Hyper-heterogeneity of the N-Terminus of Recombinant BSF-2/IL-6 Produced in CHO and NIH3T3 Cells

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Recombinant human BSF-2 (B cell stimulatory factor 2/Interleukin 6; IL-6) proteins were purified from CHO and NIH3T3 cell cultures and characterized. The lectin binding patterns suggested that the proteins have N-linked oligosaccharide(s) with tri-antennary structure of bisecting GlcNAc. Their N-termini were highly heterogeneous; at least five closely related N-termini were detected. This N-terminal heterogeneity was not generated in the cell culture because no processing activity was found in the culture medium.

BSF-2/IL-6 (B cell stimulatory factor 2/interleukin 6) is a lymphokine with many biological activities. cDNA cloning has shown its primary structure, although several heterogeneous BSF-2 molecules with dissimilar N-termini have been reported in human cell lines. Moreover, it has been suggested that natural BSF-2 is glycosylated, although the function of the glycosylation is not well understood; recombinant BSF-2 produced in E. coli is non-glycosylated. To investigate these problems, we have produced mammalian cells that synthesize human BSF-2. NIH3T3 cells showing high expression of human BSF-2 have been reported previously.

In this report, we describe the production of CHO cells, which synthesize human BSF-2 at a high level, and the characterization of the BSF-2 proteins produced by CHO and NIH3T3 cells. We found that the N-termini of the recombinant molecules were hyper-heterogeneous. No activity for BSF-2 N-terminus processing was found in the culture medium.

Materials and Methods

Cells. CHO dhfr- cells were transfected with pSD-(X)BSF2, constructed from the Xhol fragment of BSF-2 cDNA and the pSD(X) vector of SV40 promoter and the DHFR gene, and transfectants were selected in alpha MEM without nucleic acids (GIBCO, cat. No. 410-2000). The BSF-2 gene in the transfectants was co-amplified with the DHFR gene by culture in medium containing MTX.

Production of NIH3T3 cells showing high expression of BSF-2 has been described previously. BSF-2 CDNA without the 3'-untranslated region was introduced using the BMGNeo vector.

BSF-2 production and purification. The recombinant BSF-2 was produced using 10^6 CHO cells/ml of alpha-MEM and 10^6 NIH3T3 cells/ml of DMEM medium. The produced BSF-2 was purified with an affinity column of anti-BSF-2 monoclonal antibody directly from the culture medium, and further purified by RP-HPLC.

Recombinant BSF-2 and Ala-BSF-2 produced in E. coli. Ala-BSF-2 were prepared from E. coli protein fused with the human IL-2 N-terminus by kallikrein cleavage, as described previously. E. coli recombinant BSF-2 was further processed by aminopeptidase-P.

Detection of glycosylation. Saccharides were detected using lectins. One microgram of purified BSF-2 was electrophoresed into an SDS-polyacrylamide gel, western-

Abbreviations: BSF-2, B cell stimulatory factor 2; GlcNAc, N-acetylglucosamine; DHFR, dihydrofolate reductase; MTX, methotrexate; RP-HPLC, reverse-phase HPLC; Con A, concanavalin A; LCA, lentil lectin; PHA-E4, phytohemagglutinin E4; PNA, peanut lectin; RCA120 Ricinus communis lectin 120; WGA, wheat-germ lectin.
blotted onto a nitrocellulose filter, and treated with 6 types of peroxidase-linked lectin (Lectin-per. kit A, Seikagaku Kogyo Co., Ltd., Japan). The lectin being reaction was done under the conditions recommended in the instruction manual.

Search for exo-protease in the culture medium. One hundred micrograms of purified Ala-BSF-2 was added to 200 ml of a culture of 10^6/ml CHO dhfr^- cells, and cultured for 4 days. Then the BSF-2 was purified and its N-terminal sequence was analyzed.

Assay of BSF-2 productivity and specific activity. The BSF-2 was measured by ELISA (enzyme-linked immuno-sorbent assay) using mouse monoclonal and rabbit polyclonal anti-BSF-2 antibody. BSF-2 activity was assayed as the activity of IgM production by SKW-C14.

N-Terminus sequencing. Six micrograms of purified BSF-2 was sequenced using an amino acid sequencer (Applier Biosystems Inc.).

Results

Production of CHO cells with high BSF-2 expression

Gene amplification with the DHFR gene was used to obtain high BSF-2-expressing CHO cells. Transfectants requiring no nucleic acid were cultured in increasing concentrations of MTX. BSF-2 productivity increased in parallel with the increase in MTX concentration (Fig. 1). The maximum BSF-2 productivity, 1700 ng/ml, was obtained when the cells were resistant to 10^{-5} m MTX. The higher MTX concentration led to inhibition of cell growth, and a decrease of BSF-2 productivity.

