Selective Proteolytic Cleavage of Recombinant Human Interleukin 4. Evidence for a Critical Role of the C-Terminus

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ABSTRACT: Human interleukin 4 is a 129 amino acid lymphokine secreted by activated T cells that exerts pleiotropic biological effects on B and T lymphocytes and other hematopoietic cells. Structure–function relations were studied by employing selective proteolytic cleavage of purified recombinant human interleukin 4 (rhuIL-4). Limited proteolysis with endoprotease Glu-C from *Staphylococcus aureus* (V8) produced two digestion products that were observed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with apparent molecular weight values of 19K (I) and 15K (II), respectively. These species were isolated by reversed-phase HPLC. Amino acid sequencing indicated that species II was an 84 amino acid core fragment extending from Gln-20 to Glu-103 and containing a hydrolyzed peptide bond at Glu-26. On the basis of known disulfide bond assignments, it was concluded that species II was stabilized by two disulfide bonds (Cys-24/Cys-65 and Cys-46/Cys-99). Analysis of its secondary structure by circular dichroism revealed a high content of α helix. Species I was the full-length rhuIL-4 with selective cleavage at Glu-26 and Glu-103. Both species I and II were inactive in an in vitro assay based on proliferation of peripheral blood lymphocyte blasts and lacked the ability to bind to the rhuIL-4 receptor on Daudi cells. In order to elucidate further the role of the residues removed by *S. aureus* V8 protease, rabbit antisera were raised to synthetic peptides corresponding to residues 1–26 at the N-terminus and 104–129 at the C-terminus. Only antisera directed to the C-terminal peptide inhibited binding of ^{125}I-rhuIL-4 to Daudi cells. We conclude that at least a portion of the C-terminal 26 residues is critically important for the interaction of rhuIL-4 with its receptor.

The T-cell-derived lymphokine interleukin 4 (IL-4)[1] was originally described as a murine factor that could costimulate the proliferation of activated B cells (Howard et al., 1982). Subsequently, it was demonstrated that murine IL-4 could exert a variety of biological effects on B cells (Paul & Ohara, 1987; Roehm et al., 1984; Noelle et al., 1984; Coffman et al., 1986; Vitetta et al., 1985; Coffman & Carty, 1986) and other cell types including T cells (Mosmann et al., 1986; Fernandez-Botran et al., 1986; Hu-Li et al., 1987; Grabstein et al., 1987; Zlotnik et al., 1987), hematopoietic progenitor cells (Rennick et al., 1987; Peschel et al., 1987), and mast cells (Mosmann et al., 1986). On the basis of homology with the murine IL-4, the cDNA encoding huIL-4 has been cloned (Yokota et al., 1986) and expressed in both mammalian (Le et al., 1988; Sonoda et al., 1988; Takebe et al., 1988) and bacterial (van Kimmenade et al., 1988) hosts. Like murine IL-4, recombinant human IL-4 (rhuIL-4) is a pleiotropic lymphokine that acts on a variety of cell types. Thus, for example, rhuIL-4 can induce the proliferation of both activated T and B lymphocytes (Spits et al., 1987; Defrance et al., 1987a), can enhance the expression of class II major histocompatibility antigens and the low-affinity receptor for IgE on B cells (Rousset et al., 1988; Defrance et al., 1987b) and monocytes (Littman et al., 1989; te Velde et al., 1988, 1990), can induce production of IgE and other immunoglobulins (Pene et al., 1988), can induce production of colony-stimulating factors from monocytes (Wieser et al., 1989), and can activate the phagocytic activity of neutrophils (Boey et al., 1989). The expression of biological activity appears to be mediated through binding to high-affinity receptors, which have been identified in a variety of cell types (Park et al., 1987; Cabrillat et al., 1987; Mosley et al., 1989; Harada et al., 1990). The ability of rhuIL-4 to inhibit IL-2-dependent proliferation of chronic lymphocytic leukemic cells of B-cell origin has suggested a clinical application in B-cell neoplasms (Karray et al., 1988).

The availability of milligram quantities of purified rhuIL-4 has provided a basis for the initiation of structural studies of rhuIL-4 and aspects of structure–function relations. It has previously been established that the mature form of rhuIL-4 comprises 129 amino acids including 6 cysteines that form 3 disulfide linkages (van Kimmenade et al., 1988; Windsor et al., 1990, 1991). In an effort to map the receptor binding domain of rhuIL-4, we have studied the biological activity of various fragments generated by limited proteolysis. A core fragment consisting of amino acid residues 20–103 and hence devoid of both N- and C-terminal peptides was shown to be inactive in a T-cell proliferation assay and to lack the ability to bind to the cell-surface rhuIL-4 receptor. Immunochemical studies employing antisera directed to synthetic peptides supported that residues 104–129 at the C-terminus constituted at least a part of a domain critical for maintenance of receptor binding activity.

