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Disulfide Assignments in Recombinant Mouse and Human Interleukin 4

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ABSTRACT: The disulfide pairings of mouse and human interleukin 4 (IL-4) proteins have been determined. The purified proteins, synthesized by recombinant DNA technology, are fully active as judged by their ability to stimulate an appropriate biological response in a variety of functional assays. Peptide maps were produced by digesting the proteins with pepsin and separating the resulting fragments by reverse-phase HPLC using linear acetonitrile–TFA gradients. Cystine-containing peptides were identified by determining which reverse-phase peaks showed an altered elution pattern after reduction. These peptides were purified further and defined by composition and sequence analysis. Three sets of disulfide-linked peptides were consistently identified for each protein. For mouse IL-4, the first and fifth, second and fourth, and third and sixth cysteines are joined. The disulfide bonds in human IL-4 are between the first and sixth, second and fourth, and third and fifth cysteines. A large double-loop region within the central three-fifths of each protein is stabilized by these bonds. Sequence analysis of the peptides containing the third and fifth cysteines of human IL-4 also demonstrated that only one of the potential N-glycosylation sites is used by C127 mammary tumor cells. Complete alkylation of mouse IL-4 under mild conditions completely destroyed its biological activity in a hematopoietic precursor cell proliferation assay.

Developmental maturation and functional activation of cells of the immune system are regulated by a group of extracellular growth and differentiation factors known collectively as interleukins and interferons. One of these interleukins, IL-4,[1] is a T and mast cell derived cytokine that stimulates a wide variety of biological effects in cells of hematopoietic origin. It was originally identified in studies of murine B cell dif-

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[1] Abbreviations: IL-4, interleukin 4; rmIL-4, recombinant mouse IL-4; rhIL-4, recombinant human IL-4; DTT, dithiothreitol; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin.

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ferentiation. Early characterization revealed its ability to cause B cells, costimulated with anti-IgM antibodies, to proliferate (Howard et al., 1982) and to induce an increased expression of Ia antigen on resting B cells (Noelle et al., 1984; Roehm et al., 1984). Mouse IL-4 also promotes the growth of mast cells (Mosmann et al., 1986) and T cells (Fernandez-Botran et al., 1986; Hu-Li et al., 1987), stimulates the expression of the IgG1 and IgE isotypes by lipopolysaccharide-activated B cells (Coffman et al., 1986), and induces expression of the low-affinity receptor for IgE on B cells (Hudak et al., 1987). Recently, cDNA clones have been isolated from both murine (Lee et al., 1986; Noma et al., 1986) and human (Yokota et al., 1986) libraries that code for proteins that demonstrate these same properties. The amino acid sequences derived from the DNA sequence predict a mature, secreted, 120-residue mouse protein and 129-residue human protein. Although the murine polypeptide contains three potential sites for N-glycosylation and the human protein contains two potential sites, carbohydrate is not required for biological activity (Ohara et al., 1987; Park et al., 1987a,b; Le et al., 1988).

Most studies of IL-4 have addressed functional questions. However, definition of the structural characteristics of IL-4 is essential toward understanding the mechanism by which it exerts its pleiotropic actions. These types of experiments have been hampered by the limited supply of the mouse protein and the total absence of the human molecule from natural sources. The recent availability of large quantities of protein from recombinant sources makes an analysis of the conformational features more feasible. Although mouse and human IL-4 do not display cross-species receptor binding (Park et al., 1987a,b) or functional activity (Tokata et al., 1986; Grabstein et al., 1986), their folding patterns are predicted to bear a close resemblance given the similarity in their primary structures. Disulfide bonds are one feature of polypeptides that influence their stability and functionality. All of the cysteine residues of mouse IL-4 are involved in cystine bridges and these bonds are required for the biological efficacy of the protein (Ohara et al., 1987). Although both of the mature recombinant IL-4 molecules contain six cysteines, comparison of their primary structures shows that only five of the cysteine residues are conserved (Yokota et al., 1986). We were interested in determining whether this affected the pairing relationships between cysteines in these homologous proteins.

In this study, the disulfide bonding patterns of mouse and human IL-4 were elucidated by generating small peptic peptides. Fragments containing intact cystine were purified by reverse-phase HPLC and were characterized by composition and amino acid sequence analysis. Three sets of disulfide-linked peptides were found for each protein. In the course of obtaining such data, information was also gathered on the site on human IL-4 that is glycosylated by C127 mouse mammary tumor cells. This is the first direct confirmation that the putative sites for N-glycosylation are used.

Experimental Procedures

Isolation of Recombinant Interleukin 4. The rmIL-4 used in this report was synthesized in *Escherichia coli* as a 13.9-kDa protein, as revealed by silver staining and Western blot analysis. The expression vector (Olins & Rangwala, 1990) contains the amino acid sequence MTRS at the amino terminus of the mature mIL-4 coding sequence (Lee et al., 1986; Noma et al., 1986). The rhIL-4 was a 17.5-kDa glycoprotein secreted by C127 murine mammary tumor cells transfected with the pMON1123 expression vector (Ramabhadran et al., 1984). Their purification will be described elsewhere. Briefly, to purify rmIL-4 protein, *E. coli* cells expressing this protein were resuspended in 0.05 M Tris-HCl, pH 7.5, and homogenized by one pass through a Manton Gaulin homogenizer set at 8000 psi. The inclusion body pellet was washed two times in buffer. It was then dissolved and reduced in 4 M guanidine, 0.5 M NaCl, 0.05 M DTT, and 0.05 M CHES, pH 9.5, for 4–6 h. The preparation was dialyzed versus 20 volumes of 4 M guanidine, 0.5 M NaCl, 0.02 M cysteine, and 0.05 M CHES, pH 9.5. The dialysis buffer was changed to 4 M guanidine, 0.5 M NaCl, and 0.05 M CHES, pH 9.5, after 12–15 h. The latter buffer was replaced with fresh buffer after 8–12 h and refolding was complete within 24 h after changing to dialysis in this final buffer. The correctly folded structure, identified by biological activity, was purified by sequential chromatography on S Sepharose Fast Flow (Pharmacia, Piscataway, NJ) and Toyopearl HW-55 (Toyo Soda, Tokyo, Japan) columns. The S Sepharose column was equilibrated in 0.05 M glacial acetic acid, pH 5.0. After the refolded sample was loaded, this column was washed with 0.2 M glacial acetic acid, pH 5.0, until the absorbance at 280 nm returned to baseline, and it was eluted with a linear gradient from 0–1 M NaCl in 0.2 M glacial acetic acid, pH 5.0. The rmIL-4 from this column was loaded onto a Toyopearle HW-55 column equilibrated in 0.05 M Na_2HPO_4, 0.15 M NaCl, and 0.01% Tween 80, pH 7.5. The activity of rmIL-4 was determined by its ability to stimulate a murine factor-dependent hematopoietic precursor cell line, FDC-P1, to proliferate. Its specific activity was 2.5×10^7 units/mg. To purify rhIL-4 produced in cell factories in serum-free media, the conditioned media was concentrated 20-fold in an Amicon ultrafiltration cell with a YM-10 membrane. It was then desalted on a Sephadex G-25 column (Pharmacia) equilibrated in 50 mM sodium phosphate, pH 7.5. The sample was next placed on a 1-mL Mono S column (Pharmacia), equilibrated in the same buffer. rhIL-4 was eluted with a linear 0–2 M NaCl gradient in 70 min. Final purification was achieved by passage through a Brownlee C_4 reverse-phase column (Aquapore BU 300, 250 $\times$ 4.6 mm). The activity of purified rhIL-4 was measured by its ability to stimulate human peripheral blood T cells and human tonsillar B cells to proliferate. The specific activity found by using these two sources of cells was 2×10^6 and 1.5×10^7 units/mg, respectively. The concentration of the purified proteins was determined by amino acid analysis.

