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Expression, Purification, Refolding, and Characterization of Recombinant Human Interleukin-13: Utilization of Intracellular Processing

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Interleukin-13 (IL-13) is a pleiotropic cytokine that elicits both proinflammatory and anti-inflammatory immune responses. Recent studies underscore its role in several diseases, including asthma and cancer. Solution studies of IL-13 and its soluble receptors may facilitate the design of antagonists/agonists which would require milligram quantities of specifically labeled protein. A synthetic gene encoding human IL-13 (hIL-13) was inserted into the pMAL-c2 vector with a cleavage site for the tobacco etch virus (TEV) protease. Coexpression of the fusion protein and TEV protease led to *in vivo* cleavage, resulting in high levels of hIL-13 production. hIL-13, localized to inclusion bodies, was purified and refolded to yield approximately 2 mg per liter of bacteria grown in minimal media. Subsequent biochemical and biophysical analysis of both the unlabeled and ^{15}N-labeled protein revealed a bioactive helical monomer. In addition, the two disulfide bonds were unambiguously demonstrated to be Cys29–Cys57 and Cys45–Cys71 by a combined proteolytic digestion and mass spectrometric analysis.

Key Words: **fusion protein; interleukin-13; IL-13; maltose-binding protein; refolding; cytokine.**

IL-13[3] is a member of the short-four-helix-bundle cytokines, one of several classes of helical cytokines (1) that include IL-2, IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1–4). IL-13 shares a ~25% sequence identity with IL-4 and both genes are found on chromosome 5 along with those of IL-3, IL-5, IL-9, and GM-CSF (5). IL-13 is produced by T-cells, B-cells, mast cells, and basophils.

Like other cytokines, IL-13 is pleiotropic, with both proinflammatory and anti-inflammatory immune responses (5,6). Its proinflammatory effects include isotype switching to IgE production in B-cells (7) and an increase in vascular cell adhesion molecule-1 production on endothelial cells (6). The latter effect has been implicated in allergenic asthma, and the resulting inflammation may be therapeutically mediated by IL-13 (6). Two recent reports support a direct link between IL-13 treatment and airway hyperresponsiveness in murine models (8,9). Studies with anti-inflammatory cyclic nucleotide PDE inhibitors indicate that their effects are due to the downregulation of several cytokines, including IL-13 (10–14). The anti-inflammatory effects of IL-13 include downregulation of proinflammatory cytokines (i.e., IL-6, IL-12, and TNF-α) and chemokines (i.e., MIP-1α, MIP-1β, and monocyte chemoattractant protein 3) and the upregulation of 12/15-lipoxygenases (5,6,15–18).

Many functions of IL-13 overlap with those of IL-4, due to a shared receptor composed of IL-13Rα1 and IL-4Rα (gp140) that is present in synovial fibroblasts, endothelial cells, and hematopoietic cells (19–22). IL-13 binding to the IL-13Rα1/IL-4Rα heterodimer leads to a number of signal transduction events that include Janus tyrosine kinases 1 and 2 (JAK1 and JAK2), tyrosine kinase 2 (TYK2), signal transducer

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[3] Abbreviations used: NMR, nuclear magnetic resonance; CD, circular dichroism; CIP, controlled intracellular processing; DTT, dithiothreitol; IL, interleukin; IP, intracellular processing; MBP, maltose-binding protein; MIP, macrophage inflammatory protein; PBMC, peripheral blood monocytes; PDE, phosphodiesterase; TEV, tobacco etch virus; TNF-α, tumor necrosis factor-α.

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and activator of transcription 6 (STAT6), and the insulin-receptor substrate-2 (IRS-2) (5,23,24). In contrast to the aforementioned heterodimeric receptor, two other IL-13 receptors have been found that bind IL-13 alone. Both of these receptors, IL-13Rα2, a transmembrane receptor (19,25), and IL-13 binding protein (IL-13 BP), a soluble receptor found in both the serum and urine of mice (26), may regulate IL-13 activity by acting as "decoy" receptors.

Although many aspects of both production and function overlap for IL-13 and IL-4, there are several major differences between the two cytokines. For example, IL-13 is produced by Th0, Th1, and Th2 cell clones, whereas IL-4 is produced only by Th0 and Th2 cell clones (5). IL-4 is produced by multiple types of granulocytes, but IL-13 production is limited to basophils and mast cells (5,6). Differential regulation of the two cytokines is exemplified by only a 20% sequence homology between their promoter sites (5). Expression levels of the two cytokines have been observed to differ in basophils from atopic asthma patients in response to allergens, in which IL-4 and IL-13 reach peak production after 6 and 24 h, respectively (12). Anti-inflammatory PDE inhibitors were found to downregulate IL-13 without any effect on IL-4 in allergen-specific human T-lymphocytes (10). IFN-α has been shown to downregulate IL-13 mRNA levels, but not IL-4, in both Th1 and Th2 cell clones (27). With regard to cytokine function, unlike IL-4, neither murine nor human IL-13 induces IgE class switching in murine B-cells, nor does IL-13 act on T-cells (5,6). Moreover, although a number of cancer cell lines express receptors for both cytokines (28,29), cells derived from patients with glioblastoma multiforme overexpress a receptor for IL-13 that is independent of IL-4 binding (30,31) and a similar receptor has been found in AIDS-associated Kaposi's sarcoma cells (32). Whereas both cytokines induce HIV-1 resistance in monocytes, only IL-4 induces cytolysis in virally infected monocytes (5). Finally, levels of IL-13 are much higher than IL-4 in both the lymph nodes and peripheral blood monocytes (PBMC) of HIV-infected patients (33).

