of acceptor peptide (0.9 mM) with 0.9 µM Dol-P-[2–$^3$H]Man (5.61 Ci/mmol) and NC-37 cell microsomes (55 µg of protein) for 30 min at 37°C. After extraction with chloroform/methanol (3/2, v/v), the radioactivity in the aqueous phase was determined. Peptide 1 corresponds to the IL-12 derived dodecapeptide: Ac-RYYSSSWSEWAS-$NH_2$. Peptide 2 and peptide 3 correspond to the tetrapeptides Ac-WAKW-$NH_2$ and Ac-WAKA-$NH_2$, respectively. In the experiment indicated with "b" microsomes were heated at 95°C for 8 min, before the assay was performed. (**B**) The time dependence of the *in vitro* C-mannosylation reaction was examined using the peptide Ac-WAKW-$NH_2$. The reaction was stopped by extraction with chloroform/methanol (3/2, vol/vol) at the indicated time the amount of radioactivity in the upper phase was determined (solid circles). Control experiments were performed without acceptor peptide (open circles). The inset shows the incorporation of radioactivity after 30 min incubation at 37°C, as a function of the concentration of the same acceptor peptide. The values were corrected for background by subtracting the values obtained in the absence of peptide. (**C**) Dependence of the rate of reaction on the amount of added microsomes was examined using the peptide Ac-WAKW-$NH_2$. All data shown represent the average of two independent experiments.

RNase 2 and IL-12 are structurally and functionally unrelated, indicating that C-mannosylation is not restricted to a single family of proteins. Trp-319β occurs in the sequence **Trp**-x-x-Trp, which in RNase 2 has been demonstrated to be the recognition motif for the C-mannosyltransferase. This suggests that this sequence may be general and serve the same role in other proteins as well. The motif has been found in 336 secreted mammalian proteins currently present in protein databases (Krieg *et al*., 1998). It is not to be expected that all of these will be modified, however, since their availability may depend on the tertiary structure. Krieg *et al*. (1998) and Doucey *et al*. (1998) have demonstrated that the primary structure as such is recognized by the transferase, and concluded that C-mannosylation of RNase 2 must occur before folding is completed.

Lymphoblastoid cells (NC-37) secrete IL-12 and contain a microsome-associated protein transferase that carries out the C-mannosylation reaction. This shows that potentially also nonrecombinant IL-12 is C-mannosylated. The enzyme from NC-37 cells has the same requirement for a Trp residue at position +3 as a signal for C-mannosylation as the transferase from rat liver microsomes (Krieg *et al*., 1998). Also the amount of activity in microsomes from the NC-37 cells is of the same order of magnitude as that in rat liver.

rHuIL-12 from CHO cells was only partially C-mannosylated on Trp-319β. The degree of mannosylation depends on the cell line used (Krieg *et al*., 1997). With RNase 2, CHO cells were found to be the least active, yielding 49% C-mannosylation, whereas, e.g., NIH 3T3 gave 81%. No activity at all was found in insect cells, plant protoplasts, *E.coli* (Krieg *et al*., 1997) and *S.cerevisiae* (A.-M. Vicentini and M.-A. Doucey, unpublished observations). rHuIL-12 has been reported to have the same biological activity *in vitro* as the endogenous human protein (Gubler *et al*., 1991). If, however, C-mannosylation would in analogy to N-linked glycans affect parameters such as protein stability, circulatory lifetime, biodistribution, or uptake (Rasmussen, 1992), differences may be found in *in vivo* investigations. Because the therapeutic potential of IL-12 was demonstrated in several mouse models, it is important to note that in contrast to human IL-12, the protein from mouse does not contain the C-mannosylation motif. Therefore, the investigation of the therapeutic potential of rHuIL-12 in mouse models might be inappropriate.

The use of recombinant proteins in the therapy of human disease is of increasing importance. To minimize the risks of unwanted side effects substantial efforts are made to copy the human endogenous protein as faithfully as possible (Hayes *et al*., 1997). This aim is hardly reached completely, due to heterogeneity of, e.g., N- or O-linked glycans or, as demonstrated here, the presence of a new or unexpected posttranslational modification. A major question that remains to be answered concerns the presently unknown function of C-mannosylation. In view of the widespread occurrence of the C-mannosyltransferase in mammalian tissues (M.-A. Doucey, unpublished observations), and the unique stability of the C-C link between the carbohydrate and the protein, it is to be expected that a specific biological role will emerge.