Isolation of Natural IL-4. Natural murine IL-4 was obtained from PMA-induced EL-4 thymoma cells and was purified as described by Ohara et al. (1987).

Alkylation of rmIL-4. rmIL-4 (160 μg) was dried by vacuum centrifugation and dissolved in 0.5 M Tris, pH 8.5, and 6 M guanidine hydrochloride (Sigma, St. Louis, MO) at 1 mg/mL. It was incubated with 10 mM dithiothreitol (DTT) for 2 h at 37 °C and then reacted for 2 h on ice in the dark with 20 mM iodoacetamide. To remove excess reagents, the sample was next placed on a 0.21 $\times$ 22 cm C_{18} reverse-phase HPLC column equilibrated in 0.1% TFA–5% acetonitrile–95% water. The alkylating chemicals did not bind to the column under these conditions, whereas rmIL-4 did bind and could be eluted by using a linear gradient from 5% to 60% acetonitrile in 55 min.

Fragmentation of Recombinant IL-4 Proteins. rmIL-4 (100 μg) and rhIL-4 (50 μg) were separately taken to dryness in a Speed Vac concentrator (Savant, Hicksville, NY) and then dissolved in 0.1 M NH_4OAc, pH 2.45, at 0.5 mg/mL. Pepsin (Boehringer Mannheim, Indianapolis, IN) was dissolved in this buffer at 1 mg/mL and added at an enzyme to substrate ratio of 1:40 (w/w). The samples were incubated at 37 °C for 2.5 h, and then another 1:40 concentration of pepsin was

added. The reaction was incubated overnight at 37 °C. The digests were frozen at −80 °C until purification by reverse-phase HPLC.

Isolation of Disulfide-Bonded Peptides. A Spectra-Physics (San Jose, CA) system consisting of a SP8800 ternary HPLC pump, a SP8500 dynamic mixer, a Spectra Chrom 100 variable-wavelength detector, and a Chrom Jet integrator was used to purify the peptides. The columns used were a Vydac (Hesperia, CA) C_{18} (0.46 × 25 cm) and a Brownlee (Applied Biosystems, Santa Clara, CA) C_{18} Aquapore OD-300 (0.21 × 22 cm). The Vydac column was used at a flow rate of 1 mL/min and the Brownlee column at 0.5 mL/min. Digests were eluted with linear water–acetonitrile (Burdick and Jackson, Muskegon, MI) gradients containing trifluoroacetic acid (TFA) (Pierce). Solvent A was 0.1% TFA in water and solvent B was 0.09% TFA in acetonitrile. Peaks containing peptides joined by disulfide bonds were collected, dried completely to remove the TFA, dissolved in 300 μL of 10 mM NH_4OAc, pH 6.5, and repurified on the appropriate reverse-phase column equilibrated in 5% acetonitrile with 10 mM NH_4OAc, pH 6.5. The peaks were separated by using a linear acetonitrile gradient containing 10 mM NH_4OAc, pH 6.5. Solvent A was 10 mM NH_4OAc, pH 6.5, and solvent B was 50% acetonitrile containing 10 mM NH_4OAc, pH 6.5.

Amino Acid Analysis and Microsequencing. The amino acid composition of purified rIL-4 proteins and acid hydrolysates of peptic peptides was determined by using a Beckman Model 6300 auto-analyzer. Samples were hydrolyzed in 6 N HCl for 24 h at 115 °C. The analyses were performed after postcolumn derivatization of the hydrolysates using ninhydrin (Moore & Stein, 1963).

Edman degradation of peptides was performed on an Applied Biosystems (ABI, Foster City, CA) Model 470A protein sequencer. The standard degradation run (03RPTH) was used (Hunkapiller et al., 1983). Phenylthiohydantoin (PTH)–amino acids were analyzed with the Applied Biosystems Model 120A PTH analyzer utilizing a Brownlee 2.1-mm-i.d. PTH–C_{18} column.

Hematopoietic Precursor Cell Proliferation Assay. Log phase cultures of FDC-P1 cells were maintained at 37 °C in humidified 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Grand Island, NY), 10% fetal calf serum (Gibco), 2 mM glutamine, 1 mM sodium pyruvate, and 10% Wehi 3 conditioned media as a source of IL-3. Cells were washed three times in the above media lacking the Wehi 3 supplement and seeded at 10000 cells per 100 μL in a 96-well flat-bottomed microtiter plate (Costar, Cambridge, MA) to determine their proliferative response to rmIL-4. rmIL-4 was diluted into medium and added to the cells. The cells were incubated for 16 h, pulsed with 1 μCi of ^{3}H-labeled thymidine (New England Nuclear, Boston, MA), harvested after 4 h onto glass fiber filter paper, and counted in a Micromedics Taurus liquid scintillation counter. One unit is defined as the amount of IL-4 needed to stimulate a half-maximal response in a 100-μL culture.

