Functional and biophysical characterization of recombinant human hepatocyte growth factor isoforms produced in *Escherichia coli*

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Hepatocyte growth factor (HGF) is a pluripotent secreted protein that stimulates a wide array of cellular targets, including hepatocytes and other epithelial cells, melanocytes, endothelial and haematopoietic cells. Multiple mRNA species transcribed from a single HGF gene encode at least three distinct proteins: the full-length HGF protein and two truncated HGF isoforms that encompass the N-terminal (N) domain through kringle 1 (NK1) or through kringle 2 (NK2). We report the high-level expression in *Escherichia coli* of NK1 and NK2, as well as the individual kringle 1 (K1) and N domains of HGF. All proteins accumulated as insoluble aggregates that were solubilized, folded and purified in high yield using a simple procedure that included two gel-

filtration steps. Characterization of the purified proteins indicated chemical and physical homogeneity, and analysis by CD suggested native conformations. Although the K1 and N-terminal domains of HGF have limited biological activity, spectroscopic evidence indicated that the conformation of each matched that observed when the domains were components of biologically active NK1. Both NK1 and NK2 produced in bacteria were functionally equivalent to proteins generated by eukaryotic systems, as indicated by mitogenicity, cell scatter, and receptor binding and activation assays. These data indicate that all four bacterially produced HGF derivatives are well suited for detailed structural analysis.

INTRODUCTION

Hepatocyte growth factor (HGF) is a secreted protein that stimulates proliferation, migration and morphological differentiation of a wide variety of cellular targets. Among these targets are hepatocytes and other epithelial cells, melanocytes, endothelial and haematopoietic cells [1–3]. Whereas most of these cell types proliferate in response to HGF, the HGF-induced dispersion of clusters of cultured endothelial cells and certain epithelial cells, in the absence of proliferation, led to the independent discovery of HGF as ‘scatter factor’ [4,5]. HGF can also stimulate cultured cells to invade collagen gels [6], and in some instances they give rise to tubular structures in a manner analogous to branching morphogenesis *in vivo* [7,8]. Paradoxically, HGF has cytotoxic effects on some sarcoma and carcinoma cell lines [9,10]. All of these responses appear to be mediated by the *c-met* proto-oncogene product Met [11,12], a high-affinity cell-surface receptor for HGF [13–15].

HGF resembles human plasminogen (PL) in that the two proteins share 38% amino acid sequence identity and several structural motifs [16,17]. Each protein is synthesized as a single-chain polypeptide that is proteolytically processed at a conserved site to generate a biologically active disulphide-linked heterodimer [18–20]. In both instances, the heavy chain of the dimer ($\approx$ 60 kDa in HGF) is derived from the N-terminus of the precursor and contains multiple kringles (four in HGF, five in PL). Kringles ($\approx$ 80 amino acids) have a characteristic folding pattern de-

termined by three intramolecular disulphide bonds and additional conserved sequences [21]. The HGF light chain ($\approx$ 34 kDa), like that of PL, has the structure of a serine protease. However, two non-conservative substitutions within the catalytic triad and possibly other unconserved sequences render HGF devoid of proteolytic activity [16].

Multiple mRNA transcripts arise from a single HGF gene and encode at least three structurally and functionally distinct proteins. In addition to the full-length HGF protein (728 amino acids), two smaller HGF isoforms that result from alternative mRNA splicing have been identified and characterized [22–24]. Both contain the N-terminal domain (N) and kringle 1 (K1); thereafter one isoform is immediately terminated (NK1) and the other is terminated after kringle 2 (K2) (NK2). The NK1 mRNA coding sequence contains two additional amino acids downstream of the K1 domain resulting from the retention of a portion of the intron separating exons K1b and K2a [24]. The NK2 mRNA transcript is generated by the joining of the K2 exon to an alternative exon K2T, which encodes two additional amino acids followed by an in-frame stop codon [22,23].

