Structural homologies among the hemopoietins

(colony-stimulating factors/interleukins/P-cell-stimulating factor/amino acid sequence)

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ABSTRACT A group of cytokines characterized by a common set of target cells—namely, the pluripotential hemopoietic stem cells or their cellular derivatives—share similarities in the amino acid sequence at their N terminus or in the putative signal peptide immediately prior to the published N terminus. Murine P-cell-stimulating factor (PSF), murine and human interleukin 2 (IL-2), murine and human granulocyte-macrophage colony-stimulating factor (GM-CSF), human erythropoietin, and human interleukin 1 β all share alanine as the N-terminal amino acid and have some similarities in the succeeding three or four amino acids. In the case of murine PSF and GM-CSF, the six N-terminal amino acids are readily cleaved from mature molecules and are lacking from the N-terminal amino acid sequences reported initially. A sixth cytokine, colony-stimulating factor 1, has an alanine followed by a similar pattern of five amino acids at the end of the putative signal peptide. GM-CSF and IL-2 have more extensive homology, about 25% of residues being identical in three regions that comprise about 70% of the molecules. Only minor similarities of uncertain significance were found among the complete amino acid sequences of the other cytokines. Although its evolutionary origin is uncertain, the homology around the N terminus may provide a structural marker for a group of cytokines active on the pluripotential hemopoietic stem cell and its derivatives.

The pluripotential hemopoietic stem cell gives rise to the erythrocytes, granulocytes, monocytes, and lymphocytes of the blood and to a variety of tissue cells, including macrophages, mast cells, and probably dendritic cells and Langerhans cells (1). The growth and differentiation of pluripotential hemopoietic stem cells and their cellular derivatives are influenced by a series of soluble polypeptides or cytokines (1, 2), a number of which have now been purified and molecularly cloned. These include interleukin 2 (IL-2) (3–6), P-cell-stimulating factor (PSF) or interleukin 3 (IL-3) (7–10), granulocyte-macrophage colony-stimulating factor (GM-CSF) (11–13), colony-stimulating factor 1 (CSF-1) (14), erythropoietin (EPO) (15), and interleukin 1 (IL-1), in which case two related forms, IL-1 α and IL-1 β , have been cloned (16–18).

These cytokines, together with several well-characterized related factors for which amino acid sequence data are not at present available, such as granulocyte-CSF (19), B-cell growth factor (20), and eosinophil-differentiating factor (21), share a number of broad biological and structural similarities. Most are glycoproteins with an apparent M_r of 15,000–35,000 on gel filtration, although CSF-1 occurs as a large dimer (14). In most cases the biologically active polypeptide chain consists of about 140 amino acid residues. However, despite these superficial similarities in molecular structure, analyses of amino acid sequences have not yet revealed any homol-

ogy, either within this group of cytokines or with any other known protein sequences.

Here we report that, contrary to these previous conclusions, there is evidence of structural homology at or adjacent to the N terminus of a group of cytokines active on cells of hemopoietic origin. Moreover, in the case of IL-2 and GM-CSF, there is also significant homology over much of the remainder of the molecule. The homology in regions at or adjacent to the N termini of these cytokines may reflect their interaction with a common structure or related group of structures and may define a functional group of cytokines.

RESULTS

A Cleavable Hexapeptide at the N Termini of PSF and GM-CSF. One key to the present observations was the discrepancy between the reported N-terminal amino acid sequences of PSF (7) and IL-3 (8), both of which are encoded by the same gene (9, 10). Comparison of the sequences of PSF (7) and IL-3 (8) indicates that PSF has an additional six amino acids at the N terminus. As shown in Fig. 1, the N-terminal amino acid residue of PSF was determined to be alanine (solid arrow), whereas that of IL-3 was the aspartic acid at position 7 of PSF (dotted arrow). We have postulated (7) that the IL-3 form of the molecule results from proteolytic cleavage of PSF at the bond between arginine and aspartic acid at positions 6 and 7.