Results

Alkylation of rmIL-4 Destroys Biological Activity. Modification of natural mIL-4 by iodoacetamide following treatment with reducing agents was shown to completely destroy its biological activity (Ohara et al., 1987). Although this result strongly suggested that the cystine bonds, broken by such treatment, were responsible for maintaining the activity of the protein, the formal possibility exists that other important residues may have been altered as well since a relatively high concentration of iodoacetamide (88 mM) was used. We chose

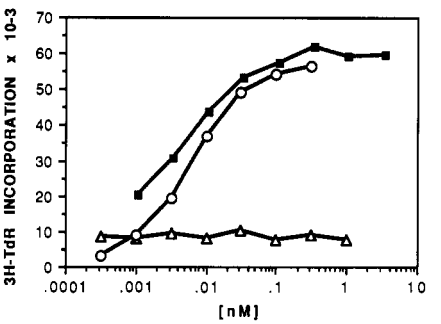

FIGURE 1: Effect of rmIL-4 on proliferation of FDC-P1 cells. Samples (500 pmol) were dried by vacuum centrifugation, dissolved in 100 μL of water, diluted into medium, and added to FDC-P1 cells for an overnight incubation. Cells were pulsed with [^{3}H]Tdr, harvested, and counted. (■) rmIL-4; (△) reduced and alkylated rmIL-4; (○) natural EL-4-derived mIL-4.

to reexamine this question with *E. coli* generated rmIL-4 under somewhat milder conditions that have been shown to modify cysteine alone (Carr et al., 1987). When alkylated rmIL-4, prepared as described under Experimental Procedures, was examined on a Vydac C_{18} reverse-phase column by using a gradient from 5% to 50% acetonitrile–TFA in 90 min, it eluted 14 min later than an untreated sample (data not shown). Alkylation is expected to increase the retention time of the protein by unfolding it and exposing more of the hydrophobic residues to the column packing. Amino acid analysis of alkylated rmIL-4 revealed all of the cysteine to be present as carboxymethylcysteine, while methionine and histidine, the two other amino acids most likely to be modified by such an alkylating agent (Gurd, 1967), were quantitatively recovered after hydrolysis (106% and 90%, respectively, of the theoretical yield). The ability of the two preparations of rmIL-4 as well as native mIL-4 to stimulate FDC-P1 cells to proliferate was assessed (Figure 1). The data confirm the lack of activity in the alkylated sample compared to the activity of untreated rmIL-4 (<0.1%) and thus lend further support to the critical importance of the cystine pairs in stabilizing the tertiary structure.

Assignment of Disulfides in Mouse IL-4. Trypsin has frequently been employed to digest proteins to assess their disulfides. However, examination of the rmIL-4 sequence indicated that complete tryptic digestion would produce a peptide containing two cysteines (Cys 87 and Cys 94 in a fragment consisting of residues 87–100, see Figure 7). Should these residues not form an intrachain link, unambiguous interpretation of the sequencing data would not be possible. Thus an enzyme had to be chosen that could produce six distinct cysteine-bearing peptides. Pepsin was used because it had the potential to accomplish this. A sample of a peptic digest of rmIL-4 was separated into its constituent peptides by reverse-phase HPLC (Figure 2A). To identify those peaks that contained cystine-linked peptides, another sample of this digest was treated with 10 mM DTT for 45 min at 37 °C and then fractionated by using identical gradient conditions (Figure 2B). Inspection of this latter separation indicated that peaks 1–4 were much reduced in size. In addition, a number of new peaks (I–V) were seen, eluting earlier in the gradient. Since treatment with reducing agent breaks disulfide bonds, this result indicated that peaks 1–4 probably contained cystine-linked peptides. The correspondence between the DTT-generated peaks and these peaks was not investigated. Instead, the remainder of the digest was resolved into peaks on the reverse-phase HPLC column by using the gradient conditions of Figure 2A, and peaks 1–4 were examined by composition and sequence analysis.

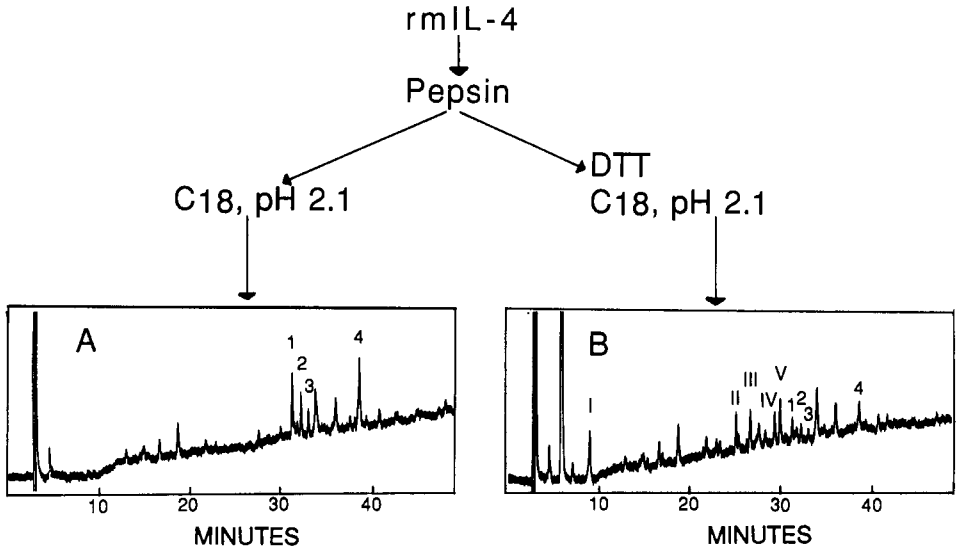

FIGURE 2: Comparison of an untreated versus a DTT-treated sample of a peptic digest of rmIL-4 separated by reverse-phase chromatography at pH 2.1. Purified rmIL-4 (7.2 nmol) was digested as described under Experimental Procedures. (A) A sample of this digest (380 pmol) was resolved on a Vydac C_{18} column. Flow rate was 1 mL/min and chart speed was 0.25 cm/min. Absorbance at 214 nm (—) was monitored at 0.005 absorbance unit full scale with an attenuation setting of 128. Solvent A was 0.1% TFA in water and solvent B was 0.09% TFA in acetonitrile. The sample was eluted with the following linear gradient: 0–3 min, 5% B; 3–73 min, 5–40% B. (B) A second 380-pmol sample was reduced with 10 mM DTT and then separated on the Vydac C_{18} column by using the same gradient conditions as in A.

Table I: Amino Acid Composition of Peptic rmIL-4 Peptides with Intact Cystine[a]

amino acid	peak 2a (pmol) (ratio)[b]	peak 3b (pmol) (ratio)[c]	peak 4b (pmol) (ratio)[d]
Asp/Asn	158.3 (2.94)	114.0 (1.16)	124.1 (1.85)
Thr	54.4 (1.01)	104.6 (1.07)	229.5 (3.42)
Ser	169.0 (3.14)	202.4 (2.06)	119.6 (1.78)
Glu/Gln	69.5 (1.29)	126.5 (1.29)	187.4 (2.79)
Pro			134.4 (2.00)
Gly	79.1 (1.47)	78.9 (0.80)	176.6 (2.63)
Ala	11.0 (0.20)	92.5 (0.94)	
Cys[e]		106.2 (1.08)	88.2 (1.31)
Val		152.9 (1.56)	82.2 (1.22)
Met		48.2 (0.49)	37.4 (0.56)
Ile	50.9 (0.95)	87.9 (0.90)	
Leu	114.5 (2.13)	93.3 (0.95)	178.6 (2.66)
Tyr			72.3 (1.08)
Phe	79.1 (1.47)		10.9 (0.16)
His	121.6 (2.26)	42.2 (0.43)	66.7 (0.99)
Lys	76.2 (1.42)	113.6 (1.16)	230.4 (3.43)
Arg	161.4 (3.00)	98.2 (1.00)	

[a] Compositional analysis determined by postcolumn derivatization with ninhydrin after a 24-h hydrolysis in 6 N HCL; 10% of each peak was analyzed. [b] Based on three arginines in the sample. [c] Based on one arginine in the sample. [d] Based on two prolines in the sample. [e] Detected as cystine.