To better understand the diverse functions of IL-13 and thereby facilitate rational design and development of agonists and antagonists, it is essential to produce large quantities of pure, bioactive IL-13. hIL-13 has previously been expressed in murine NS-O cells (34). However, in contrast to the predictive model of Bamborough *et al.* (35) which contained one disulfide and two free sulfhydryls, no free sulfhydryls were found. Far-UV circular dichroism measurements indicated high helical content and bioactivity was found independent of glycosylation (34,36). A recent report described production of hIL-13 in *Escherichia coli* (37); however, the yield was rather low. To allow both structural and dynamic studies of hIL-13 in solution by NMR spectroscopy and to study its interactions with other molecules (i.e., soluble receptors), we have produced a pMAL-c2 plasmid (New England Biolabs, Beverly, MA) with a TEV protease cleavage site followed by a sequence encoding the hIL-13 cytokine. Cotransformation of *E. coli* BL21/DE3 cells with this plasmid and one encoding TEV protease has allowed for high levels of expression and *in vivo* cleavage of the induced MBP-hIL-13 fusion protein by the constitutively expressed TEV protease. hIL-13, localized to inclusion bodies, was purified and refolded for subsequent biochemical analysis, which revealed a bioactive, helical, monomeric protein with two disulfides. The recombinant protein was characterized, and it was demonstrated that an isotopically labeled protein species can be readily produced for NMR studies.

MATERIALS AND METHODS

Materials

Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Both a prepacked Sephacryl S-200 column (2.6-cm diameter $\times$ 60-cm length) and 1-ml Resource-Q column were purchased from Pharmacia Biotech (Peapock, NJ). Amylose resin was purchased from New England Biolabs (Beverly, MA) and guanidine–HCl was purchased from J. T. Baker (Phillipsburg, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All chromatography was performed on a Pharmacia Biotech ÄKTAexplorer system. Novex NuPAGE gels were used with SeeBlue prestained standards in Mes buffer and, where applicable, the method of Pepinsky (38) was used to precipitate proteins from guanidine solutions.

Mass Spectrometry

Proteins were characterized by electrospray mass spectrometry using a Hewlett Packard 1100 HPLC-MSD instrument. Buffer and salts were removed from protein solutions via the reverse-phase HPLC columns at the input to the MSD. Isotopic incorporation was also monitored by mass spectrometry.

Disulfide Linkage Analysis

hIL-13 was buffer-exchanged into 0.1% trifluoroacetic acid using a fast desalting column from Pharmacia LKB. The fraction containing hIL-13 was pooled and lyophilized. The dry protein was reacted with 100 μl of 0.5 M cyanogen bromide (CNBr) in 70% trifluoroacetic acid for 18 h under argon. The sample was then diluted 1:10 and again brought to dryness in a speed vac. The CNBr-cleaved hIL-13 was brought up in 200 μl of 50 mM ammonium acetate buffer at pH 4.0 and digested with endoproteinase Glu-C at room temperature for 18 h. The digested fragments were then separated on a Zorbax C3 reverse-phase column using a linear gradi-

ent at 0.2 ml/min from 0 to 50% over 60 min and from 50 to 100% between 60 and 75 min. The solvents used were 5% acetic acid and 100% acetonitrile. The nonreduced digest was separated and detected on an HP1100 MSD. An equal portion of the digest was reduced by treatment with tris[carboxyethyl]phosphine hydrochloride for 15 min at 50°C, separated, and detected on the HP1100 MSD. The mass spectra before and after reduction were analyzed and fragments were assigned to various portions of the protein. The fragments that were lost upon reduction were assumed to be involved in disulfide bridges. These fragments were then assigned using software developed in-house.

Bioassay

The biological activity of hIL-13 was confirmed by its ability to completely inhibit lipopolysaccharide-induced TNF-α production by monocytes (39,40).

CD Spectroscopy

CD spectra were collected with an AVIV Model 202 spectrometer (Lakewood, NJ), equipped with a variable-temperature sample cell. Samples were contained in a quartz cell with a 1-mm path length and 30-μl volume. All spectra were the average of nine scans with baseline corrections. Samples were prepared in a buffer containing 25 mM Na_2HPO_4, pH 6.1, and 10 mM NaCl with a hIL-13 concentration of 10 μM. One sample was incubated with approximately 30 mM DTT for 24 h prior to CD data collection.