Experimental Procedures

Materials. rhuIL-4 expressed in L929 and CHO cells was purified to homogeneity as previously described (Le et al., 1988). *Staphylococcus aureus* V8 protease was purchased from Pierce (Rockford, IL). Acetonitrile (HPLC grade) was

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[1] Abbreviations: huIL-4, human interleukin 4; rhuIL-4, recombinant human interleukin 4; CHO, Chinese hamster ovary; PBL, peripheral blood lymphocyte(s); FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin.

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a product of Burdick and Jackson (Muskegon, MI). ^{125}I-Labeled Bolton–Hunter reagent was purchased from Dupont (Boston, MA). Radiolabeling of rhuIL-4 was performed essentially as described by Bolton and Hunter (1973). The specific radioactivity as determined by self-displacement analysis (Calvo et al., 1983) was 1500 Ci/mmol, corresponding to a level of incorporation of 0.68 mol of Bolton–Hunter reagent per mole of rhuIL-4. The Daudi cell line was obtained from the American Type Culture Collection (Rockville, MD). Molecular weight standards used in polyacrylamide gel electrophoresis were purchased from Sigma (St. Louis, MO).

Proteolysis. A stock solution of rhuIL-4 (0.8–2.0 mg/mL) was exhaustively dialyzed at 4 °C in 0.05 M ammonium bicarbonate, pH 7.8, containing 2 mM EDTA and 0.01% SDS. Digestion was initiated by adding a small aliquot of a stock solution of *S. aureus* V8 protease (1 mg/mL in 0.05 M ammonium bicarbonate, pH 7.8, and 2 mM EDTA). The enzyme:substrate ratio was maintained at 1:25 (w/w), and incubation at 37 °C was performed in a water bath incubator. At the indicated time, proteolysis was terminated by rapidly freezing the sample to −80 °C. For determination of T-cell proliferation activity, an aliquot of the digest was diluted in RPMI 1640 containing 10% FBS prior to freezing.

RhuIL-4 Bioassay. The biological activity of rhuIL-4 was determined in a T-cell proliferation assay employing human peripheral blood lymphocytes preactivated with phytohemagglutinin, as previously described (Yokota et al., 1986).

RhuIL-4 Receptor Binding Assay. Daudi cells (1×10^6 cells) were incubated with varying concentrations of ^{125}I-rhuIL-4 in 1 mL of RPMI 1640, 10% FBS, and 0.2% sodium azide at 4 °C for 2 h. After incubation, the cell pellet was collected by centrifugation in a microcentrifuge (Eppendorf Model 5414) and resuspended in 200 μL of cold binding buffer. The cell suspension was layered over 200 μL of dibutyl/dioctyl phthalate oil (1:1, v/v), followed by centrifugation for 45 s. The microcentrifuge tube was rapidly frozen in liquid nitrogen; the tip containing the cell pellet was cut off and placed in a vial for radioactivity determination. Nonspecific binding, which was determined simultaneously in a separate aliquot containing a 1000-fold excess of cold rhuIL-4, was less than 15% of total cell-bound radioactivity. Scatchard analysis of the data by the LIGAND program (Munson & Rodbard, 1980) showed that ^{125}I-rhuIL-4 bound to Daudi cells with a K_d value of 3.6×10^{-11} M. The number of binding sites was estimated to be 476 sites per cell.

The affinity of the proteolytic fragments for the rhuIL-4 receptor was determined in competition experiments with ^{125}I-rhuIL-4. Various concentrations of peptides were added to Daudi cells in the presence of 3.3×10^{-11} M ^{125}I-rhuIL-4. Activity was expressed as the concentration that decreases the specific binding of radiolabeled rhuIL-4 by 50% (IC_{50}).

Preparation of Rabbit Antisera to HuIL-4-Derived Peptides. Synthetic peptides representing rhuIL-4 residues 1–26 and 104–129 were purchased from Bachem (Torrance, CA). Rabbits were immunized with 1 mg of synthetic peptides emulsified in complete Freund's adjuvant (Calbiochem, San Diego, CA). Boost injections of 1 mg of peptides in incomplete Freund's adjuvant were scheduled at 28-day intervals. Antisera were obtained 1 week after the initial immunization and 1 week after each subsequent boost injection. For testing in the receptor binding assay, the antisera were diluted 4-fold in RPMI 1640 containing 10% FBS. Preimmune sera obtained 1 day prior to immunization were used as negative controls in all receptor binding assays. Binding of the antisera from the last bleeds to the synthetic peptides and the cognate sequences in rhuIL-4 was demonstrated by direct ELISA as described (Seelig et al., 1988). Titers of 50000 and 100000 units were obtained for antisera to residues 1–26 and 104–129, respectively, when either the synthetic peptides or rhuIL-4 was used as antigen. One ELISA unit was defined as the reciprocal of the dilution of antisera that resulted in a 50% decrease of the color intensity at saturation binding.