Preliminary attempts to determine the sequences of the peptides in these peaks demonstrated the presence of two major sequences and one or two minor sequences. Because the contaminating fragments made it difficult to quantitate yields, the digest was repeated and refractionated and peaks 2–4 were purified further on a reverse-phase column equilibrated at pH 6.5 instead of pH 2.1 (Figure 3). Each sample was separated into three or more components by using this second chromatographic step. Such results illustrate the danger of presuming that sharp, well-defined HPLC peaks are comprised of a single component. A sample of each major peak from the pH 6.5 separations was analyzed for composition to identify the peptides of interest. The compositions of two cystine containing peaks (3b, 4b) are shown in Table I. Peak 2a is also included in this table, although no cystine was detected, because of the high histidine content. Analysis of the sequence of rmIL-4 in Figure 7 indicates that three of the four histidines

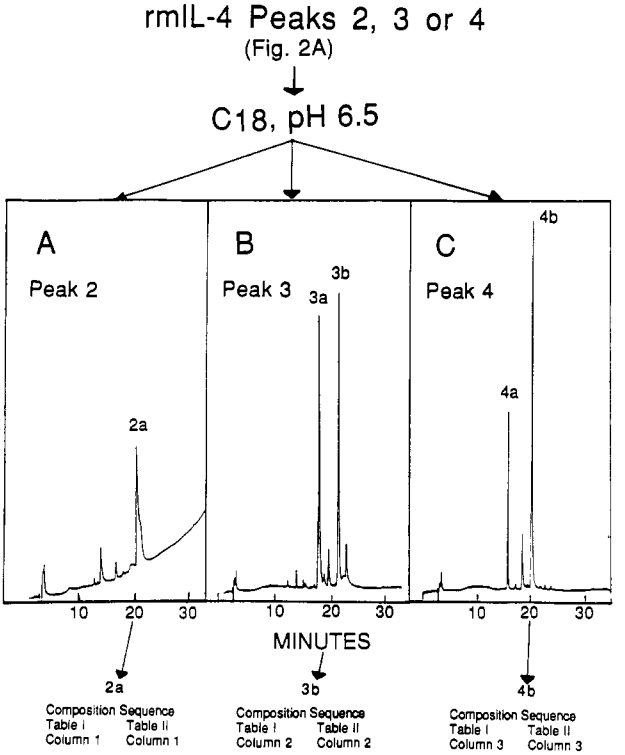

FIGURE 3: Rechromatography of rmIL-4 peaks 2–4 on a Vydac C_{18} column equilibrated at pH 6.5. Purified rmIL-4, 7.2 nmol, was digested with pepsin as described under Experimental Procedures and separated on a C_{18} column as in Figure 2A. Peaks 2–4 were separately collected, dried completely, dissolved in 10 mM NH_4OAc, pH 6.5, and applied to the column equilibrated at pH 6.5. The absorbance unit full-scale setting was 0.1 and the attenuation setting was 16. The samples were eluted with a linear gradient between solvent A (10 mM NH_4OAc, pH 6.5) and solvent B (50% acetonitrile containing 10 mM NH_4OAc, pH 6.5) as follows: 0–45 min, 10–100% B. A sample of each peak was analyzed for composition and peaks 2a, 3b, and 4b were used for Edman degradation: (A) peak 2; (B) peak 3; (C) peak 4.

are located in the first nine residues of the mature protein. Peptic cleavage of rmIL-4 would be expected to generate a fragment containing these histidines as well as the first cysteine (Cys 5). Our inability to detect cystine in the sample may

Table II: Amino Acid Sequences of Isolated Peptic rmIL-4 Peptides[a]

cycle	peak 2a residue(yield)[b]	peak 3b residue(yield)[b]	peak 4b residue(yield)[b]
1	M(393.1); F(369.1)[c]	I(1337.9); L(1370.6)	N(395.5) Y(410.2)
2	T(230.2); R(454)	S(353.3); V(1122.2)	E(242.6); L(318.6)
3	R(430); Y(21.7)[d]	Y(720.2)[d]	V(79.2); K(85.8)
4	S(99.8); L(152.1)	T(286.4); R(91.4)	T(58.9); H(58.3)
5	H(116.1); D(135.3)	M(1017); A(977)	G(66.0)
6	I(98.5); S(45.4)	N(555.3); S(252)	E(20.6); K(26.6)
7	H(33.6); S(45.4)	E(127.5); K(342.3)	G(11.0); T(20.8)
8	G(75.5)	V(95.2)	T(11.0); P(13.1)
9	Y(4.8)[d]		P(7.4); *d*
10	D(52.9)		*d*; L(6.1)
11	K(34.4)		T(4.6); K(3.0)
12	N(31.8)		E(3.5); K(+3.0)
13	H(23.8)		M(1.1); N(3.1)
14	L(22.0)		S[e]
15	R(27.5)		

[a] The conventional one-letter code for amino acids is used. [b] The yield is expressed in picomoles. [c] Cycle 1 also contained D (191.2). [d] Cysteine is predicted here, see text. [e] Indicates residue present but not quantified.

be explained by the instability of cystine to acid hydrolysis (Martin & Synge, 1945). A more definitive identification of cystine could have been achieved had the sample been oxidized by performic acid to cysteic acid, which is stable toward acid hydrolysis (Schram et al., 1954). An assessment of the peptide constituent(s) of peaks 3a and 4a (Figure 3B,C) was difficult because, like peak 2a, no cystine was detected (data not shown). Nevertheless, their proline, tyrosine, and histidine content resembled that of peak 4b and suggested that these peaks were also composed of two cystine-containing peptides. These peptides may differ only in the site of peptic cleavage since pepsin could cleave at numerous locations near to the cysteines (refer to Figure 7).