NMR Spectroscopy

All NMR spectra were collected on a Varian Unityplus 500 spectrometer (Palo Alto, CA). The $^{1}H-^{15}N$ heteronuclear single-quantum correlation (HSQC) spectrum was acquired using coherence selection with water flip-back pulses (41). A typical sample was 0.5 mM hIL-13 in 25 mM Na_2HPO_4, 50 mM NaCl, 1 mM EDTA, pH 6.1, in 95% H_2O/5% D_2O contained within a Shigemi (Allison Park, PA) microcell in 300-μl volume. ^{15}N T_1/T_2 ratios were used to estimate the rotational correlation time, τ_c, of hIL-13 (42). Spectra were collected using sensitivity-enhanced experiments (43) with T_1 delays of 20, 55, 100, 180, 255, 365, 465, 555, 700, 890, 1110, and 1380 ms and T_2 delays of 14, 43, 58, 72, 86, 101, 115, 130, 144, 156, and 173 ms. Two-dimensional spectra yielded a total of 108 resolved peaks, from which T_1/T_2 ratios were used to calculate τ_c. All spectra were processed and analyzed using NMRPipe software (44).

Plasmid Expression Vectors

A gene encoding the 112-residue hIL-13 was synthetically engineered to include several unique restriction enzyme sites, which would readily allow for future mutation studies. Seven oligonucleotides and their complementary strands, each with nine base pair overhangs, were ligated to yield a single double-stranded fragment containing a 5′ *Nco*I overhang and a 3′ *Hin*dIII overhang. The synthetic gene was initially inserted into the pUC12N vector (a generous gift from Dr. Paul Boyer of the National Cancer Institute). The proper sequence of this final hIL-13 gene was confirmed by DNA sequencing. The DNA encoding hIL-13 was subsequently subcloned from this vector into pPROEX-1 (Life Technologies, Inc., Rockville, MD). This cloning was accomplished by a triple ligation of a 5′ *Sac*I and 3′ *Eco*RI 305-bp fragment encoding hIL-13 derived from the aforementioned pUC12N vector, a relatively short "linker" oligonucleotide with a 5′ blunt end and a 3′ *Sac*I end and an *Ehe*I- (producing another blunt end) and an *Eco*RI-digested pPROEX-1 vector. Finally, the sequence encoding for both the TEV protease cleavage site and hIL-13 was PCR amplified from this vector, digested with *Eco*RI and *Eco*RV (producing a blunt end), and inserted into the pMAL-c2 vector digested with *Xmn*I (producing another blunt end) and *Eco*RI. The resulting plasmid, pMAL-IL-13, encodes the fusion protein, MBP-hIL-13, with a TEV protease cleavage site between the two domains, and induction is controlled by IPTG. Cleavage results in hIL-13 with a single extra N-terminal glycine.

To generate cells capable of intracellular processing (IP), BL21/DE3 cells were cotransformed with the pMAL-IL-13 expression vector and TEV protease expression vector, pRK603 (45). The cells were grown on LB plates containing 100 μg/ml ampicillin (selecting for pMAL-IL-13) and 30 μg/ml kanamycin (selecting for pRK603). This system allows for the *in vivo* cleavage of the MBP-hIL-13 fusion protein by the constitutively expressed TEV protease.

In Vitro Cleavage with TEV Protease

MBP-hIL-13 cleavage reactions with TEV protease were processed in a buffer containing 50 mM Tris, pH 7.6, 200 mM NaCl, 1 mM EDTA, and 2 mM reduced glutathione (46). The ratio of TEV protease to the fusion protein was approximately 1:100. The TEV protease used for the in vitro cleavage was overexpressed as described by Kapust and Waugh (45,47) and purified in-house (unpublished data).

Expression and Purification of Denatured hIL-13

Two approaches were tested to achieve high-yield production of isotopically labeled hIL-13: (1) expression and purification of the MBP-hIL-13 fusion protein followed by in vitro cleavage to yield the target hIL-13, and (2) expression of the MBP-hIL-13 fusion protein under IP conditions, followed by purification and refolding of hIL-13. Isotopic labeling is routinely achieved by expressing proteins in M9 minimal media

(48,49), where the nitrogen and carbon sources are well controlled and can be enriched in either ^{15}N, ^{13}C, or both.

For each approach, cells were grown and harvested in a similar manner. Drug-resistant colonies containing the appropriate plasmid(s) were grown overnight to saturation at 37°C in 10 ml of LB broth (50) supplemented with 100 μg/ml ampicillin. For a typical 2-L preparation, an aliquot was taken from the 10-ml culture grown in LB broth and added to a 100-ml volume of M9 medium to an OD_{600} of 0.1. The M9 medium was modified to contain a total of 3 g per liter of glucose and 100 μg/ml ampicillin. The 100-ml culture was grown at 37°C until $OD_{600} \geq 2.0$ (approximately 8–12 h). The culture was diluted into 2 L of M9 medium, yielding an initial OD_{600} of approximately 0.1. The cells were grown to an OD_{600} of 0.3, at which time IPTG was added to a final concentration of 1.0 mM. After 24 h at 37°C, the cells were harvested by centrifugation, yielding approximately 3 g of wet cell paste per liter of culture and frozen at −80°C.