A similar discrepancy in data on the N-terminal amino acid sequence of GM-CSF indicated that the cleavage of a small peptide from the N terminus was not unique to PSF. As shown in Fig. 1, the N-terminal amino acid sequence of murine asialo-GM-CSF (11, 22) (dotted arrow) differed from that subsequently reported for human GM-CSF (12, 13) (solid arrow) (Fig. 1). Based upon the N-terminal amino acid sequence of human GM-CSF it was suggested that murine GM-CSF might in fact include an additional six amino acids (12, 13). Subsequent analysis of murine GM-CSF indicated that this was indeed the case and that, as in the human, the N-terminal residue of murine GM-CSF was an alanine (23). This alanine occurred six residues N-terminal to the isoleucine that was initially reported as the N terminus (11, 23) and it was concluded that the original result reflected cleavage of the N-terminal six amino acid residues during production or purification of the GM-CSF (23).

CSF-1. Based upon the amino acid sequence deduced from the cDNA clone, Kawasaki *et al.* (14) concluded that CSF-1 was unrelated to other cytokines or proteins. However, as shown in Fig. 1, at the end of the putative signal peptide there is a short region with some similarities to the N terminus of PSF. Thus, six residues from the glutamic acid, which Kawasaki *et al.* identified as the N terminus, there is an alanine and, of the next three residues, two are serines that can be aligned with the serines at positions 2 and 4 in PSF

PSF (M)	..Leu Gln <u>Ala</u> - Ser Ile Ser Gly Arg <u>Asp</u> ..
	CTC CAA GCT TCA ATC AGT GGC CGG GAT
CSF-1 (H)	..Leu Leu <u>Ala</u> - Ser Arg Ser Ile Thr <u>Glu</u> ..
	CTC CTG GCG AGC AGG AGT ATC ACC GAG
GM-CSF (M)	..Leu Ser <u>Ala</u> Pro Thr Arg Ser Pro - <u>Ile</u> ..
	CTC TCA GCA CCC ACC CGC TCA CCC ATC
GM-CSF (H)	..Ile Ser <u>Ala</u> Pro Ala Arg Ser Pro - Ser..
	ATC TCT GCA CCC GCC CGC TCG CCC AGC
IL-2 (M)	..Asn Ser <u>Ala</u> Pro Thr Ser Ser Ser Thr Ser..
	AAC AGC GCA CCC ACT TCA AGC TCC ACT TCA
IL-2 (H)	..Asn Ser <u>Ala</u> Pro Thr Ser Ser Ser Thr Lys..
	AAC ACT GCA CCT ACT TCA AGT TCT ACA AAG
IL-1 β (H)	..His Asp <u>Ala</u> Pro Val Arg Ser Leu Asn Cys..
	CAC GAT GCA CCT GTA CGA TCA CTG AAC TGC
EPO (H)	..Leu Gly <u>Ala</u> Pro Pro Arg Leu Ile Cys Asp..
	CTG GGC GCC CCA CCA CGC CTC ATC TGT GAC

FIG. 1. Sequences at or adjacent to the N termini of six cytokines of human (H) or murine (M) origin. N-terminal residues are underlined and the cleavage sites of the signal peptides are indicated by solid arrows. Broken underlining and arrows indicate the alternative N termini that have been reported for IL-3 (8) or murine asialo-GM-CSF (11, 22).

(Fig. 1). This observation raises the possibility that, as in the case of PSF and murine GM-CSF, a hexapeptide had been cleaved from the material that had been sequenced and that the N terminus of mature CSF-1 may be alanine.

The N Termini of IL-2 and EPO. In the cases of murine and human IL-2 the N-terminal amino acid was once again alanine (3–6). Moreover, there was a striking pattern of homology with the N termini of murine and human GM-CSF. Of the four amino acids following the alanine, three were identical in murine and human IL-2 and murine GM-CSF, forming the pattern Ala-Pro-Thr-Xaa-Ser. Human GM-CSF differed slightly in that a single base change had resulted in the substitution of an alanine for the threonine (Fig. 1).

Inspection of the N-terminal amino acid sequence of human EPO once again revealed that the N-terminal amino acid was alanine and that the second residue was proline, as in the case of IL-2 and GM-CSF. The fourth residue, arginine, was identical with the fourth residue of GM-CSF and IL-1 β (see below) (Fig. 1).