## Materials and methods

### *Tissue culture*

NC-37 cells (ATCC CCL214) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies/Gibco BRL, Gaithersburg, MD) (Stern *et al*., 1990).

*Protein chemistry*

rHuIL-12 from CHO cells was a gift from Dr. Alvin Stern, Roche Research Center, Hoffman-La Roche, Inc., Nutley, NJ (Stern *et al*., 1990). The protein was reduced and carboxymethylated according to the method described previously (Hofsteenge *et al*., 1991). Digestion with trypsin (Worthington, Freehold, NJ) and fractionation of the peptides by reversed phase LC-ESIMS were performed as described previously (Krieg *et al*., 1997). The C-terminal β-chain peptide and its modified counterpart were detected by extraction of the MS data for ions with *m/z* 898 and 979 (the $[M+2H]^{2+}$ ions), respectively. Final purification was achieved by $C_8$ reversed phase HPLC in 10 mM trimethylamine-acetate, pH 6.0 (Hofsteenge *et al*., 1991). Nanospray ESIMSMS (Wilm *et al*., 1996) and solid-phase Edman degradation (Pisano *et al*., 1993) were performed according to published methods.

*NMR spectroscopy*

The peptide comprising residues 316β-322β was obtained by cleaving the tryptic peptide from 9 mg rHuIL-12 (see above) with chymotrypsin (Worthington, Freehold, NJ), followed by purification on a $C_{18}$ HPLC column. The identity and homogeneity of the peptide (mass: 1029 Da) were confirmed by Edman degradation. The peptide was dissolved in $D_2O$ and transferred into a susceptibility matched 5 mm NMR tube (Shigemi Co., BMS-005V) with a sample volume of 200 µl. The concentration was ~35 µM. Clean-TOCSY spectra (Griesinger *et al*., 1988) with mixing times of 40 ms and 80 ms were recorded on a Varian Unity*plus* 600 MHz spectrometer equipped with a $^1H$, $^{13}C$, $^{15}N$ triple resonance probe head with z-gradients. The 2-dimensional spectra were folded once in $t_1$, and were acquired with 160 scans and 176 increments using phase sensitive data acquisition in both dimensions. Data were processed using the VNMR program.

In vitro *C-mannosylation and product characterization*

Microsomes from rat liver and NC-37 cells were prepared as described previously (Graham, 1992), except that in the case of the cells, the 10,000 × *g* centrifugation was omitted. The microsomes were washed with 50 mM phosphate, pH 7.4 containing 150 mM NaCl, 1 mM $MgCl_2$, 20% glycerol, 2 µg/ml benzamidine, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, and 2 mM EGTA, frozen, and stored at -80°C. Before use the microsomes were washed with 500 mM NaCl in the same buffer to remove proteases and endogenous acceptor proteins.

The IL-12 peptide (Ac-RYYSSSWSEWAS-$NH_2$) was C-mannosylated *in vitro* using rat liver microsomes as a source of C-mannosyltransferase and Dol-P-[2–$^3$H]Man as the sugar donor (Doucey *et al*., 1998). The reaction mixture contained in a final volume of 24 µl: 0.9 µM Dol-P-[$^3$H]Man (5.61 Ci/mmol), 0.9 mM of peptide, rat liver microsomes (150 µg of protein), 20 mM Hepes-NaOH pH 7.2, 110 mM K-acetate, 2 mM Mg-acetate, proteases inhibitors (2 µg/ml benzamidine, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 mM EDTA), 0.2% Triton X-100. The reaction was performed at 26°C for 20 h and stopped by adding 2 ml of chloroform/methanol 3:2 (v/v) and 0.48 ml of water. The long incubation period was necessary to obtain sufficient quantity of modified peptide for detailed structural analysis. The aqueous phases of 12 experiments were combined and dried. The peptide was isolated by chromatography using a $C_{18}$ Sep-Pak cartridge (Waters, Milford, MA) and purified by reversed phase HPLC at pH 6.0 (see above). The purified peptide was digested with trypsin and fractionated by $C_8$ reversed phase LC-ESIMS (Krieg *et al*., 1997). Final purification was achieved by re-chromatography. The purified radioactive peptide was subjected to nanospray ESIMSMS (Wilm *et al*., 1996) and to solid-phase Edman degradation (Pisano *et al*., 1993).