Reversed-Phase HPLC. Reversed-phase HPLC was performed with instrumentation obtained from Waters (Milford, MA) utilizing an Aquapore RP-300, 100 × 2.1 mm, column (Brownlee Labs, Santa Clara, CA). The column was developed with a gradient of acetonitrile (40–60%) in 0.1% (v/v) trifluoroacetic acid.

Amino Acid Sequencing. Automated Edman degradation was performed on an Applied Biosystems (Foster City, CA) Model 470 protein sequencer equipped with on-line HPLC analysis of phenylthiohydantoin-amino acids.

Electrophoresis. SDS–PAGE was performed essentially as described by Laemmli (1970). The method of Oakley et al. (1980) was employed for silver staining of polyacrylamide gels.

CD Analysis. Far-UV CD spectra were obtained with a Jasco J-500A spectropolarimeter. Measurements were taken at room temperature in a 0.1-cm cuvette for the wavelength range of 190–260 nm. The spectrum for each sample was corrected with an appropriate buffer blank. The mean residue ellipticity for rhuIL-4 was calculated on the basis of a mean residue weight of 115 for both intact and truncated proteins. Analysis of secondary structure was performed with the PROSEC program (Chang et al., 1978).

Results

Selective Proteolysis in the Presence of SDS. RhuIL-4 produced by L-929 cells is composed of a single polypeptide chain that exhibits microheterogeneity based on variations in the nature and extent of glycosylation. SDS–PAGE of highly purified preparations revealed the presence of a predominant species with an apparent molecular weight of 19K and several minor species of apparent M_r between 18K and 22K (Le et al., 1988). In preliminary studies of the digestion of rhuIL-4 with *S. aureus* V8 protease, we observed that complete digestion could be achieved in ammonium bicarbonate buffer (50 mM, pH 7.8). Proteolysis proceeded with little selectivity, and discrete large fragments could not be obtained. Selective fragmentation occurred only when a low concentration of SDS was introduced into the digestion mixture. Incubation at 37 °C in the presence of 0.01% SDS and *S. aureus* V8 protease caused distinct alterations in the electrophoretic mobility of all the glycosylation variants (Figure 1). These changes were apparent on SDS–PAGE of both reduced and nonreduced samples. Under nonreducing conditions, a slight decrease in mobility was observed for rhuIL-4 within 30 min of incubation. This slower migrating species was designated species I. This effect was accompanied by the appearance of increasing amounts of a fragment of M_r 14–15K during longer times of incubation (Figure 1, upper panel, lanes d–g), which was designated species II.

SDS–PAGE of the same sample following reduction with 2-mercaptoethanol demonstrated that the majority of the glycosylated variants were converted to a species of apparent M_r 14–15K. The conversion appeared to be completed by 4 h of incubation (Figure 1, lower panel, lane f). These data indicated that although *S. aureus* V8 protease treatment apparently resulted in two distinct molecular weight species (species I and II), treatment with 2-mercaptoethanol converted virtually all of the protein into one or more forms with the

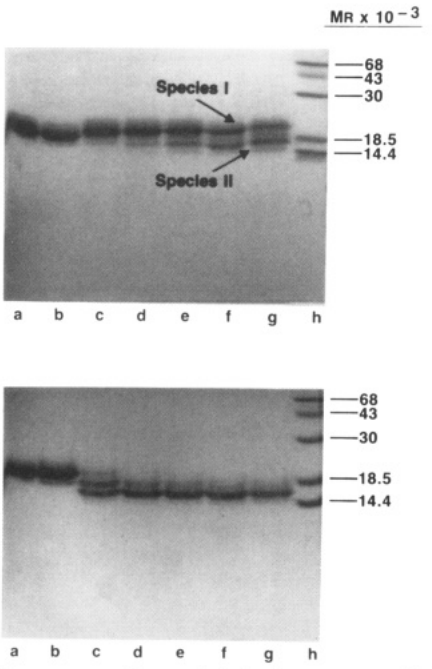

FIGURE 1: Time course of proteolysis in the presence of 0.01% SDS. RhuIL-4 (0.8 mg/mL) was digested at 37 °C in 0.05 M ammonium bicarbonate and 2 mM EDTA, pH 7.8, containing 0.01% SDS. The digestion was terminated at various time intervals between 0 and 6 h. Aliquots of the digest were analyzed by SDS–PAGE using a 20% acrylamide separating gel before (upper panel) and after (lower panel) treatment with 5% 2-mercaptoethanol dissolved in 0.125 M Tris·HCl, pH 6.8, 2.5% SDS, 25% glycerol, and 0.0025% bromophenol blue. The gel was stained with Coomassie Blue R250. Lane a, undigested rhuIL-4; lanes b–g, rhuIL-4 digested for 0, 0.5, 1, 2, 4, and 6 h, respectively; lane h, molecular weight standards (bovine serum albumin, 68K; ovalbumin, 43K; carbonic anhydrase, 30K; β-lactoglobulin, 18.5K; lysozyme, 14.3K).

lower apparent molecular weight (i.e., 14–15K).