Both truncated HGF isoforms, purified from the conditioned medium of cultured eukaryotic cell lines, can bind directly to Met, and both can be effectively displaced from this receptor by HGF, as shown by covalent affinity cross-linking experiments [23,24]. Both truncated isoforms also appear to retain some degree of scatter activity on MDCK cells [24,25]. Interestingly, the HGF isoforms differ in their respective abilities to stimulate

Abbreviations used: EGF, epidermal growth factor; Gdn-HCl, guanidine hydrochloride; HGF, human hepatocyte growth factor; IGF-I, insulin-like growth factor-I; K1 or K2, kringle 1 or 2 of HGF; MDCK, Madin–Darby canine kidney; N, N-terminal domain of HGF; NK1, the N-terminal domain through K1 of HGF; NK2, the N-terminal domain through K2 of HGF; PL, human plasminogen; t-PA, tissue plasminogen activator.

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DNA synthesis in HGF target cells. NK1 exhibits a lower maximum activity than full-length HGF, and can thus partially block HGF-induced mitogenesis when present in sufficient molar excess [24]. NK2, in contrast, lacks detectable mitogenic activity and can completely block HGF-stimulated DNA synthesis when added in sufficient molar excess [23]. To characterize these and other diverse biological properties of the HGF isoforms, and to produce sufficient quantities of protein for structural studies, we expressed NK1 (residues 31 to 210) and NK2 (residues 31 to 290) in *Escherichia coli*. We report here high-yield prokaryotic expression of both truncated HGF isoforms, and characterization of their physicochemical and conformational properties, and we demonstrate comparable specific biological activities between the prokaryotically expressed proteins and their eukaryotically expressed counterparts. We also describe the expression in *E. coli* of the HGF N-terminal peptide (N; residues 31–127) and the HGF K1 domain (residues 123–210) and show that overall conformation of each was similar whether analysed individually or as components of NK1.

MATERIALS AND METHODS

Construction of expression plasmids

NK1 and NK2, products of alternatively spliced HGF transcripts [22–24], were produced in *E. coli* using the T7 expression system developed by Studier et al. [26]. The DNA encoding the desired polypeptides was generated as an *Nde*I–*Bam*HI fragment using the PCR as previously described [27], and cloned into pET11a [26]. The construct pET11-HGF/N encodes the polypeptide MG³¹Q³²R³³...I¹²⁵R¹²⁶N¹²⁷; the construct pET11-HGF/K1 encodes MD¹²³Y¹²⁴I¹²⁵...E²⁰⁸G²⁰⁹K²¹⁰; the construct pET11-HGF/NK1 encodes the polypeptide MG³¹Q³²R³³...E²⁰⁸G²⁰⁹K²¹⁰; and the construct pET11-HGF/NK2 encodes the polypeptide MG³¹Q³²R³³...C²⁸⁸E²⁸⁹T²⁹⁰. The amino acid numbering scheme corresponds to that of full-length HGF [28]; residues 1–30 constitute the signal sequence and are absent in the mature protein. pET11-HGF/NK2 C214A was derived from pET11-HGF/NK2 using the PCR to change the codon for cysteine-214 to one encoding alanine [29]. The DNA sequences of all HGF coding regions were verified using an Applied Biosystems 373A DNA sequencer using procedures and reagents supplied by the manufacturer.

Expression of HGF-derived proteins in *E. coli*

Plasmid DNAs were transformed into *E. coli* strain BL21 (DE3; [26]). Fermentations were in a MD2 2 litre bench-top fermentor (Braun Biotech) in the following medium: 2% (w/v) glucose, 2% (w/v) Bacto-Tryptone, 1% (w/v) yeast extract, 0.5% (w/v) sodium citrate, 1% (w/v) dibasic potassium phosphate, 10 mM magnesium sulphate, 200 mg/l carbenicillin and trace metals. Cells were grown at 37 °C and 30% pO₂ (equivalent to an aeration rate of 2 l/min). When the cells reached an absorbance of 5–10 (usually 10) at 600 nm, protein expression was induced with 2 mM IPTG for 3.5 h. Each fermentation yielded about 50 g wet weight of cells, which were stored at –80 °C until needed for processing.