Homology at or Adjacent to the N Termini of Other Proteins and Cytokines. Based upon these observations, which suggested that a group of cytokines was characterized by the presence of a common pattern of amino acids around the N-terminal amino acid sequence, we used the SEARCH computer program (24) to locate other proteins containing peptides of patterns APTXS, APSXS, ATXS, ASXS, and APXR, where the letters represent the single-letter amino acid code and X represents an unspecified residue. Homology was scored using the mutational data matrix for 250 accepted point mutations (25). The 100 top-scoring sequences were selected for further visual examination.

In only 1 of the 3477 sequences available in the NIH protein-sequence database of The Protein Identification Resource was one of the searched patterns located at the N

terminus of a molecule. Remarkably, the sequence involved was that of a cytokine, IL-1 β , which had the N-terminal amino acid sequence Ala-Pro-Val-Arg-Ser (Fig. 1).

We also examined the sequences of a number of other cytokines. Interferon γ showed no evidence of homology at the N terminus or in the signal peptide region. Likewise, a homologous peptide was absent in lymphotoxin, tumor-necrosis factor, and also insulin, insulin-like growth factors, and relaxin. The pentapeptide Ala-Pro-Ser-Arg-Ala, however, occurs at the end of the putative signal peptide of the human tissue inhibitor of metalloproteinases (26), which has also been termed erythroid-potentiating activity (EPA) (27) because it promotes the formation of colonies of erythroid cells in bone marrow cultures. Although EPA has been classified as a hemopoietin (27), this point requires clarification as a direct action on erythroid progenitors has not been demonstrated and it is conceivable that its effects on erythropoietic cells *in vitro* are indirect and related to its action as a proteinase inhibitor. Moreover, according to the empirical rules of van Heijne (28), it is unlikely that cleavage of a signal peptide would occur between the first alanine of this pentapeptide and the isoleucine of the preceding sequence—Trp-Leu-Ile-. Thus, on balance, EPA is not likely to be a member of this group of cytokines.

Homology in Other Regions of GM-CSF and IL-2. When the sequences of IL-2 and GM-CSF of murine and human origin were aligned at the N and C termini and gaps introduced, there were multiple identities (Fig. 2).

There were matches between at least one of the two GM-CSF sequences and one of the two IL-2 sequences at about 28% of residues. If human GM-CSF was compared with murine IL-2 or human IL-2 there were identities in 17% and 18% of positions, respectively. Figures were similar if mouse GM-CSF was compared with human IL-2 or mouse IL-2 (20%) and (17%), respectively.

If the regions where the sequences appear to differ radically (i.e., those corresponding to residues 35–48 and 73–95 of human GM-CSF) were excluded, in the remaining 70% of the molecules there were identities between at least one of the GM-CSF sequences and one of the IL-2 sequences at 40% of positions. Table 1 shows in more detail the degree of homology between GM-CSF and IL-2 in these three regions. The similarities between the sequences of GM-CSF and IL-2 were even greater if instances were taken into account where amino acid differences could be accounted for by single base changes at the codon level or involve functionally related amino acids.

DISCUSSION

These observations on a group of cytokines that has as its targets the pluripotential hemopoietic stem cell or its cellular derivatives fall into two main categories. (i) There is the demonstration that there is a group of cytokines that share a short homologous peptide at the N terminus that has the pattern Ala-Ser-Xaa-Ser or Ala-Pro-Xaa-Arg(or Ser)-Ser(or Leu). In the case of GM-CSF and PSF the six N-terminal amino acids are readily cleavable, raising the possibility that a homologous hexapeptide at the end of the putative signal peptide of CSF-1 also represents an N-terminal hexapeptide that is readily cleavable from the native molecule. (ii) There is the demonstration that two cytokines in this group, IL-2 and GM-CSF, show significant homology over the greater part of the molecules. Both sets of observations demand reassessment of the prevailing view that these cytokines lack homology and raise the possibility of functional interrelationships between the members of this group.