Purification of Fragments and Determination of Biological Activity. A sample of a 4-h digest was analyzed by reversed-phase HPLC (Figure 2, top panel). Elution of the C-4 resin with a shallow acetonitrile gradient resolved two peaks of UV absorbance exhibiting retention times of 28 min (peak I) and 25 min (peak II), respectively. Analysis of the eluates by SDS–PAGE without prior reduction (Figure 2, bottom panel) showed that peak I consisted of species I (M_r ca. 20K) whereas peak II consisted of a lower molecular weight species II (M_r ca. 15K). However, similar to the data described above for the unfractionated digest, when samples were reduced with 2-mercaptoethanol prior to SDS–PAGE, fractions corresponding to peak I and II appeared virtually identical, exhibiting the lower apparent molecular weight of 15 000 (data not shown).

Biological activity of rhuIL-4 as measured by the T-cell proliferation assay was rapidly abolished by the limited proteolysis with *S. aureus* V8 protease (Table I). After a 4-h incubation in 0.01% SDS, the specific activity of the digest was 5×10^5 units/mg, corresponding to nearly 2 orders of magnitude decrease in comparison with the specific activity of a control sample incubated in the absence of enzyme (2×10^7 units/mg). Control experiments indicated that the presence of a low concentration of SDS (0.05% or less) in the digest buffer also had no effect on the T-cell proliferation activity.

Assay of the HPLC eluates in a T-cell proliferation assay revealed that species II (retention time, 25 min) had no detectable activity within the limits of sensitivity of the assays (Table I). This lack of activity was apparently due to a failure

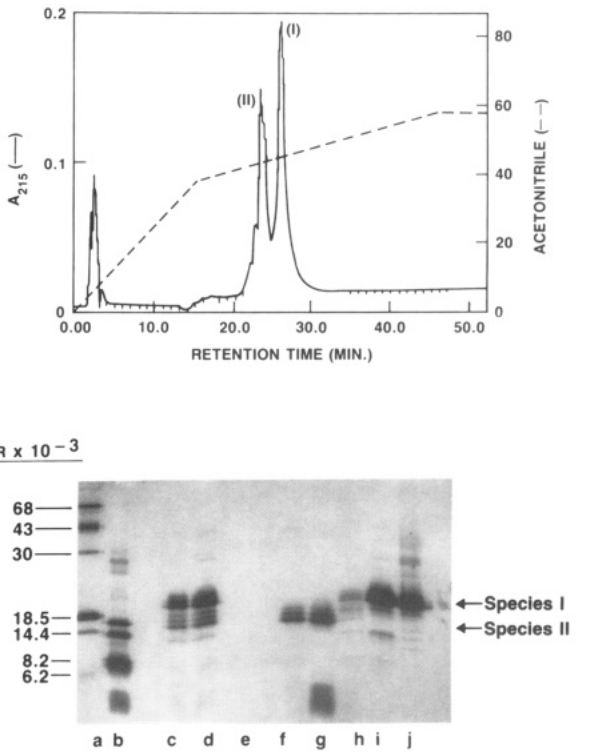

FIGURE 2: Reversed-phase HPLC of a limited digest of rhuIL-4. An aliquot (0.15 mg) of rhuIL-4 digested with *S. aureus* V8 protease for 4 h in the presence of 0.01% SDS was chromatographed on an Aquapore RP 300 C-4 reversed-phase column. The column flow rate was maintained at 0.5 mL/min, and a gradient was established from 40 to 60% acetonitrile in 0.1% trifluoroacetic acid. The absorbance at 215 nm was continuously monitored. Aliquots from 50 fractions (0.5 mL per fraction) were processed for electrophoresis and biological activity determination. Upper panel, absorbance at 215 nm. Lower panel, SDS–PAGE. 100-μL aliquots were dried in vacuo and redissolved in electrophoresis sample buffer (0.125 M Tris·HCl, pH 6.8, 2.5% SDS, 25% glycerol, and 0.0025% bromophenol blue). Electrophoresis was performed under nonreducing condition using a 15% separating acrylamide gel. Lane a, molecular weight standards (bovine serum albumin, 68K; ovalbumin, 43K; carbonic anhydrase, 30K; β-lactoglobulin, 18.5K; lysozyme, 14.4K); lane b, molecular weight standards (myoglobin, 17K; myoglobin fragment I + II, 14.4K; myoglobin fragment I, 8.2K; myoglobin fragment II, 6.2K; myoglobin fragment III, 2.5K); lanes c and d, *S. aureus* V8 digest of rhuIL-4; lanes e–j, reversed-phase HPLC fractions 23, 24, 25, 26, 27, and 28, respectively.