Protein purification

The following procedure was used for all the HGF-derived proteins (N, K1, NK1, NK2 and NK2 C214A). Cells (≈ 50 g wet weight) were resuspended into 200 ml of 100 mM Tris/HCl, pH 8.0, containing 5 mM EDTA and 5 mM benzamidine/HCl (break buffer) and were passed once through a French pressure cell (SLM-Aminco) operated at 18000 psi. The suspension was

briefly sonicated, 0.1% (w/v) Triton X-100 was added, then centrifuged at 10000 *g* for 45 min. The pellet was resuspended in 250 ml of break buffer and recentrifuged. The washed pellet was suspended with 35–40 ml of 50 mM Tris/HCl, pH 8.0, containing 8 M guanidine hydrochloride (Gdn-HCl) and 100 mM dithiothreitol. The slightly cloudy solution was clarified by centrifugation at 100000 *g* for 30 min and applied at 5 ml/min to a column (6 cm diameter × 60 cm length) of Superdex 200 (Pharmacia-Biotech) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 4 M Gdn-HCl and 10 mM dithiothreitol. The column was eluted at 5 ml/min, and 20 ml fractions were collected. Fractions were analysed by SDS/PAGE after removal of the Gdn-HCl using the method of Pepinsky [30]. Selected fractions containing purified HGF proteins were pooled and concentrated to 30–40 mg/ml in an ultrafiltration stirred cell using Diaflo PM-10 membranes (Amicon). The Gdn-HCl and dithiothreitol concentrations were adjusted by the addition of solid reagents to ≈ 8 M and 20 mM respectively, and the protein was stored at –80 °C until required.

Protein folding

The protein solution in aliquots of 2.5–3.0 ml was slowly added (during ≈ 1 h) at 20 °C to 1 litre of the following folding buffer: 100 mM Tris/HCl, pH 7.5, containing 2.5 M urea, 5 mM reduced glutathione and 1 mM oxidized glutathione (final protein concentration ≈ 0.1 mg/ml). The solution was incubated with gentle stirring for 12 h, then transferred to dialysis tubing and dialysed for 12 h against 5 litres of 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl. The stirring bar was caged in a perforated plastic container to prevent it from rubbing against the dialysis tubing. Dialysis was continued for at least another 12 h against 20 litres of the same buffer with two changes of buffer. All dialysis stages were performed at 4 °C. The proteins were concentrated by ultrafiltration to about 50 ml using an Amicon Model 2000 stirred cell (capacity 2 litre) with a Diaflo PM-10 membrane, clarified by filtration or centrifugation, then concentrated further to 5–6 ml using either Centriprep-10 concentrators (Amicon) or an ultrafiltration stirred cell (200 ml capacity) with a PM-10 membrane. The concentrate was applied to a Superdex 75 column (2.6 diameter × 60 cm length; Pharmacia-Biotech) equilibrated in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl. The column was eluted at 1 ml/min, and 4 ml fractions were collected. Fractions containing HGF-derived protein (see the Results section) were pooled, concentrated to 1 mg/ml or higher using Centriprep-10 units, then sterilized using 0.22 µm Millex-GV filter units (Millipore) and stored at –80 °C.

Determination of protein concentration

Purified proteins were quantified by measuring their absorbance at 280 nm in a 1 cm path-length cell using a double-beam, diode-array Hewlett-Packard 8450A UV/VIS spectrophotometer. The molar absorption coefficients ($\epsilon_{\text{mg/ml}}$) used, calculated from the respective amino acid compositions according to Wetlaufer [31], were: 0.76 for N; 1.21 for K1; 1.25 for NK1 and 1.65 for NK2 and NK2 C214A.

SDS/PAGE

SDS/PAGE was performed using precast 4–20%-polyacrylamide gels according to the manufacturer's instructions (Novex). Samples analysed under reducing conditions were dissolved in the SDS sample buffer plus 20 mM dithiothreitol. Samples analysed under non-reducing conditions were pretreated with 20 mM iodoacetamide for 15 min at 22 °C before the addition of the SDS sample buffer minus reductant.