Purification of recombinant BSF-2

Figure 2 shows SDS-PAGE of recombinant BSF-2 purified from CHO and NIH3T3. The purified BSF-2 gave a single peak in RP-HPLC. The recombinant BSF-2 formed a broad band at approximately 23 kDa and an additional band at 31 kDa. The broad 23-kDa band was found to consist of several bands (less in BSF-2; data not shown). The proteins therefore seemed to be several closely related types of molecules.

Detection of saccharides of purified BSF-2

The main 23-kDa molecule of BSF-2 pro-

<table>
<thead>
<tr>
<th>BSF-2</th>
<th>conA</th>
<th>LCA</th>
<th>PHA-E4</th>
<th>PNA</th>
<th>RCA120</th>
<th>WGA</th>
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<tbody>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHO</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

--; no response, +, response, ++; marked response. The types of lectins used are given in “Abbreviations.”
The Heterogeneous N-Terminus of Recombinant BSF-2

Table II. N-Terminal Amino Acids of Recombinant BSF-2

<table>
<thead>
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<th>3</th>
<th>4</th>
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<td>Pro (46)</td>
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<td>Glu (8)</td>
<td>Val (3)</td>
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<tr>
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<td>Asp (3)</td>
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<td>Asp (9)</td>
<td>Lys (2)</td>
</tr>
</tbody>
</table>

The numbers represent quantity of amino acid detected (pmol). The quantities of serine residues * were not calculated because they were not quantitatively recovered.

Produced by NIH3T3 or CHO was glycosylated, because of its responsiveness to some of the lectins used (Table I). Non-glycosylated E. coli BSF-2 showed no responsiveness to any of the lectins. The form of the saccharide was suggested to have N-linked oligosaccharide(s) with a triantennary structure or bisecting GlcNAc, according to the binding pattern (binding to PHA-E4 and RCA120, but not to ConA). The binding of BSF-2 from CHO was somewhat weaker than that of NIH3T3 BSF-2.

N-Terminal heterogeneity

The N-terminal amino acids of purified BSF-2 detected are shown in Table II. The complex pattern indicates that these proteins have several closely related N-termini. All of these N-terminal amino acids were included in the sequence of BSF-2, Ala-Pro-Val-Pro-Gly (Fig. 3).

The composition of the BSF-2 species of each N-terminus in the mixture was not analyzed because of the proline-rich nature of the sequence; the peptide bonds after proline are not cleaved completely. However, the composition was suggested to differ between CHO and NIH3T3. The major N-terminal amino acid in CHO was Val or Gly, but in NIH3T3, it was Ala or Val. The fourth Pro was not detected in NIH3T3.

No protease activity in the culture medium for BSF-2 N-terminus processing was found; only alanine was detected at the N-terminus of E. coli Ala-BSF-2 in CHO cell culture (Materials and Methods).

Discussion

We have described here the characterization of recombinant BSF-2. First, a cell line expressing a high level of BSF-2 was produced. BSF-2 productivity in CHO transfectants was increased in parallel with the MTX concentration up to 1700 ng/ml at 10^{-5} M MTX. Further MTX addition resulted in inhibition of cell growth. SDS-PAGE showed a 31-kDa BSF-2 molecule as well as the 23-kDa molecules observed in several previous studies.5,6,12,13}
This heterogeneity may be due to heterogeneous glycosylation or phosphorylation.

It has been reported that the 26-30-KDa molecules were tunicamycin-sensitive, and are suggested to be N-glycosylated, but the 23-25-kDa molecules were not. However, we detected responsiveness of 23-25-kDa molecules to lectins, and a previous study also revealed uptake of labelled glycosamine by these molecules. The lectin binding pattern found in this study suggested N-glucosylation. In view of their degree of responsiveness to lectins, the saccharides of BSF-2 derived from CHO and NIH3T3 may differ. In fact, it has been reported that the saccharide moiety of recombinant beta interferon differs among the cells producing it.

The N-terminus of purified BSF-2 was hyper-heterogeneous. Five or six molecular species were found in the purified BSF-2. Some of the N-termini have been reported previously; included Ala-, a mixture of Ala- and Val-, or a Pro-Val-Pro-Pro-sequence. Three other species were found for the first time in this study.

No processing was observed in the E. coli Ala-BSF-2 in the CHO cell culture, so much heterogeneity was not created in the culture medium. It may be created at some stage in the secretion process, due to processing by putative aminopeptidase or differences in the cleavage site of the signal peptidase. No other examples of N-terminal heterogeneity in proteins produced by mammalian cells have been reported, although in yeast processing at the signal junction can be variable.

The specific activity of the mixture of these recombinant BSF-2 molecules was 4100 U/μg, identical to that of natural or E. coli-derived BSF-2, and thus no difference is apparent between the activities of glycosylated and non-glycosylated BSF-2 species.

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References