C-Mannosyltransferase activity in microsomes from NC-37 cells was assayed with the IL-12 derived peptide, as well as the general acceptor peptide Ac-WAKW-$NH_2$ . In the standard assay the reaction mixture contained in a final volume of 24 µl: 0.9 mM of peptide, 0.9 µM Dol-P-[2–$^3$H]Man (5.61 Ci/mmol) and microsomes from NC-37 cells (55 µg of protein) in the buffer described above (Doucey *et al*., 1998). The Triton X-100/protein ratio was always kept constant at 0.34 (w/w). The reaction was stopped after 30 min at 37°C by adding 2 ml of chloroform/methanol 3:2 (v/v) and 0.48 ml of water. After centrifugation, the upper, aqueous phase contained the peptide and the lower, organic phase the Dol-P-[$^3$H]Man. The radioactivity in 0.2 ml of the upper phase was determined by scintillation counting. As controls, either the peptide or microsomes were omitted, or the peptide was substituted with a homolog missing the C-mannosylation motif (Ac-WAKA-$NH_2$). Furthermore, microsomes were heated or treated with chymotrypsin as described previously (Doucey *et al*., 1998).

## Acknowledgments

We thank Dr. Alvin Stern, Roche Research Center, NJ, for his generous gift of rHuIL-12, Renate Matthies for amino acid sequencing and Drs. Jack Rohrer and Yoshikuni Nagamine for reading the manuscript. We are grateful to Dr. Wolfgang Gläsner for stimulating discussions.

## Abbreviations

($C^2$-Man-)Trp, $C^2$-α-mannopyranosyltryptophan; Dol-P-Man, dolichyl-phosphate-mannose; ESIMS, electrospray ionization mass spectrometry; EBV, Epstein-Barr virus; IL-12, interleukin-12; LC, liquid chromatography; PTH, phenylthiohydantoin; rHuIL12, recombinant human interleukin 12; RNase 2, ribonuclease 2; TOCSY, total correlated spectroscopy.

## References

Bergwerff,A.A., Stark,W., Fendrich,G., Knecht,R., Blommers,M.J., Märki,W., Kragten,E.A. and van Oostrum,J. (1998) Identification of Manα1–3Manα1–2Man and Man-linked phosphate on O-mannosylated recombinant leech-derived tryptase inhibitor produced by *Saccharomyces cerevisiae* and determination of the solution conformation of the mannosylated polypeptide. *Eur. J. Biochem*., **253,** 560–575.

de Beer,T., Vliegenthart,J.F.G., Löffler,A. and Hofsteenge,J. (1995) The hexopyranosyl residue that is C-glycosidically linked to the side chain of tryptophan-7 in human RNase Us is α-mannopyranose. *Biochemistry,* **34,** 11785–11789.

Doucey,M.-A., Hess,D., Cacan,R. and Hofsteenge,J. (1998) Protein C-mannosylation is enzyme-catalysed and uses dolichyl-phosphate-mannose as a precursor. *Mol. Biol. Cell*., **9,** 291–300.

Gately,M.K. and Mulqueen,M.J. (1996) Interleukin-12: potential clinical applications in the treatment and prevention of infectious diseases. *Drugs*, **52 (Suppl. 2),** 18–26.

Graham,J. (1992) Isolation of subcellular organelles and mebranes. In Rickwood,D. (ed.), *Centrifugation: A Practical Approach.* IRL Press, Oxford, pp. 161–182.

Griesinger,C., Otting,G., Wüthrich,K. and Ernst,R.R. (1988) Clean TOCSY for proton spin system identification in macromolecules. *J. Am. Chem. Soc*., **110,** 7870–7872.