Protein sequencing

Samples were applied to PVDF membranes using the ProSpin preparation cartridge (Perkin-Elmer). Automated Edman degradation was performed using the Blott Cartridge and an Applied Biosystems Model 477A protein sequencer (Perkin-Elmer).

MS

Samples (0.3–0.5 mg/ml) were dialysed or subjected to gel filtration into 5% (v/v) acetic acid. The clear solutions were diluted to about 15–20 μ M, and 25 μ l portions were mixed 1:1 with hexafluoroisopropanol in preparation for electrospray MS. The protein solutions were infused at a rate of 0.5 μ l/min into an Analytica electrospray source fitted to a JEOL JMS-SX 102 mass spectrometer operated at 5 kV accelerating voltage. The electrospray instrument was calibrated against lysozyme, and molecular masses of the measured proteins were obtained by deconvolution of the resultant mass/charge peak distributions. By this method the determined mass accuracy was about 0.01%.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed using a Beckman Optima XL-A analytical ultracentrifuge with an An-60 Ti rotor and standard double-sector centerpiece cells. For equilibrium measurements, samples (90 μ l) were centrifuged for 14–20 h at 20 °C at the following values of g : 57058 for K1, 45486 for N, 38500 for NK1, and 23580 for NK2 and NK2 C214A. Sedimentation velocity measurements (400 μ l samples) were made at 147375 g for 2–3 h at 20 °C, with data collection every 15 min. Data was analysed using both the standard Beckman XL-A data analysis software (v.3.0 for DOS) and the Beckman-Origin software (v.2.0 for Windows). Protein partial specific volumes were calculated from the amino acid compositions [32]. Values (g/ml) of 0.735 for N; 0.716 for K1; 0.727 for NK1 and 0.719 for NK2 and NK2 C214A were used. Solvent densities were either calculated as described by Laue et al. [33], or the values were taken from the International Critical Tables [34].

CD

CD spectra were recorded on a JASCO J-720 spectropolarimeter. Measurements in the near- (340–240 nm) and far- (260–180 nm) UV regions were made using 1 cm and 0.02 cm path-length cells respectively. A 1 nm bandwidth was used for both spectral regions. Protein solutions were 0.75–1 mg/ml in 50 mM sodium phosphate, pH 7.5. Protein buffers were exchanged using Sephadex G-25M (PD-10 columns; Pharmacia-Biotech). Immediately before use, solutions were filtered with 0.22 μ m pore size Millex-GV filter units and briefly degassed. Secondary structures were estimated using the methods of Provencher and Gloeckner [35], Toumadje et al. [36], and Sreerama and Woody [37].

Tissue culture

B5/589 human mammary epithelial cells, Madin–Darby canine kidney (MDCK) cells, and Balb/MK mouse keratinocytes were each maintained as described previously [20,38].

Mitogenicity and scatter assays

DNA synthesis was assayed by incorporation of [3 H]thymidine into a trichloroacetic acid-precipitable cell fraction as described previously [38]. Epidermal growth factor (EGF; recombinant

murine) was from Collaborative Research, and full-length HGF (recombinant human) was produced in insect cells as previously described [24]. MDCK cell movement, observed as the dispersion or scatter of single cells from tightly grouped colonies, was assayed as described previously [39]. Treatments were made over several serial dilutions, and cell scatter was observed by light microscopy of fixed and stained cells [20].

Ligand/receptor covalent affinity cross-linking

125 I-Labelled NK2 and 125 I-labelled NK1 were prepared as described previously [13,24]. 125 I-labelled ligands, in the presence and absence of excess unlabelled HGF isoforms, were cross-linked to HGF receptors on B5/589 or Balb/MK cells and observed by SDS/PAGE and autoradiography as described previously [13,24]. Cross-linked complexes were immunoprecipitated from Balb/MK cell lysates with 1 μ g/ml of anti-(mouse Met) polyclonal antiserum in the presence or absence of 10 μ g/ml competing peptide (antiserum and peptide from Santa Cruz Biotechnology). Insulin-like growth factor-I (IGF-I) was from Pepro Tech.