Table I: Effect of Limited Proteolysis on the T-Cell Proliferation Activity and Receptor Binding Activity of RhuIL-4[a]

	T-cell proliferation activity (units/mg × 10^{-5})	receptor binding activity, IC_{50} (ng/mL)
control	200	1
S. aureus V8 treated	5	ND[b]
species I	7	70
species II	0	>14400

[a] RhuIL-4 was digested with *S. aureus* V8 protease in 0.01% SDS and 0.05 M ammonium bicarbonate, pH 7.8. After a 4-h incubation at 37 °C, aliquots were diluted in RPMI-1640 containing 10% FBS and were stored at −80 °C for assay of biological activity. The remaining digest was chromatographed on reversed-phase HPLC for the isolation of species I and II, as described in Figure 2. The control sample consisted of rhuIL-4 incubated at 37 °C for 4 h in 0.01% SDS and 0.05 M ammonium bicarbonate, pH 7.8. An additional control for the T-cell proliferation consisted of *S. aureus* V8 protease and rhuIL-4 in culture medium containing 10% fetal bovine serum assayed without prior incubation at 37 °C. The specific activity of such a preparation (2×10^7 units/mg) was identical with the control shown above. [b] ND = not determined.

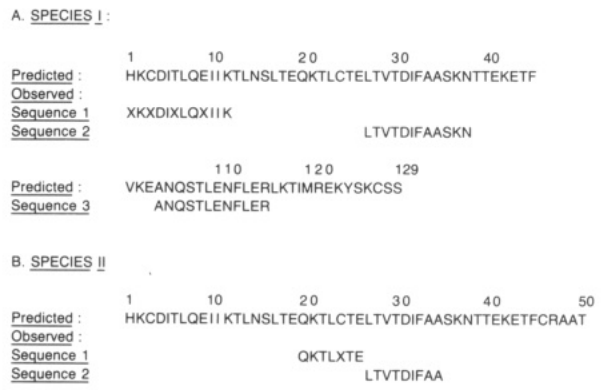

FIGURE 3: Amino acid sequencing of rhuIL-4 species I and II. HPLC eluates containing fragments of rhuIL-4 were pooled and evaporated in vacuo to approximately 0.1 mL. Small aliquots (330 pmol) were applied to polybrene-coated glass fiber sequenator disks. Edman degradation was performed with an Applied Biosystems (Foster City, CA) Model 470 protein sequencer. The yield of PTH-amino acids, which ranged from 25 to 50%, reflects in part the partial loss of sample during processing for sequencing. The repetitive yield was 93%. The presence of multiple sequences was confirmed by the simultaneous appearance of equivalent amounts of three and two PTH-amino acids per cycle for species I and species II, respectively. The numbering of amino acid was based on the mature protein sequence (Le et al., 1988). X represents the amino acid residues that were not identified unambiguously in the observed sequences. Species I, the rhuIL-4 fragment eluting at 26–28-min retention time (Figure 2, upper panel) and exhibiting an apparent molecular weight of 20K by SDS–PAGE (Figure 2, lower panel); species II, the rhuIL-4 fragment eluting at 24–25-min retention time and exhibiting an apparent molecular weight of 15K.

to bind to its cell-surface receptor since HPLC-purified species II did not compete for binding of ^{125}I-rhuIL-4 to Daudi cells at a concentration as high as 14.4 μg/mL (Table I). Residual activities in the digest chromatographed in two fractions (retention times, 27 and 28 min, respectively). In other experiments, we demonstrated that intact rhuIL-4 eluted at a similar retention time (data not shown) with full recovery of specific activity in the T-cell proliferation assay (Le et al., 1988). Consequently, the residual activity detected in the *S. aureus* V8 digest may arise from the presence of trace amounts of undigested rhuIL-4.

N-Terminal Sequence Determinations and Identification of Cleavage Sites. Species I and II isolated by reversed-phase HPLC were subjected to automated Edman degradation for analysis of primary structure (Figure 3). Assignments of amino acid residues could be performed for up to 12 cycles during the Edman degradation of species I. Three PTH-amino acids were observed per cycle. On the basis of the reported cDNA structure of rhuIL-4 (Yokota et al., 1986), the following three sequence assignments were made: residues 1–12, 27–38, and 104–115, respectively. We infer from the data that species I is a rhuIL-4 polypeptide that has been cleaved at Glu-26 and Glu-103. In the Edman degradation of species II, amino acid assignments were performed for eight cycles. Two sequences corresponding to residues 20–26 and 27–34, respectively, were observed. Premature signal termination at Glu-26 was consistent with the simultaneous presence of sequence 27–34. Thus, on the basis of sequence determination, we could assign in species II at least two cleavage sites at Glu-19 and Glu-26.