In the first approach, the intact fusion protein (MBP-hIL-13) was expressed by selecting single, ampicillin-resistant colonies containing only the pMAL-IL-13 plasmid to seed the initial 10-ml culture. After lysis via sonication (Sonics and Materials Vibracell, 1/2″ SM0896 horn at 60% amplitude with 0.5-s pulses and delays for a 30-s duration), the expressed fusion protein was soluble and could be purified from the cell lysate by amylose column chromatography and subjected to *in vitro* cleavage by TEV protease.

In the IP approach, drug-resistant (ampicillin and kanamycin) colonies containing both plasmids (pMAL-IL-13 and pRK603) were used to seed the initial 10-ml culture. Under the IP conditions, expressed MBP-hIL-13 was processed *in vivo* to release hIL-13, which was driven into inclusion bodies. Inclusion bodies from a 1-L preparation were separated from the cell debris and extensively washed by repeated suspension in 30 ml of wash buffer with sonication (Sonics and Materials Vibracell, 1/2″ SM0896 horn at 90% amplitude with 0.5-s pulses and delays for a 2-min duration) and centrifugation at 4000*g* for 15 min. The wash buffer contained 2 M urea, 50 mM Tris, pH 7.6, 200 mM NaCl, and 1 mM EDTA. This process was repeated until the supernatant was clear (7–10 cycles). The washed inclusion bodies were then solubilized using 8 M guanidine, 100 mM Na_2HPO_4, pH 6.0, and 100 mM DTT to a volume corresponding to approximately 2 ml per liter of original cell culture. This solution was stirred for at least 5 h at room temperature and filtered through a 0.45-μm membrane. Two milliliters of solubilized inclusion bodies were applied to a Sephacryl S-200 column (a total column volume of approximately 318 ml) at a flow rate of 0.25 ml per min. The column was equilibrated and eluted with 4 M guanidine, 50 mM Na_2HPO_4, 1 M NaCl, 1 mM EDTA, and 20 mM β-mercaptoethanol. Peaks containing only hIL-13, as verified by SDS–PAGE, were pooled and stored at −80°C until further use.

Refolding and Purification of hIL-13

The pooled fractions from the Sephacryl S-200 column, approximately 20 ml per run, were added dropwise into 1 L of a refolding buffer over the course of 4–5 h at room temperature, corresponding to a final dilution of 1:50. Unless otherwise noted, the refolding buffer was a modification of that used by van Kimmenade *et al.* (51) and consisted of 50 mM Tris, pH 8.0, 50 mM NaCl, 1 M glycine, 1 mM EDTA, and 5 mM β-mercaptoethanol. Any precipitated protein was removed by filtration through a 0.22-μm membrane, and the clear solution was stirred overnight. The solution was concentrated to less than 50 ml, using Amicon stirred cell filtration at room temperature, and dialyzed against 2 L of 25 mM Na_2HPO_4, pH 6.1, 10 mM NaCl, and 1 mM EDTA with three buffer changes. This solution was further concentrated to 5 ml. If nucleic acids were still present, as determined by a low OD_{280}:OD_{260} ratio (e.g., <1), a final purification of the protein was achieved by passage through a 1-ml Resource-Q column at a flow rate of 0.5 ml per min, equilibrated and eluted with the same buffer. hIL-13 did not bind to the Resource-Q resin because its p*I* is relatively high (calculated p*I* of ~8.4). This last step was used to remove any residual nucleic acids and/or lipids remaining in the refolded protein solution.

RESULTS

Expression of hIL-13 and MBP-hIL-13 Fusion Protein Failed to Produce a Viable System

Our attempts to express *de novo* hIL-13 in an unfused (native) form using the synthetic DNA construct resulted in no overexpression with either pUC12N or pPROEX-1. Therefore, we tested the pMAL-c2 system and showed that it could yield overexpression of an MBP-hIL-13 fusion protein (Fig. 1A). The soluble fusion protein could be purified using amylose resin; however, upon in vitro cleavage with TEV protease, all of the recombinant hIL-13 precipitated within a 48-h period, despite testing a variety of conditions. It was presumed that the target hIL-13 was either incompletely or improperly folded in the context of the fusion, and the cleaved target precipitated due to aggregation and/or disulfide cross-linking. Alternatively, the hIL-13 may have precipitated because of a lack of disulfide bonds that are needed to stabilize its native conformation. Attempts were made to alleviate this problem by refolding the fusion protein prior to cleavage. Although attempts to refold fusion proteins have proven successful (52,53), refolding of some MBP fusion proteins has been only partially successful (54–