HGF receptor phosphorylation

B5/589 cells grown to confluence in 10-cm-diameter dishes were serum deprived for 16 h, then treated with each HGF isoform at the concentration indicated, or left untreated, and incubated for 10 min at 37 °C. The culture medium was then aspirated. Triton X-100 extracts were prepared and phosphotyrosyl proteins were immunoprecipitated using monoclonal antiphosphotyrosine antibodies (anti-pY; Upstate Biotechnology). Anti-pY immunoprecipitates were subjected to SDS/PAGE, transferred to PVDF membranes, and immunodetected using antiserum to human Met (Santa Cruz Biotechnology) and 125 I-labelled Protein A. Alternatively, the HGF receptor was immunoprecipitated with anti-Met, resolved by SDS/PAGE, transferred to PVDF, and immunoblotted with anti-pY. The anti-pY-precipitable HGF receptor was revealed by autoradiography using a Phosphor-Imager (Molecular Dynamics), or by conventional autoradiography using Kodak XOMAT AR film and intensifying screens at –70 °C.

RESULTS

Protein expression and purification

The HGF-derived proteins were all well expressed in *E. coli*, each accumulating to about 10–20% of the total protein, as evidenced by SDS/PAGE of cell extracts. All the expressed proteins were insoluble in bacterial lysates and were extracted with 8 M Gdn-HCl. Solubilized proteins were fractionated by gel filtration also in the presence of Gdn-HCl. Purified proteins (fully denatured and reduced) were folded into biologically active species using an equilibrium dialysis scheme (see the Material and methods section) that can be easily scaled up or down depending on requirements and equipment available. In the first folding buffer urea was included to prevent protein aggregation during the removal of the Gdn-HCl. Also included in the buffer was a glutathione-based oxido-shuffling system to promote the formation of disulphide linkages from the reduced protein by thiol–disulphide exchange reactions (for a review see [40]). The ratio of reduced to oxidized glutathione (5:1) and the concentration of urea (2.5 M) used were optimized empirically.

After the dialysis stage, the proteins were concentrated by ultrafiltration and fractionated by gel filtration. Typical chromatograms for the separation of NK1 and NK2 are shown in Figure

Figure 1 Gel filtration of the NK1 and NK2 HGF isoforms

Following the protein folding (see the Materials and methods section), concentrated protein was applied to a Superdex 75 column (2.6 diameter $\times$ 60 cm length) equilibrated in 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl. The fraction size was 4 ml. Chromatograms for the NK1 ($\circ$) and NK2 ($\bullet$) proteins are shown. The separated peaks are labelled and are referred to throughout the text.

Figure 2 SDS/PAGE of recombinant HGF-derived proteins

The Figure shows a 4–20%-polyacrylamide gel; samples were analysed under either reducing or non-reducing conditions (see the Material and methods section). Lanes A, F and I, standards (BioRad; molecular masses indicated at the right); lane B, NK1, reduced; lane C, NK1, non-reduced (the trailing left edge of the band is due to diffusion of reductant from lane B and clearly indicates the mobility shift); Lane D, NK2, non-reduced (as in lane C, the large mobility shift of left portion of band is due to lateral diffusion of reductant); Lane H, NK2, reduced; lane E, N-domain, non-reduced; lane G, N-domain, reduced.

1. For NK1, there are two main protein peaks, b_1 and c_1 , and a third one, d, that contained non-proteinaceous material eluting at the included volume. As peaks b_1 and c_1 contained monomeric

Table 1 Molecular-mass determination of recombinant HGF-derived proteins

SDS/PAGE (Figure 2) was carried out under reducing (R) or non-reducing (NR) conditions as described in the Material and methods section, and the indicated molecular masses, especially under non-reducing conditions, are apparent values. MS and sedimentation equilibrium were also carried out as described in the Materials and methods section. Predicted molecular masses were derived from translation of the respective DNA coding sequences. Values indicated are for the fully oxidized proteins in which the initiating N-terminal methionines are removed, except for K1, which retained methionine. nd, not determined.