A number of arguments makes it extremely unlikely that the pattern of homology around the N termini of these molecules has occurred by chance alone. Merely the obser-

FIG. 2. Amino acid sequence homology of human (H) and murine (M) GM-CSF and IL-2. In aligning the four sequences, gaps were introduced to maximize homology. Sequences are numbered from the N-terminal alanine of the mature proteins.

vation that the five cytokines PSF, GM-CSF, IL-2, EPO, and IL-1 β share alanine as their N-terminal amino acid residue suggests that this part of the molecule may be under some common selective pressure. Based upon the NIH protein-sequence database the frequency of alanine in proteins of higher primates is 0.065. Accordingly, probability that four randomly selected human molecules would resemble PSF in sharing alanine as the N-terminal amino acid, as do GM-CSF, IL-2, EPO, and IL-1 β , is only 1.8×10^{-4} . The fact that the N-terminal alanine is conserved across species in the human and mouse in the case of GM-CSF and IL-2 (Fig. 1) adds further weight to the notion that the alanine at this position has a particular functional significance. There is no evidence

that alanine is in general overrepresented at the N terminus of secreted proteins, and alanine occurred at a frequency of about 0.08 in the collection of secreted proteins studied by van Heijne (28).

Similarities in the pattern of amino acids following the alanine in Fig. 1 further strengthen the notion that the N termini of PSF, GM-CSF, IL-2, and IL-1 β are homologous. Some indication of the very low probability that this situation could occur by chance alone can be gained by considering the likelihood of identities at particular positions in two randomly chosen peptides of six amino acids. Based on the relative frequencies of the various amino acids in rodents and higher primates, the probability of any match at a particular position

Table 1. Homologous regions of GM-CSF and IL-2

Region*	% identical amino acids in homologous regions				
	GM-CSF (M or H) and IL-2 (M or H)	GM-CSF (H) and IL-2 (M)	GM-CSF (H) and IL-2 (H)	GM-CSF (M) and IL-2 (M)	GM-CSF (M) and IL-2 (H)
	(M or H)	IL-2 (M)	IL-2 (H)	IL-2 (M)	IL-2 (H)
1-34	44	24	29	26	26
49-72	42	25	25	25	33
96-127	34	25	22	22	31
All†	40	24	26	24	30

H, human; M, murine.

*Regions corresponding to the indicated residues of human GM-CSF as aligned in Fig. 1.

†Sum of regions indicated above.

is 0.0579 for mice and 0.0581 for humans. By using the relevant binomial distributions, the probability that two such random hexapeptides would be identical at greater than or equal to three positions is 1.5×10^{-4} (for higher primate sequences) and 1.6×10^{-4} (for rodent sequences). The probability of four or greater than four identities is 4×10^{-6} for higher primates and rodents. Of the six N-terminal amino acids of GM-CSF and IL-2, four are identical in the mouse ($P = 4 \times 10^{-6}$) and three in the human ($P = 1.5 \times 10^{-4}$). The six N-terminal residues of GM-CSF and IL-1 β in the human are identical at four positions ($P = 4 \times 10^{-6}$) and the six N-terminal residues of human IL-2 and IL-1 β are identical at three positions ($P = 1.5 \times 10^{-4}$). If, as in Fig. 1, we align the reported N termini and the preceding six amino acids of the putative signal peptides of CSF-1 (14) and IL-3 (8), there are identities at three positions. It should be noted that none of the identities considered above depends upon the insertion of the gap in the sequences.

The fact that a computer search of a database of 3477 proteins located a related sequence at or adjacent to the N terminus of only one other protein further supports the notion that the occurrence of these patterns around the N terminus of PSF, GM-CSF, IL-2, CSF-1, and EPO is not coincidental. This conclusion is enhanced by the fact that the sequence located was that of another cytokine, IL-1 β .

The relationship of IL-1 β to the other molecules shown in Fig. 1 is worthy of comment. Unlike other cytokines and indeed all other secreted proteins, IL-1 β lacks a signal peptide with the characteristic stretch of hydrophobic amino acids (16). IL-1 β may also differ from the other cytokines in Fig. 1 in having other targets in addition to derivatives of the pluripotential hemopoietic stem cell (16). The published data on the biological activity of IL-1 β derived from cloned cDNA relate to its action on T lymphocytes, but further experiments should establish whether IL-1 β acts directly on cells of nonhemopoietic origin such as hepatocytes or fibroblasts. Information on this question and data on the amino acid sequences at the N terminus of IL-1 β in other species may help in deciding whether the striking similarity of IL-1 β with the other cytokines in Fig. 1 is coincidental.