The amino acid sequence of the reduced form of the rhuIL-4 digest was examined by Edman degradation (Figure 4). Electrophoresis followed by electroblotting of the reduced digest onto a Immobilon membrane revealed two major Coomassie-stained regions corresponding to apparent molecular weight values of 15 000 (A) and 3000–4000 (B), respectively. Bands A and B were excised and subjected to 40 cycles of gas phase Edman degradation. For band A, amino acid residues were assigned for 27 cycles. A single sequence corresponding to residues 27–53 of rhuIL-4 was identified. Sequencing of band B yielded 2 PTH-amino acids per cycle up to 12 cycles. The sequencing data were consistent with the presence of two polypeptides consisting of residues 1–12 and 104–129, respectively. Thus, the detection of peptide fragments representing part of a core region (i.e., band A) and the N- and C-terminal regions (i.e., band B) confirmed the cleavage of rhuIL-4 at Glu-26 and Glu-103. This pattern of cleavage is consistent with data obtained in the sequencing of

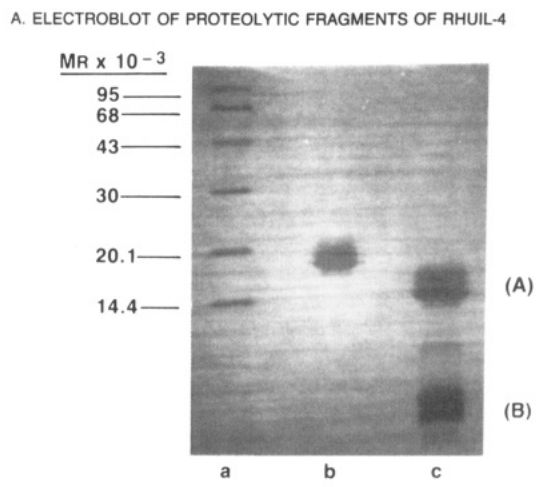

FIGURE 4: Amino acid sequencing of an electroblot of proteolytic fragments of rhuIL-4 separated by SDS–PAGE. RhuIL-4 was digested with *S. aureus* V8 protease for 4 h at 37 °C in 0.01% SDS, 0.05 M ammonium bicarbonate, and 2 mM EDTA, pH 7.8. The digest (50 μg) was boiled for 2 min in sample buffer containing 2% 2-mercaptoethanol, followed by electrophoresis in a 20% separating polyacrylamide gel. The electroblot was obtained following electroblotting onto a Immobilon membrane (Millipore, Bedford, MA) and staining with Coomassie Blue R-250. Lane a, molecular weight standards (phosphorylase *b*, 95K; bovine serum albumin, 68K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 20.1K; lysozyme, 14.4K); lane b, undigested rhuIL-4; lane c, *S. aureus* V8 digest of rhuIL-4. Bands A and B were transferred to the Applied Biosystems gas phase sequenator. The repetitive yield of sequencing was 93%. The PTH-amino acid yield was not calculated due to uncertainty in the efficiency of transfer of peptides to the Immobilon membrane during the electroblotting process.

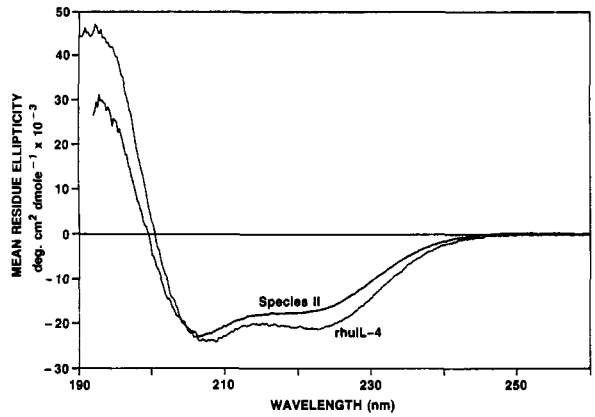

FIGURE 5: Far-UV CD spectra of native huIL-4 and species II. CD measurements were performed in 0.05 M sodium phosphate, pH 7.4, at protein concentrations of 0.30 and 0.11 mg/mL for rhuIL-4 and species II, respectively.

Table II: Neutralization of Receptor Binding Activity by Rabbit Antisera to RhuIL-4 N- and C-Terminal Peptides[a]

	inhibition of receptor binding activity (%) at week[b]			
immunogen	4	12	24	36
rhuIL-4 (1–26)	0	0	25	7
rhuIL-4 (104–129)	30	60	80	90

[a] Antisera were obtained at the indicated time intervals after initial immunization with peptides 1–26 and 104–129 in complete Freund's adjuvant. The antisera were diluted 4-fold in RPMI-1640 containing 10% fetal bovine serum for determination of inhibitory activity in the receptor binding assay employing ^{125}I-rhuIL-4 and Daudi cells. The results are expressed as the mean of triplicate determinations. Preimmune sera for both peptides had no inhibitory effects when tested simultaneously with the antisera. [b] Time of bleeding.

nonreduced species I and II obtained by reversed-phase HPLC, as described above. However, no sequencing data to confirm the third cleavage site at Glu-19 were obtained. This result was not unexpected since residues 20–26 constitute an oligopeptide that might not have been retained efficiently during electroblotting.