Protein	Method ...	Molecular mass (kDa)				
		SDS/PAGE		MS	Sedimentation equilibrium	cDNA
		R	NR			
N		15	15	11.371	11.50	11.372
K1		10	≈ 7	nd	10.30	10.135
NK1		24	21	20.693	19.50	20.691
NK2		36	29	30.642	32.50	30.335
NK2 C214A		36	29	30.305	30.00	30.303

protein (determined by analytical ultracentrifugation; results not shown), we ascribe the difference in elution position to conformational differences (described below). As also shown below, peak c_1 contained active folded protein, and was used for all further characterization unless stated otherwise. In the NK2 chromatogram, an additional peak (a) was observed that eluted at the void volume and contained highly aggregated protein, whereas peaks b and c correspond to the monomeric peaks b_1 and c_1 of NK1, except they eluted earlier, as expected for larger proteins (Figure 1). Hence, peak c contained the active folded protein. The elution profiles for N and K1 of HGF (results not shown) were qualitatively similar to those shown in Figure 1; only material eluting in the peak corresponding to peak c (or c_1) was analysed further.

Protein sequencing and molecular mass

Thiol titration of the four HGF-derived proteins with Ellman's reagent [41] under denaturing conditions [1 % (w/v) SDS] indicated that all proteins were fully oxidized, containing no detectable free cysteine residues. Purified proteins all exhibited single bands when analysed by SDS/PAGE under reducing conditions (Figure 2). Under non-reducing conditions, all proteins also exhibited single bands, indicating that they lacked intermolecular disulphide bonds (Figure 2). That NK1 and NK2 contain intramolecular disulphide bonds is clearly shown by the increased mobility of non-reduced versus reduced proteins (Figure 2 and Table 1). Non-reduced K1 of HGF also exhibited increased mobility compared with reduced protein (Table 1). There was little or no change in the mobility of reduced versus oxidized N-domain although this protein contains two intramolecular disulphides (Figure 2). The above findings indicate that the HGF derivatives contain only intramolecular disulphides; however, the actual cysteine–cysteine linkage patterns have not been determined.

Amino acid sequencing of N, NK1 and NK2 for 20 cycles indicated that all proteins have the same N-terminal sequence, as predicted from the respective DNA coding sequences. Furthermore, for each protein the N-terminal methionine from initiating N-formylmethionine was removed by the *E. coli* processing enzymes. In contrast, K1 of HGF fully retained

Figure 3 CD spectra of HGF-derived proteins

(A, B) N-domain in pH 7.5 buffer (spectrum 1) or in 5% (v/v) acetic acid ($\approx$ pH 2.5; spectrum 2); (C, D) NK1: peak c_1 (spectrum 1); peak b_1 (spectrum 2); peak c_1 plus 5 M Gdn-HCl (D, spectrum 3); (E, F) NK2: peak c (spectrum 1); peak b (spectrum 2); peak c plus 5 M Gdn-HCl (E, spectrum 3); peak c plus 10 mM dithiothreitol (F, spectrum 3). Peak designations refer to the Superdex 75 chromatography shown in Figure 1. The buffer was 50 mM sodium phosphate, pH 7.5. Spectra are shown in far-UV region to limits of reliable detection as monitored by photomultiplier voltage (usually less than 600 V). The ordinates for the far- and near-UV CD spectra are the mean residue ellipticity $[\theta]_{MWR}$ and the differential molar ellipticity $\Delta\epsilon$ respectively.

methionine, as predicted for protein with aspartic acid encoded in the second codon of the open reading frame [42].