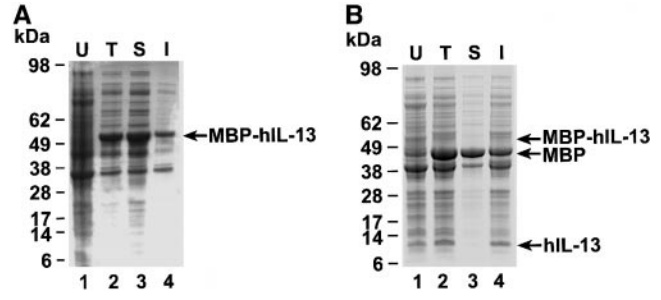

FIG. 1. Overexpression of MBP-hIL-13 and intracellular processing of the MBP-hIL-13 fusion protein by TEV protease. (A) BL21/DE3 cells overexpressing MBP-hIL-13 were as follows: lane 1, uninduced control (U); lane 2, induced total protein (T) homogenized by sonication; lane 3, soluble protein (S) remaining after centrifugation; lane 4, insoluble protein (I) resuspended in lysis buffer and homogenized via sonication. (B) Same as in A, but with constitutive expression of TEV protease in addition to the overexpressed MBP-hIL-13 fusion protein. Cells were lysed by sonication in 50 mM Tris, 200 mM NaCl, 1 mM EDTA at pH 7.6, with longer sonication times in B compared to A (see text).

56). Refolding conditions were tested by using variations from that of human IL-4 (51) at pH 8.0. The use of 5 mM β-mercaptoethanol as a reducing agent, instead of a 5 mM reduced:1 mM oxidized glutathione redox buffer, increased the yield of refolded fusion protein by a factor of two. The addition of either 1 M glycine or 0.5 M arginine resulted in approximately a 50% increase in the yield of soluble fusion protein. The final, optimized refolding buffer consisted of 50 mM Tris, pH 8.0, 50 mM NaCl, 1 M glycine, 1 mM EDTA, and 5 mM β-mercaptoethanol. Cleavage of the refolded fusion protein by TEV protease yielded a soluble fraction and precipitate, which could be separated by centrifugation and/or filtration. Three species were always found in both the soluble fraction and the precipitate: (1) the intact fusion protein, resulting from incomplete cleavage; (2) MBP; and (3) hIL-13. Cleavage of the refolded soluble fusion protein in the presence of reduced glutathione alone as opposed to DTT, β-mercaptoethanol, or a mixture of 2 mM reduced:1 mM oxidized glutathione resulted in the highest yields of intact soluble hIL-13 (data not shown). Several separation protocols proved unsuccessful either in purifying soluble hIL-13 from the uncleaved fusion protein and MBP or in the amount of hIL-13 recovered. The overall yield of this approach remained low; therefore, this expression system was not pursued further.

MBP-hIL-13 and TEV Protease Coexpression Led to a Functional IP System

A variation of the controlled intracellular processing (CIP) method (45) was investigated for expression of hIL-13. BL21/DE3 cells were transformed with both the pMBP-hIL-13 vector and a TEV protease vector, pRK603. The combination of these two plasmids enabled *in vivo* cleavage of the MBP-hIL-13 fusion protein encoded by the pMAL-hIL-13 vector, resulting in high levels of denatured hIL-13 found in inclusion bodies. This is a modified version of CIP, termed IP, since TEV protease is constitutively expressed in BL21/DE3 cells that lack the gene encoding TetR (45). The performance of this approach is illustrated in Fig. 1B. When TEV protease is coexpressed from pRK603, the fusion protein is efficiently processed in vivo. hIL-13 is found only in the insoluble fraction (Fig. 1B, lane 4), representing a solution of homogenized inclusion bodies. Steps that were previously needed for *in vitro* cleavage and subsequent isolation, which resulted in concomitant reduction in yield, could now be avoided by using this IP expression system. All subsequent efforts were directed toward purification and refolding of hIL-13 produced using this expression system. The presence of proteins other than hIL-13 in the insoluble fraction was most likely a result of the harsh lysis conditions (see Materials and Methods). These conditions were selected based on the observation that the final purified hIL-13 was dramatically cleaner, in terms of the OD_{280}:OD_{260} ratio, following this protocol. For example, by increasing the sonication times (from 30 s to 2 min) and amplitude (from 60 to 90%) during the washings, the final OD_{280}:OD_{260} ratio changed from 0.9 to 1.4, indicating an increased removal of cellular debris and enabling a simpler purification strategy.

Purification of Denatured hIL-13

hIL-13 was first separated from most other major contaminants found in the inclusion bodies (Fig. 2A). Repeated washing of inclusion bodies with urea is often used as an initial purification step to remove impurities such as lipids and nucleic acids, as well as proteins that only loosely associate with the inclusion bodies (57–59). Urea concentrations as high as 5 M have been used with up to 90% recovery of protein (60). In the case of hIL-13, numerous washings resulted in only marginal loss of the target protein and substantially reduced the proportion of contaminating proteins (Fig. 2A). Proteins that could not be removed by washing, such as the uncleaved fusion protein (~55 kDa) and MBP (~43 kDa), were most likely trapped within the inclusion bodies. The initial purification, under denaturing conditions, was readily achieved by a Sephacryl S-200 column, and a typical elution profile is shown in Fig. 2B. The late-eluting peak in this chromatogram was composed of hIL-13, as determined by SDS–PAGE (Fig. 2A, lanes 3–7). The use of 1 M NaCl was observed to be essential in preventing elution of hIL-13 in earlier fractions. This behavior was most likely due to ionic interactions between hIL-13 and both the highly acidic MBP (calculated p*I* of ~4.9) and the uncleaved MBP-hIL-13 fusion (calculated p*I* of ~5.5). Approximately 11

