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

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Introduction

Only one C-mannosylated protein, human RNase 2, has been reported so far (Hofsteenge et al., 1994; de Beer et al., 1995; Lößfler et al., 1996). C-Mannosylation was discovered in endogenous RNase 2 purified from human urine and human blood cells (Lößfler 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).

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

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift (ppm)</th>
<th>J-coupling (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>RNase 2a</td>
<td>IL-12</td>
</tr>
<tr>
<td>residue 319β</td>
<td>residue 7</td>
<td>residue 319β</td>
</tr>
<tr>
<td>H1'</td>
<td>5.18</td>
<td>5.22</td>
</tr>
<tr>
<td>H2'</td>
<td>4.44</td>
<td>4.42</td>
</tr>
<tr>
<td>H3'</td>
<td>4.07</td>
<td>4.09</td>
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<tr>
<td>H4'</td>
<td>3.93</td>
<td>3.96</td>
</tr>
<tr>
<td>H5'</td>
<td>3.83</td>
<td>3.87</td>
</tr>
<tr>
<td>H6'</td>
<td>4.18</td>
<td>4.21</td>
</tr>
<tr>
<td>H7</td>
<td>7.65</td>
<td>7.67</td>
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<tr>
<td>H5</td>
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<td>7.14</td>
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<tr>
<td>H6</td>
<td>7.20</td>
<td>7.20</td>
</tr>
<tr>
<td>H7</td>
<td>7.42</td>
<td>7.41</td>
</tr>
</tbody>
</table>

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. *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-RYYSSWSEWAS-NH2 with Dol-P-[2–3H]Man and rat liver microsomes (55 µg of protein) for 30 min at 37°C resulted in the incorporation of 4.2 × 10^5 c.p.m., compared to 1.37 × 10^5 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 [2–3H]Man had been transferred from Dol-P-[2–3H]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).
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-[2-3H]Man and the IL-12 derived peptide (Ac-RYYSSSSWEAS-NH₂), or the general acceptor tetrapeptide Ac-WAKW-NH₂, resulted in the transfer of radioactivity from the Dol-P-[2-3H]Man to the peptides (Figure 5A). The reaction was linear with time (Figure 5B) and its rate increased with the micosomal 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-WAKA-NH₂, 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 (C²-Man-)Trp at position 319 of the β-chain. Initial evidence was obtained by ESIIMS 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 (C²-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 (C²-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 (C²-Man-)Trp residue in the peptide from IL-12 [S-S-S-(C²-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-(C²-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. 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.

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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 mannansylation 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 therapeutical 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 therapeutical 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 carbohydrase 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 C₁₈ reversed phase HPLC in 10 mM trimethylamine-acetate, pH 6.0 (Hofsteenge et al., 1991). Nanospray ESIMS/MSMS (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 C₁₈ HPLC column. The identity and homogeneity of the peptide (mass: 1029 Da) were confirmed by Edman degradation. The peptide was dissolved in D₂O and transferred into a 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 Unityplus 600 MHz spectrometer equipped with a 1H, 13C, 15N triple resonance probe head with z-gradients. The 2-dimensional spectra were folded once in t₁, 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 NaCl, 1 mM MgCl₂, 20% glycerol, 2 μg/ml benzamidine, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, and 2 mM EDTA, 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-RYYSSSWSEW AS-NH₂) was C-mannosylated in vitro using rat liver microsomes as a source of C-mannosyltransferase and Dol-P-β-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