Edman degradation was performed on the remaining material from the three samples analyzed in Table I. The results are shown in Table II. Only two amino acids were observed at most sequencer cycles. Almost all of the amino acids in these peptides were unambiguously identified. Cycles in which only one residue was detected were indicative of the presence of cysteine in one of the fragments since, without alkylation, cysteine is destroyed during derivatization with phenyl isothiocyanate. An exception to this interpretation of the data occurred in the fifth Edman cycle of peak 4b where only glycine was seen. Since each peptide should have glycine at this position, the glycine content of cycle 5 was attributed to both peptides. Another unusual result was obtained in cycle 3 of peak 3b. Tyrosine alone was detected. Because tyrosine has been discovered at positions of cysteine residues (Kellaris & Ware, 1989) and because cysteine was expected at this location in each of the fragments, cysteine was assigned to both. Additional confirmation for this assignment came from the compositional results (Table I), which showed the presence of cystine but not tyrosine. Tyrosine was also observed in the third and ninth Edman cycles of peak 2a where cysteine was anticipated. The two peptides present in peak 2a were determined to be MTRSHIHG(C)DKNHLR (residues −4 to 11) and FR(C)LDSS (residues 85–91). The fragments in peak 3b were identified as IS(C)TMNE (residues 92–98) and LV(C)RASKV (residues 47–54), while the peptides NEVTGEGTP(C)TEM (residues 18–30) and YLKHGKTP-(C)LKKNS (residues 59–72) were found in peak 4b. These findings agree quite well with the compositional data in Table I. The data demonstrate disulfide bridges between Cys 5 and Cys 87 (peak 2a), Cys 27 and Cys 67 (peak 4b), and Cys 49

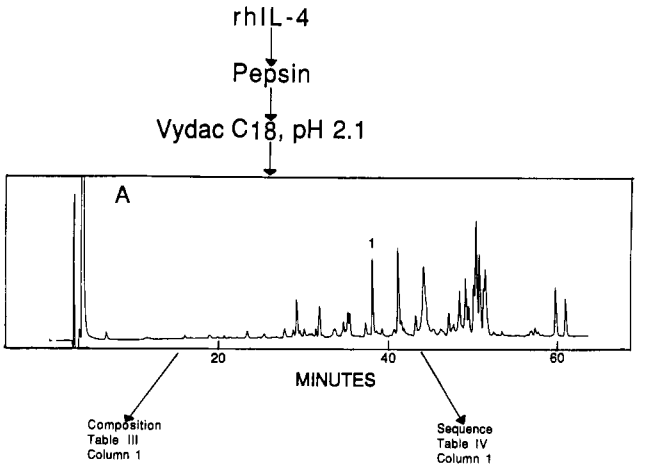

FIGURE 4: pH 2.1 reverse-phase separation of peptides from a peptic digest of rhIL-4. RhIL-4 (2.8 nmol) was digested as described under Experimental Procedures. (A) The peptides were resolved on a Vydac C_{18} column using the following linear gradient: 0–3 min, 0% B; 3–73 min, 0–35% B. Solvents A and B were the same as in Figure 2. Chart speed was 0.5 cm/min and the attenuation setting was 32. Peak 1 was analyzed for composition and then sequenced without additional purification.

Table III: Amino Acid Composition of Peptic rhIL-4 with Intact Cystine[a]

amino acid	peak 1[b] (pmol) (ratio)[d]	peak 3b[b] (pmol) (ratio)[d]	peak 4c[c] (pmol) (ratio)[e]
Asp/Asn	58.4 (1.02)	80.0 (2.92)	81.3 (2.61)
Thr	59.8 (1.05)	103.4 (3.77)	87.2 (2.80)
Ser	159.2 (2.78)	69.2 (2.53)	132.6 (4.26)
Glu/Gln	161.7 (2.83)	112.4 (4.10)	177.2 (5.69)
Pro		43.8 (1.60)	
Gly	17.6 (0.31)	839.2	211.4 (6.79)
Ala		128.7 (4.70)	65.4 (2.10)
Cys[f]	147.8 (2.58)	9.5 (0.35)	
Val		6.9 (0.25)	
Met			
Ile	59.9 (1.05)		26.0 (0.83)
Leu	53.6 (0.94)	27.8 (1.01)	102.0 (3.27)
Tyr	30.8 (0.54)		53.4 (1.71)
Phe			52.9 (1.70)
His	56.4 (0.99)	14.5 (0.53)	57.1 (1.83)
Lys	158.7 (2.77)	79.9 (2.92)	67.0 (2.15)
Arg	57.2 (1.00)	27.4 (1.00)	62.3 (2.00)

[a] Compositional analysis determined by post column derivatization with ninhydrin after a 24-h hydrolysis in 6 HCl. [b] 10% of sample was analyzed. [c] 20% of sample was analyzed. [d] Based on one arginine in the sample. [e] Based on two arginines in the sample. [f] Detected as cystine.

and Cys 94 (peak 3b). The link between Cys 5 and Cys 87 was also observed when peak 1 (Figure 2A) was repurified at pH 6.5 and analyzed for sequence (data not shown). Therefore, the first and fifth, third and sixth, and second and fourth cysteines participate in disulfide bonds in this preparation of rmIL-4 (Figure 7).

Assignment of Disulfides in Human IL-4. The cystine bonds in rhIL-4 were determined by using a comparable scheme. Pepsin was used to digest the protein since it had worked well with the mouse molecule. The peptic digest was fractionated into peptides on a Vydac C_{18} reverse-phase column (Figure 4A). Peaks containing cystine were identified by amino acid analysis. Peak 1 in Figure 4A was sequenced without further purification. It consisted of two sequences present in equimolar concentration (Table IV: HK(C)DI-TLQE, residues 1–9, and REKYSK(C)SS, residues 121–129). A cysteine residue was assumed to have been present in the third and seventh Edman cycles where only a single amino acid

Table IV: Amino Acid Sequences of Isolated Peptic rhIL-4 Peptides[a]

amino acid	peak 1 residue(yield)[b]	peak 3b residue(yield)[b]	peak 4c residue(yield)[b]
1	H(76.0); R(63.9)[c,d]	A(456.2); N(295.9)[e]	T(27.5); R(28.5)[f]
2	K(389.6); E(548.3)	A(456); S(138.6)	E(12.9); Q(36.4)
3	*g*; K(493)	S(138); *g*	Q(29.3); F(45.3)
4	D(257.7); Y(402.4)	K(216.2); P(186.7)	K(7.0); Y(31.8)
5	I(319.9); S(128)	*h*; V(175.9)	T(6.5); S(15.)
6	T(135); K(368.6)	T(92.3); K(171.4)	L(4.2); H(13.0)
7	L(246.1); *g*	T(92); E(123.1)	*g*; H(17.8)
8	Q(175.2); S(63.3)	E(148.1); A(209.4)	T(1.1); E(8.7)
9	E(43.9); S(+63.3)	K(125.8); N(130.5)	E(8.7); K(3.7)
10		E(63.7); Q(53.7)	D(3.7)
11		T(47.5)	T(2.3)
12		F(87.5)	R(4.8)
13		*g*	*g*
14		R(28.5)	L(2.2)
15		A(38.7)	G(2.8)
16			
17			
18			

[a] The conventional one-letter code for amino acids is used. [b] The yield is expressed in picomoles. [c] Cycle 1 also contained these amino acids: D(19.6); A(11.5); I(18.8); K(7.3). [d] Linear regression analysis of the data indicated an initial yield of 471.9 (H) and 645.2 (R). [e] Cycle 1 also contained D(456.2) which was likely to be a buffer contaminant. [f] Cycle 1 also contained D(151); G(292); L(16.4). [g] Cysteine is predicted here. [h] Asparagine is predicted here; see text.