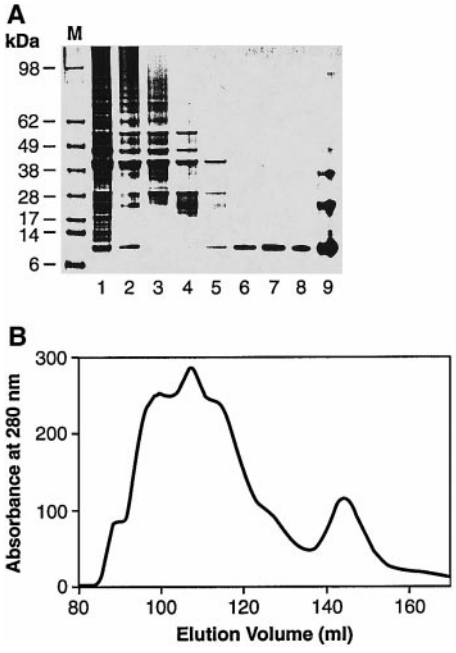

FIG. 2. Purification of hIL-13. (A) SDS–PAGE of the homogenized inclusion bodies, Sephacryl S-200 eluted fractions, and refolded hIL-13. Lane M, molecular weight markers of indicated mass in kilodaltons; lanes 1 and 2, homogenized inclusion bodies before and after washing, respectively; lanes 3–7, Sephacryl S-200 eluted fractions sampled at elution volumes of approximately 100, 120, 130, 140, and 145 mL; lane 8, refolded hIL-13 prior to the Resource-Q column; lane 9, precipitate collected during refolding. (B) Sephacryl S-200 elution profile of solubilized inclusion bodies.

mg of denatured protein was obtained per liter of cell culture (Table 1).

Refolding hIL-13

After expression using the IP system and purification of denatured hIL-13 by Sephacryl S-200 column chromatography, the hIL-13 was refolded. Refolding conditions were tested using the refolding buffer that had been optimized earlier for the MBP-hIL-13 fusion protein (50 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 M glycine, and 5 mM β-mercaptoethanol). The refolding efficiency was assessed at pH 7, 8, 9, and 10. A 3-[cyclohexylamino]-1-propanesulfonic acid (Caps) buffer, with all other components remaining the same, was substituted for Tris in the trial at pH 10. All pH trials utilized aliquots from the same batch of hIL-13 (obtained from a single pool of Sephacryl S-200 fractions), and each refolding mixture was purified with the Resource-Q column. Refolding yield was assessed by densitometry of SDS–PAGE Coomassie-stained gels and final OD_{280} values. Renaturation at pH 7 and 8 gave similar results, while a slight decrease in yield was detected at pH 9 and 10 (data not shown). The latter refolding conditions also produced more covalently linked dimer, as detected by silver staining of Coomassie-stained SDS–PAGE gels. The final conditions selected were 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 M glycine, and 5 mM β-mercaptoethanol. Typical yields for refolding were approximately 30% (Table 1). The final yield of pure, refolded hIL-13 was 2–4 mg per liter of cell culture, depending on the need for passage through the Resource-Q column. The final purification and refolding protocol is shown schematically in Fig. 3.

TABLE 1

Purification of Overexpressed hIL-13[a]

Step	Total hIL-13 (mg)	Recovery (%)	Purification[b] (fold)
Overexpression (total cells)	93[c]	100	1.0
Insoluble fraction	86[c]	93	1.3
Washed insoluble fraction	51[c]	55	2.1
Sephacryl S-200	11[d]	12	200
Refolded	4[d]	4	
Resource-Q	2[d]	2	

[a] Data are normalized to quantities obtained per liter of growth medium that produced approximately 3.5 g wet weight of cells.

[b] Total protein estimated from SDS–PAGE gel band intensity.

[c] Quantity determined from densitometric scanning of an SDS–PAGE gel, for which the sample was homogenized by sonication prior to loading.

[d] Quantity determined by OD_{280} using an extinction coefficient of 5678 M^{-1} cm^{-1} (34).