CD Analysis of Species II. Species II obtained by preparative reversed-phase HPLC was analyzed for its content of secondary structure by CD spectroscopy in the far-UV region. The resulting spectrum was compared with a spectrum from a sample of native rhuIL-4 (Figure 5). In sodium phosphate buffer (50 mM), pH 7.4, species II exhibited bands of ellipticity that were both qualitatively and quantitatively different from native rhuIL-4. However, calculation of the percent secondary structure revealed that species II had retained substantial α-helical structure (50% α, 50% random) as compared to native rhuIL-4 (75% α, 25% β). Thus, species II was characterized by a significant degree of secondary structure.

Activity of Polyclonal Antisera Directed to the N- and C-Terminal Fragments. Since specific cleavage of rhuIL-4 near the N- and C-terminal sequences resulted in inactivation of biological activity, the importance of these domains was further assessed by immunochemical methods. Rabbits were immunized with synthetic peptides representing the N-terminal sequence 1–26 and the C-terminal sequence 104–129. The antisera were obtained after booster injection, and a series of four booster injections was performed over a 9-month period. Neutralizing activities were determined as the inhibition of binding of radiolabeled rhuIL-4 to its receptor on Daudi cells (Table II). Rabbit antisera directed against the C-terminal peptide 104–129 inhibited receptor binding following each of the four booster injections. In parallel immunization experiments, the N-terminal peptide 1–26 failed to produce any inhibition of receptor binding for two consecutive early booster injections, and only negligible inhibitory effects were observed following the third and fourth injections. The specificity of the antisera to peptide 104–129 was further evaluated in experiments in which the peptide was added to a dilution of antisera that caused 50% inhibition of receptor binding activity. Complete reversal of the inhibitory effect could be demonstrated when the diluted antisera were incubated with 1 μM peptide prior to the receptor binding assay (data not shown). These results imply that residues 104–129 at the C-terminus play a more critical role than the N-terminus in receptor recognition.

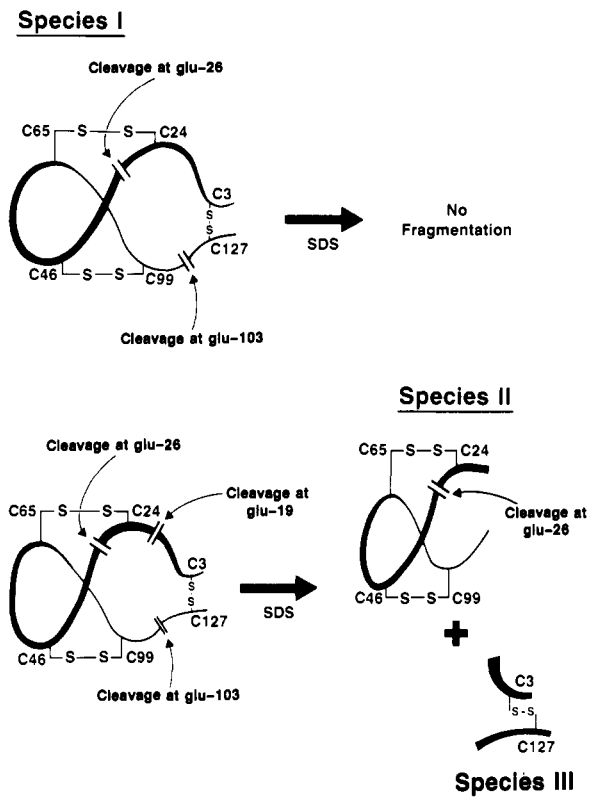

FIGURE 6: Schematic representation of rhuIL-4 fragments obtained by limited proteolysis with *S. aureus* V8 protease.

Discussion

We report here that limited digestion of rhuIL-4 with *S. aureus* V8 protease resulted in the release of a large core fragment, which was characterized as being composed of residues 20–103 (species II; Figure 6). This species was stabilized by the presence of two disulfide bonds, one of which (Cys-24/Cys-65) linked two polypeptide chains and exhibited a high degree of α-helical structure, as determined by CD spectroscopy. The estimated percentage of α-helical structure in species II was 50%, compared to 75% in the parent rhuIL-4. However, species II was essentially inactive in a T-cell proliferation assay and failed to compete with ^{125}I-rhuIL-4 in a receptor binding assay. The retention of two disulfide bonds and the high α-helical content suggested that extensive denaturation due to exposure to acetonitrile and trifluoroacetic acid was not responsible for the observed inability of species II to interact with the rhuIL-4 receptor. Hence, these data suggest that the N- or C-terminal sequences at residues 1–19 and 104–129, respectively, might be present at or near receptor binding regions. In support of this hypothesis, we showed that rabbit polyclonal antisera directed against the C-terminal peptide 104–129 inhibited the binding of ^{125}I-rhuIL-4 to re-

ceptors on Daudi cells. In contrast, the polyclonal antisera directed against the N-terminal peptide 1–26 were either not inhibitory or weakly inhibitory following multiple booster injections. These data support a critical role for C-terminal amino acid residues in receptor binding, although the participation of at least a portion of N-terminal amino acid residues in binding has not been rigorously excluded.