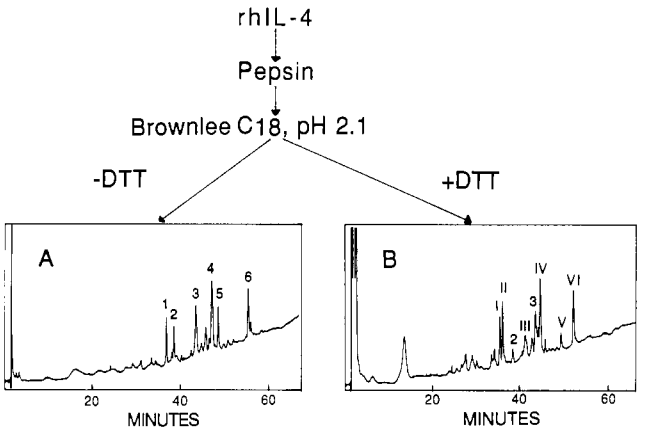

FIGURE 5: Comparison of an untreated versus a DTT-treated sample of a peptic digest of rhIL-4 separated by reverse-phase chromatography at pH 2.1. A second digest of 2.8 nmol of rhIL-4 was performed as described under Experimental Procedures. Samples were resolved on a Brownlee C_{18} column at a flow rate of 0.5 mL/min. Solvents A and B and gradient conditions were identical with those of Figure 4A. The attenuation setting was 4. (A) A 95-pmol sample of the digest was applied to the column. (B) Another 140-pmol sample was treated with 10 mM DTT and then separated on the Brownlee C_{18} column.

was detected. The compositional data (Table III) concur with the data obtained from sequencing. These results revealed that the first cysteine (Cys 3) is bonded to the sixth (Cys 127).

Sequence analysis of two other peaks containing cystine showed that they were composed of disulfide-linked peptides in low yield and several minor contaminating sequences. To improve the yield of these peptides, a smaller Brownlee C_{18} reverse-phase HPLC column (2.1 × 220 mm versus 4.6 × 250 mm) was initially used to fractionate a portion of a second peptic digest of rhIL-4 (Figure 5A). Although the overall

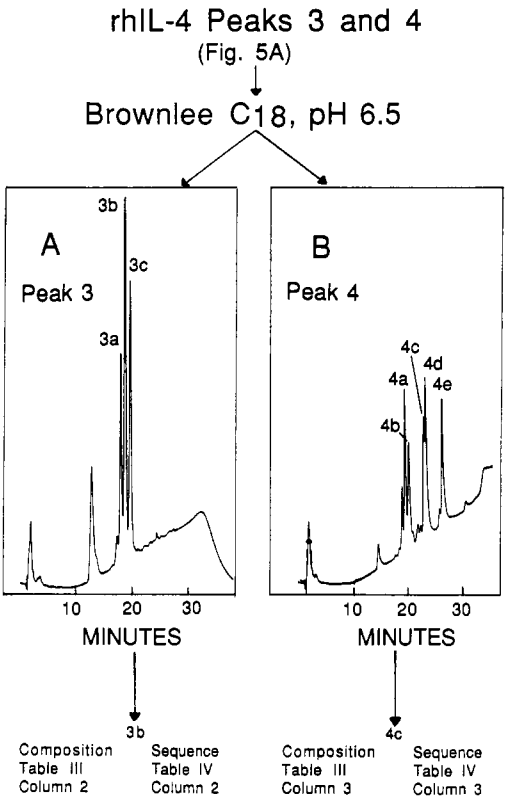

FIGURE 6: Rechromatography of rhIL-4 peaks 3 and 4 on a Brownlee C_{18} column equilibrated at pH 6.5. The remainder of the second peptic digest of rhIL-4 was separated on the Brownlee column as described in Figure 5. Peaks 1–6 were individually collected, dried, resuspended in 10 mM NH_4OAc, pH 6.5, and applied to the same column equilibrated at pH 6.5. The attenuation setting was 8 for peak 3 and 32 for peak 4 and the flow rate was 0.5 mL/min. Solvents A and B and gradient conditions were the same as in Figure 3. (A) peak 3; (B) peak 4. A sample of each peak was analyzed for composition and peaks 3b and 4c were analyzed for sequence.

pattern of peaks separated on this column resembled that of the first digest of rhIL-4 (Figure 4A), the differences were such that it was necessary to determine the cystine-bonded peaks again. Another sample of the digest was eluted from the small column after treatment with DTT as had been accomplished with rmIL-4 (Figure 2B) and the chromatograms were compared (Figure 5, part A versus part B). Peaks 1–6 either disappeared or were diminished in size as a consequence of their exposure to the reducing agent, and several new peaks (I–VI) were observed. No attempt was made to define the relationship between peaks 1–6 and peaks I–VI. The remainder of the digest was separated on the Brownlee column, and all six peaks were next repurified on the same reverse-phase column equilibrated at pH 6.5. As occurred with the mouse samples, each peak was separated into multiple components. The elution profiles of peaks 3 and 4 are presented in Figure 6. In data not shown, peak 5 eluted in this buffer system as one major peak (5a) preceded by two or three minor peaks, while two peaks of equal size (6a and 6b) were generated from peak 6 at this pH. The recovery of peaks 1 and 2 was poor from this second elution scheme and so these samples were not analyzed further. All of the peaks from the pH 6.5 separation of peaks 3–6 were then analyzed for composition. The results suggested that peaks 3a–c and 4a were comprised of one class of disulfide-linked peptides and that peaks 4b–d and 5a were made up of a different set of cystine-linked peptides. The composition of peak 4e was consistent with a single peptide (residues 83–87). Peaks 6a and 6b were also likely to be single peptides that comigrated with

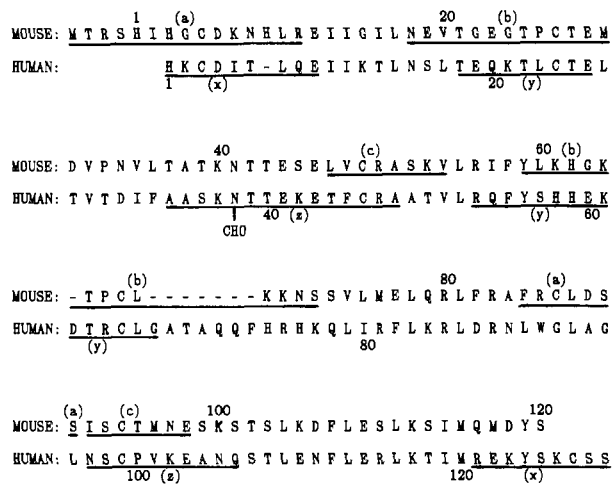

FIGURE 7: Alignment of rmIL-4 and rhIL-4 sequences. Sequences deduced from cDNA clones are aligned to maximize homology by using the Genetics Computer Group sequence analysis software package (Devereux et al., 1984). Peptic peptides identified by sequencing are underlined and disulfide-bonded pairs are denoted by the same letter, a, b, or c for mIL-4 and x, y, or z for hIL-4. The glycosylation site for hIL-4 is indicated (CHO).