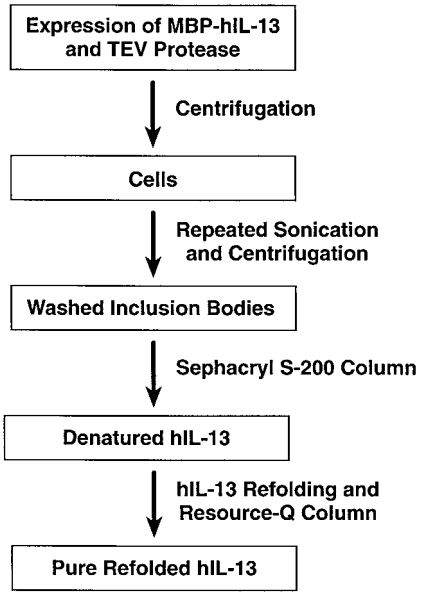

FIG. 3. Schematic protocol of the optimized purification of recombinant hIL-13 from the IP expression system.

Biochemical and Biophysical Analysis of Refolded hIL-13

The refolded hIL-13 was characterized by several methods. First, the identity of the purified protein was

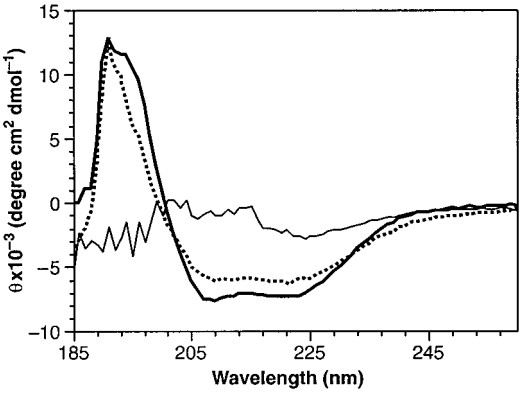

FIG. 4. CD spectra of 10 μM hIL-13 in 25 mM Na_2HPO_4, 10 mM NaCl, pH 6.1, at 25°C (thick solid line), at 60°C (dotted line), and after the addition of excess DTT at 25°C (thin solid line).

confirmed both by N-terminal sequence analysis and by electrospray mass spectrometry. The first six residues were determined to be GGPVPP, in agreement with the constructed sequence of hIL-13. The mass spectrum yielded a parent mass of 12,404 Da, which is in agreement with the theoretical mass of 12,403.7 Da; this result confirmed that there were no chemical modifications as a result of exposure to guanidine and urea. Second, the protein was characterized using SDS–PAGE analysis and mass spectrometry. The SDS–PAGE analysis of purified, soluble hIL-13 showed a single band at approximately 12,500 Da (Fig. 2A, lane 8). In contrast, the precipitate isolated from the refolding reactions (*vide supra*) exhibited bands corresponding to multiples of this mass, indicative of insoluble, cross-linked oligomers (Fig. 2A, lane 9). The proper establishment of two disulfide bonds, comprising the four cysteine residues, in monomeric hIL-13 was initially supported by the mass spectra of unfolded and folded hIL-13. The mass differed by 4 Da, representing the loss of four protons in the formation of two disulfide bonds in the folded state. Third, we unambiguously determined the existence of and location of the two disulfide bonds via combined proteolytic digestion and mass spectrometric analysis. These studies revealed that the four cysteine residues formed two disulfide bonds, Cys29–Cys57 and Cys45–Cys71. Fourth, the refolded hIL-13 was very stable and had a high helical content, as expected for a four-helix-bundle cytokine. The CD spectrum exhibited a maximum at 193 nm and minima at both 208 and 222 nm at 25°C (Fig. 4). The spectrum is similar to that reported previously for hIL-13 (34,37). The CD spectral features persisted at high temperature (Fig. 4). The value of $\theta_{222nm}/\theta_{208nm}$ fell from 0.97 to 1.04 at temperatures of 25 and 60°C, respectively. This reduction might have been due to a change in the coiled-coil structure within the four-helix-bundle predicted for hIL-13 (61,62). The thermal stability was most likely due to the two disulfide bonds, since the addition of excess DTT drastically altered the CD spectrum (Fig. 4) as observed in other four-helix bundles (62,63). Interestingly, a precipitate was observed upon the addition of DTT in the absence of NaCl (i.e., 25 mM Na_2HPO_4 at pH 6.1), but not in the presence of 10 mM NaCl.

NMR spectroscopy on an ^{15}N-labeled hIL-13 was used to assess the monomeric nature of the protein in solution and to compare the conformation of our refolded hIL-13 to authentic, biologically active hIL-13. ^{15}N-Labeled hIL-13 was prepared according to the same procedures with the single modification of $^{15}NH_4Cl$ incorporated into the M9 minimal medium. Mass spectrometric analysis revealed a 12,546-Da species, indicating 95% ^{15}N incorporation. The ^{15}N HSQC spectrum of hIL-13 is shown in Fig. 5. This spectrum is characteristic of a well-folded, helical protein, and we were able to resolve 108 amide H^N–N correlations in the two-dimensional spectrum. From these peaks, we measured the T_1/T_2 ratios and estimated the overall correlation time for molecular tumbling, τ_c, to be 7.2 ns. The oligomeric state of hIL-13 could be inferred from a comparison with other proteins, since to a rough approximation, τ_c is proportional to molecular weight (64). Specifically compared with other cytokines, native MIP-1β exists as a 138-residue homodimer and has a measured τ_c of 8.6 ns. A truncated mutant exists as a monomer, MIP(9), and has a measured τ_c of 4.5 ns (65). An IL-3 variant of 112 residues that is also a short four-helix bundle has a measured τ_c of 6.5 ns (2). Thus,