The fact that in the cases of PSF and GM-CSF the homologous peptide is present on the secreted molecule, but appears to be readily cleaved off, raises the question of whether a similar phenomenon occurs with the other factors in Fig. 1. It will be interesting to determine whether the published N-terminal amino acid sequence of CSF-1 also represents the sequence of a cleaved form of the secreted protein. The published N terminus is consistent with the empirical rules governing the cleavage sites of signal peptides proposed by van Heijne (28). However the cleavage sites established for other cytokines in Fig. 1 make it reasonable to postulate a cleavage site between the leucine and the alanine aligned with the N-terminal alanines of the other

cytokines in Fig. 1. A related question is whether there are shorter forms of IL-2 molecules that lack an N-terminal peptide, the published sequences of IL-2 corresponding to a long, more abundant form.

The function of the homologous regions at the N termini of these cytokines is unknown but presumably reflects interaction of these molecules with a common structure or closely related set of structures. One possibility is that cleavage of the homologous peptide from these cytokines allows the further metabolic processing or excretion of the molecule. It is interesting in this respect that the N-terminal amino acid sequence of CSF-1 was determined on material obtained from urine (14). Certainly it is unlikely that the homologous peptide is involved in the primary biological activity of the various molecules because the short forms of PSF (IL-3) (8) and GM-CSF (11, 22) lacking the homologous peptide are fully biologically active, at least *in vitro*.

The question of whether the similarities at the N termini reflect convergent evolution or divergent evolution cannot be resolved on the data available. The more extensive homology between GM-CSF and IL-2, however, favors a common evolutionary origin of at least these two members of the group. We have observed some other homologies between CSF-1, PSF, GM-CSF, and IL-2, but more refined analyses and additional data on sequences from other species will be necessary to establish whether they have any significance.

Analysis of the three cysteines that may be conserved in human IL-2 and GM-CSF (the latter having an additional cysteine at position 88) may assist in the elucidation of the secondary structure of GM-CSF. Thus, in IL-2 the cysteines at positions 58 and 105 are paired (29) and the cysteine at position 125 can be substituted with a serine without impairing biological activity (30). By analogy, it might be predicted that in GM-CSF the corresponding cysteines at positions 54 and 96 are paired and are critical for maintaining the biologically active structure.

The fact that a group of cytokines that share a common set of targets, the pluripotential hemopoietic stem cell or its derivatives, also share similarities at the N termini provides a new parameter for classifying cytokines. The evidence of structural homology between GM-CSF, which acts on myeloid cells, and IL-2, generally regarded as acting between lymphocytes, highlights the unsatisfactory nature of the present nomenclature, which has tended to distinguish between factors studied by hematologists and those by immunologists. One option is that the general term "hemopoietin" be adopted for this entire group of molecules. Individual members could then be identified by numbers (hemopoietin 1, etc.).

It could be argued that hemopoietin is an unsuitable term for molecules that affect lymphocytes. Lymphocytes, however, are a major cellular product of the bone marrow (1) and, like other blood cells, are derived from the pluripotential hemopoietic stem cell. There are many structural similarities between lymphocytes and other derivatives of the pluripotential hemopoietic stem cell, such as Thy-1 and Ly-5 antigens (1). The close developmental relationship between these derivatives of the pluripotential hemopoietic stem cell is also emphasized by evidence that limited rearrangement of DNA in the region of the genes for the T-cell receptor can occur in myeloid cells (31) and that transcription of the μ heavy chain gene can occur in macrophages (32). Moreover, IL-2 receptors have been detected on certain lines of macrophage (33) and mast cell origin (34), suggesting that IL-2 may not act exclusively on the lymphoid component of the hemopoietic system. A simple unified system of nomenclature would have much to recommend it; the present data suggest that structural similarities may be helpful in defining a group of molecules.

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