the DTT-sensitive peptides at pH 2.1. Examination of the predicted hIL-4 sequence (Figure 7) indicates that there are numerous peptic cleavage sites near each cysteine residue. The multiple peaks associated with a given pair of disulfide-bonded cysteines are likely to represent the differential fragmentation by pepsin around the residues of interest. The compositional analysis for peak 3b (Figure 6A), representing one distinct class of cystine-bonded peptides, is presented in Table III. Peak 4c is included in the table for much the same reason that rmIL-4 peak 2a was included in Table I. For this sample, the tyrosine, phenylalanine, and histidine contents suggested the presence of cystine. Analysis of the rhIL-4 sequence (Figure 7) indicates a potential peptic fragment containing cysteine as well as these other amino acids between residues 55 and 66. The lack of detectable cystine can again by attributed to its instability during acid hydrolysis (Martin & Synge, 1945). The sequencing data for peaks 3b and 4c are in Table IV. The information in Tables III and IV agrees well. Only two amino acids were observed in most of the Edman cycles. Except for the fifth cycle of peak 3b, the detection of a single amino acid was indicative of a cysteine residue in one of the peptides. The sequencing data established that Cys 24 is connected to Cys 65 since the two peptides in peak 4c were identified as TEQKTL(C)TE (residues 18–26) and RQFYSHHEKDTR-(C)LG (residues 53–67). Furthermore, Cys 46 couples to Cys 99 because the fragments AASKXTTEKETF(C)RA (residues 34–48) and NS(C)PVKEANQ (residues 97–106) were found in peak 3b. The "X" in the peptide starting at alanine 34 indicates that the expected asparagine was not identified in that sequencer cycle. For rhIL-4, therefore, cystine bridges exist between the first and sixth, second and fourth, and third and fifth cysteines (Figure 7).

An asparagine was anticipated in the fifth cycle of peak 3b as predicted by the cDNA sequence for hIL-4 (Yokota et al., 1986). Since this particular asparagine is one of the two potential glycosylation sites in hIL-4, an inability to detect it demonstrates that this site is glycosylated because N-linked residues are not detected by Edman chemistry. Furthermore, the compositional data (Table III) indicate that there are three Asp/Asn residues rather than the two Asn residues that were directly determined by sequencing the sample. The data from peak 3b are instructive not only as regards the glycosylation of Asn 38 but also with regard to the potential glycosylation of Asn 105. Unlike the former residue, the latter asparagine was observed in the ninth Edman cycle of peak 3b. Therefore it is not glycosylated by C127 mammary tumor cells. Only one of the putative glycosylation sites is modified by carbohydrate.

Discussion

In this paper, we present the first description of the three disulfide bridges in human and mouse IL-4. Such a study could not readily have been accomplished before the advent of recombinant DNA technology because of the paucity of naturally occurring IL-4. Misfolding of recombinant proteins during purification is a potential problem associated with the characterization of the cystine linkages in recombinant proteins. While incorrect folding was unlikely for rhIL-4, since that protein was synthesized by mammalian cells where misfolding is not often observed, it was a concern with the rmIL-4 produced in *E. coli*. However, because the specific activity of our preparation of rmIL-4 was similar to that of mIL-4 isolated from natural sources (Figure 1; Grabstein et al., 1986; Ohara et al., 1987), the procedures used to refold the molecule are likely to have resulted in the correct formation of the native disulfide bonds. We have also demonstrated that the formation of the disulfide bridges in mouse IL-4 is critical for its biological activity (Figure 1). Although the specific activity of natural hIL-4 is not known, the activity of our preparations is comparable to that of rhIL-4 purified from other hosts (Park et al., 1987b; Le et al., 1988; Scott et al., 1990). It too is a reliable source of protein.

One concern in identifying disulfide bonds is that they may be reshuffled during handling of the protein with the unfortunate consequence that incorrect cystine assignments may be made. Since the potential for disulfide shuffling is reduced in acidic conditions (Spackmann et al., 1960), pepsin, which works optimally at low pH, was chosen for this study. The same three cystine bonds were consistently characterized in two sequencing experiments of rmIL-4. These disulfide bonds were identified in a third experiment by composition when the peptides were separately isolated after DTT treatment of the material in peaks 1–4 of Figure 2A (data not shown). No alternate pairing of cysteines was observed in any of these digests. Therefore it seems unlikely that disulfide interchange had occurred.

In a similar manner, only three sets of cystine linkage were identified in peptic digests of rhIL-4. Two of the bonds were repeatedly identified in digests separated on C_{18} reverse-phase columns and on a microbore C_8 column (data not shown). Although a peak comprised of peptides joined through the first and sixth cysteines was isolated in only one digest (peak 1 in Figure 4A and Table IV), this was not unexpected since different reverse-phase columns were employed to elute the rhIL-4 peptides. It has been shown that the column used for peptide separations greatly influences their recovery (Banes et al., 1985). Such a disulfide bond, joining the amino and carboxyl termini of the polypeptide, is a favorable means of stabilizing folded proteins (Thornton, 1981).

Alignment of the cysteines within the primary structure of rmIL-4 and rhIL-4 reveals that the first four are present at equivalent positions, while the sixth cysteine of rmIL-4 matches the fifth of rhIL-4 (Figure 7). Thus, disulfide pairings between the second and fourth cysteines of both proteins and between the third and sixth cysteines of rmIL-4 and the third and fifth cysteines of rhIL-4 determined in this report are consistent with the one-dimensional alignment of amino acids. These results demonstrate that the cystine links between conserved cysteines of homologous IL-4 proteins are conserved as well.