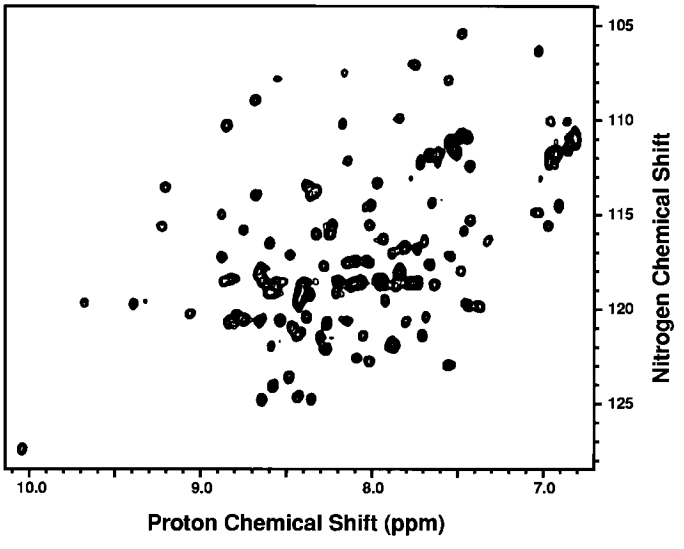

FIG. 5. ^{1}H–^{15}N HSQC spectrum at 500 MHz of 1.0 mM ^{15}N-labeled hIL-13 in 25 mM Na_2HPO_4, 50 mM NaCl, 1 mM EDTA at pH 6.1 with 95% H_2O/5% D_2O. Acquisition parameters: 128-ms acquisition time, 2.5-ms flip-back pulse, 32 scans per t_1 increment, 64 t_1 increments, and States-TPPI for quadrature detection in t_1. Data were processed using time-domain solvent subtraction in t_2 and were linear predicted, zero-filled, and multiplied by a 90°-shifted squared sine-bell before transformation.

the measured τ_c of 7.2 ns for hIL-13 is commensurate with a monomeric protein of approximately 12,000–13,000 Da. The ^{15}N HSQC spectrum is identical to the spectrum recorded on a sample of ^{15}N-labeled hIL-13 obtained from R&D Systems (a generous gift of Monica Tsang, Biotech Division, R&D Systems), which was shown to exhibit biological activity equal to that of wild-type hIL-13 (data not shown). Both the ^{15}N-labeled hIL-13 obtained from R&D Systems and that produced in our laboratory were also shown to be equivalent to unlabeled hIL-13 by comparison of ^{1}H two-dimensional spectra using total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY). These results confirm the biological functionality of the recombinant, refolded hIL-13.

DISCUSSION

We have developed an efficient method to produce isotopically labeled, biologically active hIL-13 that will enable investigations of the structural biology of this important cytokine. Our strategy utilizes the intracellular processing system as described by Kapust and Waugh (45), in which a soluble fusion protein, composed of MBP and hIL-13, is cleaved *in vivo* at a unique site by a constitutively expressed TEV protease. Although the released hIL-13 is misfolded and packs into inclusion bodies, a straightforward purification and renaturation procedure yields large quantities of a monomeric, biologically active protein. We have demonstrated by a variety of biophysical and biological means that the protein is equivalent to wild-type hIL-13. Our data establish unambiguously the identification of the two disulfide bonds in hIL-13 as those formed between Cys29–Cys57 and Cys45–Cys71. A recent report concurs with our disulfide analysis (66). This finding resolves an earlier confusion based on molecular modeling (35). Previous reports of the production of recombinant hIL-13 include expression in murine NS-O cells (34) and *E. coli* (37). In terms of NMR studies, the NS-O expression system would not be amenable to isotopic labeling, and the reported *E. coli* system produces a two- to fourfold lower yield than that reported here, even in rich media. Hence, the development of an efficient expression and purification protocol is critical to our ongoing studies of hIL-13. A related purification protocol has been reported for human IL-5 (67), which supports our results and confirms that this approach can be a rapid and efficient means of producing medium-sized proteins.

The availability of large amounts of recombinant hIL-13 will enable detailed studies of its structure, dynamics, and interactions with other macromolecules. In particular, we will be able to investigate the detailed differences between IL-4 and IL-13, with the aim of elucidating the cross-reactivity of IL-13 with IL-4 receptors. The recent structure of hIL-4 in complex with the soluble extracellular domain from IL-4Rα (68) presents an opportunity to examine structural differences and initiate rational mutational studies to probe these interactions. IL-13 and its receptors are potential therapeutic targets for a number of disorders that include both asthma and cancer (28,29,31,69). The ability to embark on any rational drug design effort to target these features requires detailed structural biology investigations that can now be pursued with the availability of adequate amounts of protein in any desired labeling pattern. These studies are currently underway in our laboratory.

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