Gubler,U., Chua,A.O., Schoenhaut,D.S., Dwyer,C.M., McComas,W., Motyka,R., Nabavi,N., Wolitzky,A.G., Quinn,P.M., Familletti,P.C. and Gately,M.K. (1991) Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA*, **88,** 4143–4147.

Hayes,T. and Ryffel,B. (1997) Safety considerations of recombinant protein therapy: introductory comments. *Clin. Immunol. Immunopathol*., **83,** 1–4.

Hendrzak,J.A. and Brunda,M.J. (1995) Biology of disease. Interleukin-12, biologic activity, therapeutic utility, and role in disease. *Lab. Invest*., **72,** 619–637.

Hofsteenge,J., Servis,C. and Stone,S.R. (1991) Studies on the interaction of ribonuclease inhibitor with pancreatic ribonuclease involving differential labeling of cysteinyl residues. *J. Biol. Chem*., **266,** 24198–24204.

Hofsteenge,J., Müller,D.R., de Beer,T., Löffler,A., Richter,W.J. and Vliegenthart,J.F.G. (1994) New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase Us. *Biochemistry*, **33,** 13524–13530.

Hofsteenge,J., Löffler,A., Müller,D.R., Richter,W.J., de Beer,T. and Vliegenthart,J.F.G. (1996) Protein C-glycosylation. In Marshak,D.R. (ed.), *Techniques in Protein Chemistry VII*. Academic Press, New York, pp. 163–171.

Krieg,J., Gläsner,W., Vincentini,A., Doucey,M.-A., Löffler,A., Hess,D. and Hofsteenge,J. (1997) Protein C-mannosylation is an intracellular process, performed by a variety of cultured cells. *J. Biol. Chem*., **272,** 26687–26692.

Krieg,J., Hartmann,S., Vicentini,A., Gläsner,W., Hess,D. and Hofsteenge,J. (1998) Recognition signal for C-mannosylation of Trp-7 in RNase 2 consists of sequence Trp-x-x-Trp. *Mol. Biol. Cell*., **9,** 301–309.

Löffler,A., Doucey,M.-A., Jansson,A.M., Müller,D.R., de Beer,T., Hess,D., Meldal,M., Richter,W.J., Vliegenthart,J.F.G. and Hofsteenge,J. (1996) Spectroscopic and protein chemical analyses demonstrate the presence of C-mannosylated tryptophan in intact human RNase 2 and its isoforms. *Biochemistry*, **35,** 12005–12014.

Pisano,A., Redmond,J.W., Williams,K.L. and Gooley,A.A. (1993) Glycosylation sites identified by solid-phase edman degradation: O-linked glycosylation motifs on human glycophorin A. *Glycobiology*, **3,** 429–435.

Rasmussen,J.R. (1992) Effect of glycosylation on protein function. *Curr. Opin. Struct. Biol*., **2,** 682–686.

Stern,A.S., Podlaski,F.J., Hulmes,J.D., Pan,Y.-C.E., Quinn,P.M., Wolitzky,A.G., Familletti,P.C., Stremlo,D.L., Truitt,T., Chizzonite,R. and Gately,M.K. (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA*, **87,** 6808–6812.

Tahara,H. and Lotze,M.T. (1995) Antitumor effects of interleukin-12 (IL-12): applications for the immunotherapy and gene therapy of cancer. *Gene Therapy*, **2,** 96–106.

Trinchieri,G. and Scott,P. (1994) The role of interleukin 12 in the immune response, disease and therapy. *Immunol. Today*, **15,** 460–463.

Vliegenthart,J.F.G., Dorland,L. and van Halbeek,H. (1983) High-resolution proton nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins. *Adv. Carbohydr. Chem. Biochem*., **41,** 209–374.

Wilm,M. and Mann,M. (1996) Analytical properties of the nanoelectrospray ion source. *Anal. Chem*., **68,** 1–8.

Zitvogel,L. and Lotze,M.T. (1995) Role of interleukin-12 (IL12) as an anti-tumour agent: experimental biology and clinical application. *Res. Immunol*., **146,** 628–638.