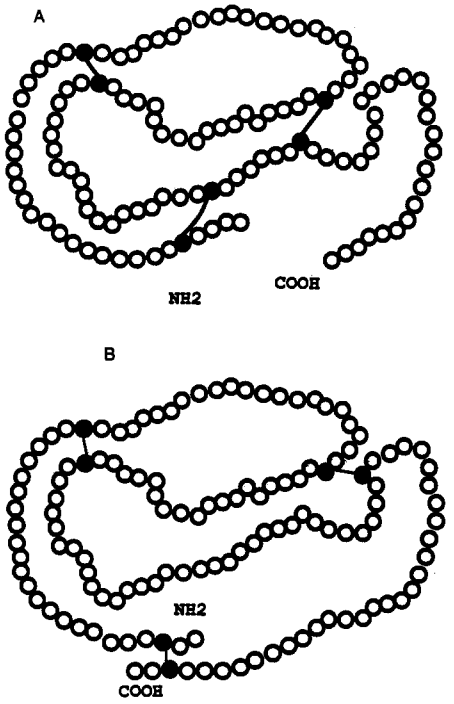

FIGURE 8: Proposed two-dimensional structures of (A) rmIL-4 and (B) rhIL-4, showing the locations of the disulfide bonds within each molecule.

Thus IL-4 behaves like other homologous proteins in the conservation of cystines. This result provides additional evidence that disulfide shuffling did not occur in these experiments and that the cystine bridges have been correctly identified.

The information on disulfides was used to propose a two-dimensional model depicting the folding of these proteins. As shown in Figure 8, the conserved cystines stabilize a large double-loop region within the central three-fifths of the IL-4 molecule. Several issues are raised by these models. First, because the disulfide bonds appear to place analogous restrictions on the polypeptides, it is tempting to speculate that secondary and tertiary structures within the central loop region resemble each other as well. However, the size of the loops, with the concomitant potential for the amino acids to rotate freely, does not support this possibility. Furthermore, since there is less homology in the middle of the molecule than elsewhere (Yokota et al., 1986), there is a greater chance for different microenvironments that lead to different folding patterns. Finally, the inability of mouse and human IL-4 to cross the species barrier strongly suggests that the folds within the central loop must be distinct. While predictions of secondary structure can be made by using the Chou-Fasman (1978) or Kyte-Doolittle (1982) algorithms, significant characterization requires spectroscopic and crystallographic analysis. Plentiful supplies of both recombinant proteins will allow such studies to proceed.

A second prediction of the model is that the region contained between the conserved cystines may be crucial for the biological activity of IL-4. At the same time, it may be inferred that the 25–30 residues at the amino and carboxyl termini are not as important because analogous constraints are not placed on them. In support of this hypothesis, we have demonstrated that the addition of four noncoded amino acids to the amino terminus of the mature mIL-4 sequence does not alter its biological activity in vitro (Figure 1). The proposal that the amino terminus of mIL-4 may not play a role in the biological functions of IL-4 is also confirmed by Mosley et al. (1989) who were able to derivatize the amino terminus of mIL-4 onto a solid support without losing its receptor binding activity. In addition, we conjecture that the function of the bond between the conserved (first) cysteine and nonconserved (mouse fifth or human sixth) cysteine, which limits the motion of at least the amino terminus of both proteins, may not be to create a distinct structural feature but to reduce the reactivity of an unpaired cysteine. While both our results (Figure 1) and those of Ohara et al. (1987) agree in demonstrating that some of the disulfide pairs must remain intact for mIL-4 to function, these experiments give an all or none answer and do not distinguish between the cystines. Having determined the disulfide links, we can now ask functional questions about specific bridges. This could be accomplished genetically by mutating an appropriate set of cysteines and then examining the activity of the resultant protein. In a similar fashion, site-directed mutagenesis can be used to assess the importance of the central region of IL-4 homologues. The definition of the disulfide bonds in recombinant IL-4 proteins allows for a greater understanding of the relationship between the structure and function of this molecule.

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A Novel Cell-Permeable Cromoglycate Derivative Inhibits Type I Fc_ϵ Receptor Mediated Ca^{2+} Influx and Mediator Secretion in Rat Mucosal Mast Cells†

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ABSTRACT: Type I Fc_ϵ receptor ($Fc_\epsilon RI$) mediated Ca^{2+} uptake and secretion of rat serosal mast cells have been shown to be inhibited by disodium 1,3-bis[(2′-carboxylatochromon-5′-yl)oxy]-2-hydroxypropane (disodium cromoglycate, DSCG), which is widely employed in the treatment of allergic asthma [Foreman et al. (1977) *Br. J. Pharmacol. 59*, 473P–474P; Cox (1967) *Nature (London) 216*, 1328–1329]. This drug was also found to modify the protein phosphorylation pattern of these mast cells [Theoharides et al. (1980) *Science 207*, 80–82]. We have isolated by affinity chromatography on a water-insoluble cromoglycate-carrying matrix a cytosolic enzyme recently identified as a nucleoside 5′-diphosphate kinase. In order to examine a possible intracellular activity of the drug, a cell-permeant cromoglycate derivative, 1,3-bis[[2′-[[(acetoxymethyl)oxy]carbonyl]chromon-5′-yl]oxy]-2-hydroxypropane [bis(acetoxymethyl) cromoglycate, CG/AM], has been synthesized, and its uptake and effect on the $Fc_\epsilon RI$-mediated exocytosis of mast cells was investigated. A tritium-labeled CG/AM derivative, used as radioactive tracer, was found to permeate mucosal mast cells of the rat line RBL-2H3 and accumulate intracellularly up to 40-fold its extracellular concentration following hydrolysis by cytoplasmic hydrolases. A CG/AM dose dependent inhibition of the $Fc_\epsilon RI$-induced mediator secretion was observed in RBL-2H3 cells loaded with this compound ($I_{50} \approx 40$ μM extracellular CG/AM). A similar dose-dependent inhibition was observed for both the $Fc_\epsilon RI$-mediated transient rise in the concentration of cytosolic free Ca^{2+} ions ($[Ca^{2+}]_i$) and the net Ca^{2+} influx, as monitored by the fluorescent indicator Quin2 and the radioactive tracer $^{45}Ca^{2+}$, respectively. These results clearly show that cell-permeant cromoglycate inhibits the $Fc_\epsilon RI$-mediated Ca^{2+} influx into the cell and further underscore the dominant role of this process in the coupling of stimulus to secretion in RBL cells. Furthermore, with the identification of nucleoside 5′-diphosphate kinase as a potential intracellular target for CG activity, distinct mechanisms of action may be inferred for cell-permeant and nonpermeant forms of CG.

Mast cells and basophils express the type I cell surface receptors ($Fc_\epsilon RI$)[1] for the Fc domains of class E immunoglobulin (IgE). Aggregation of the $Fc_\epsilon RI$ either by multivalent antigen and IgE or by other agents that bind receptor–IgE complex epitopes initiates a cascade of processes which culminates in the release of inflammatory mediators (Ishizaka & Ishizaka, 1981). In certain serosal mast cell subtypes,

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[1] Abbreviations: CG, cromoglycate; CG/AM, cromoglycate bis(acetoxymethyl) ester; DSCG, disodium cromoglycate; IgE, immunoglobulin class E; $Fc_\epsilon RI$, type I cell surface receptor for the Fc domain of IgE; RBL, rat basophilic leukemia cells of the line 2H3.

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