Digestion with *S. aureus* V8 was performed at or near neutral pH at a concentration of SDS (0.01%) that does not adversely affect the catalytic activity of the enzyme (Drapeau, 1977). The requirement for the presence of SDS to achieve preferential cleavage at certain glutamoyl sites is not understood. However, it is notable that the amino acid residues on the C-terminal side of the glutamic acid residues that were preferentially cleaved were either hydrophobic or uncharged (i.e., Leu-27, Ala-104, and Gln-20). In contrast, four out of five of the remaining glutamic acid residues contained either charged or hydrophilic residues on their C-terminal side (i.e., Lys-42, Thr-44, Lys-61, and Lys-123). Since the binding of SDS to proteins is principally through hydrophobic interactions, these observations suggest that interaction of SDS with hydrophobic side chains that are in close proximity to certain glutamoyl bonds may increase their susceptibility to hydrolysis by the *S. aureus* V8 protease.

The time course of fragmentation in the presence of SDS indicated that the glutamoyl bonds at positions 26 and 103 were most readily cleaved by the *S. aureus* V8 protease. RhuIL-4 cleaved at these two sites (i.e., species I) was readily observable within the first 30 min of incubation with the enzyme. The additional cleavage at Glu-19, which resulted in the formation of species II (M_r ca. 15K), appeared to occur at a slower rate since detectable quantities of species II were obvious only after 4–6 h of incubation. As discussed above, this result may be a consequence of the more polar nature of the amino acid following Glu-19. The selective hydrolysis at Glu-26 and Glu-103 compared to Glu-19 could imply that in the presence of a low concentration of SDS these two sites become more accessible to the enzyme. Interestingly, the secondary structure algorithm of Garnier (Garnier et al., 1978) predicts that Glu-103 occurs in a β-turn region, indicating that Glu-103 in the native protein could already be at least partially accessible to solvent.

The observed fragmentation is consistent with the reported disulfide bonding pattern in rhuIL-4, i.e., Cys-3/Cys-127, Cys-24/Cys-65, and Cys-46/Cys-99 (Windsor et al., 1990). On the basis of this pairing assignment, cleavage of rhuIL-4 at Glu-19, Glu-26, and Glu-103 is expected to yield two fragments (species II and III), each containing two chains linked by a disulfide bond (Figure 6). The larger fragment would be predicted to be stabilized by the Cys-46/Cys-99 disulfide bond. The fact that Edman degradation of species I indicated the correct N-terminal sequence, as well as the presence of sequences commencing at residues 27 and 104, is consistent with the disulfide assignments. Analysis of the predicted fragmentation pattern that would result from other types of disulfide assignments indicates that there is no other potential arrangement of disulfide bonds that can explain the generation of species I and II. However, direct evidence for the indicated disulfide pairing in these fragments has not yet been obtained.

Species I and II were essentially inactive in both a proliferation assay and a receptor binding assay. The fact that proteolysis at only two glutamoyl sites is sufficient to inactivate the biological activity of species I might indicate that the native conformation of rhuIL-4 is highly dependent upon the integrity of its polypeptide backbone. Consistent with this observation, it was noted that although antibodies to the C-terminal peptide 104–129 of rhuIL-4 neutralize its activity, the peptide itself did not efficiently block the binding of rhuIL-4 to cell-surface receptors when tested at a concentration as high as 1000-fold the K_d concentration (data not shown). The potential involvement of the C-terminal sequence in receptor binding constitutes a notable feature shared with several other cytokines. Immunochemical studies of human interferon γ and analyses of the biological activities of mutants have supported the potential existence of a receptor binding domain at or near the C-terminus (Seelig et al., 1988; Dobeli et al., 1988; Wetzel et al., 1990). Site-specific mutagenesis of human interleukin 2 (Ju et al., 1987; Zurawski & Zurawski, 1988) has demonstrated that residues 121–133 of the C-terminus are essential for receptor binding and biological activity. Similarly, a receptor binding region on human granulocyte–macrophage colony-stimulating factor has been definitively located at the C-terminal 15 amino acid residues (Kaushansky et al., 1989; Scheffler et al., 1991), consistent with previous studies on synthetic truncated variants (Clark-Lewis et al., 1988).

The studies described here demonstrate the utility of limited proteolysis for the elucidation of the structure–activity relationship of rhuIL-4. Identification of the fragments generated by *S. aureus* V8 protease has provided further confirmation for the pattern of disulfide bonds previously established by tryptic mapping and FAB-MS analysis. In addition, the limited proteolysis provided us with a facile technique for evaluating the biological properties of two large fragments of huIL-4 containing intact disulfide bonds. These results together with the analysis of antisera directed to the N- and C-terminal fragments establish the importance of the latter in receptor binding.

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