Further identification of the HGF-derived proteins was obtained by electrospray MS (Table 1). There was excellent agreement between the determined masses and those predicted from the respective cDNA coding sequences, with the exception of NK2, whose mass was 307 Da higher (Table 1). By sequence alignment with human PL, all of the cysteine residues (17 in total) in NK2 can be assigned to various disulphides except cysteine-214 which in full-length HGF is thought to form an intramolecular disulphide with cysteine-347 located in K3. It follows that a likely explanation for the mass discrepancy is that the unpaired cysteine-214 forms a stable disulphide linkage with glutathione (mass of reduced glutathione: 307), which is used in the refolding protocol. To test this prediction, a mutant NK2 C214A, in which cysteine-214 was replaced by alanine, was constructed to prevent the formation of the putative glutathione adduct. Protein expression and folding of NK2 C214A were carried out as described for the wild-type protein, and subsequent analysis by MS showed that its mass conformed to the predicted value for the fully oxidized protein (Table 1).

The molecular mass of the proteins under native conditions was determined by sedimentation equilibrium measurements using an analytical ultracentrifuge. All four HGF derivatives

were monomeric under the solvent conditions used (50 mM Tris/HCl, 100 mM NaCl, pH 7.5, or 50 mM sodium phosphate, pH 7.5) with no observed tendency for self-association or aggregation over the concentration ranges studied (0.1–1.0 mg/ml; Table 1).

Protein conformation

As the various HGF derivatives were all produced from unfolded and reduced proteins, it was necessary to characterize their conformation; CD was chosen for this purpose. In the far-UV region (240–180 nm), the spectrum for the N-domain of HGF (Figure 3A, spectrum 1) was qualitatively similar to that of a α -helix-containing protein [43], although the overall ellipticity was low. Determination of the secondary structure by several methods [35–37] indicated that the helix content was in fact only 5%, or less, and that the predominant secondary structure was β -sheet ($\approx$ 50%) and β -turn ($\approx$ 20%). Treatment of the N-domain with 5% (v/v) acetic acid resulted in a spectrum with intense negative ellipticity at $\approx$ 200 nm (Figure 3A, spectrum 2), typical of denatured protein [44]. In folded or native proteins, the CD bands in the near-UV region (240–340 nm) arise mainly from the asymmetric orientation of aromatic residues (Trp and Tyr) and are weak or not detected in denatured protein [45,46]. Loss of secondary structure on acid treatment is mirrored in the near-UV region, where the prominent CD bands at 291 nm (positive) and 286 nm and 277 nm (negative) are eliminated (Figure 3B, spectrum 2), thus also indicating loss of tertiary structure.

The most active fractions from the gel-filtration chromatography of NK1 and NK2 (peaks c_1 and c in Figure 1) had similar far-UV CD spectra (Figures 3C and 3E, spectra 1). These spectra are unusual in having a broad unresolved peak of positive ellipticity centred at $\approx$ 230 nm with weak negative ellipticity at 205–207 nm, and a well-resolved CD band at 197–198 nm (positive). Positive ellipticity in the 230 nm region, usually originating from aromatic residues with possible contribution from disulphide bonds [47], is derived from folded protein, as emphasized by the distinctly different spectra of proteins denatured with Gdn-HCl (e.g. Figure 3E, spectrum 3).

In the near-UV region, the active fractions exhibited several positive CD bands in the region 275–290 nm characteristic of folded protein (Figures 3D and 3F, spectra 1). By comparison, these CD bands are reduced in intensity (40–50%) in protein from the earlier eluting fractions b_1 (NK1) and b (NK2), indicating partial loss of tertiary structure (Figures 3D and 3F, spectra 2). It is evident that the proteins are not fully unfolded by comparing with sample (e.g. peak c_1 of NK1) denatured with Gdn-HCl (Figure 3D, spectrum 3). This appears inconsistent with the far-UV data (Figures 4C and 4E, spectra 2), which suggest these proteins are more extensively denatured. For acid-denatured N of HGF (Figure 3A, spectrum 2), fairly typical of denatured protein [44], the prominent negative CD band at $\approx$ 200 nm is almost twice as intense as for either peak b_1 of NK1 or peak b of NK2. Therefore, these proteins are distinguishable from fully unfolded protein in both the far- and near-UV CD regions, the latter being of more diagnostic importance [43].

Molecular-mass determination by sedimentation equilibrium indicated that peak b_1 of NK1 and peak b of NK2 are monomeric proteins (results not shown), with masses similar to those determined for folded counterparts (Table 1). However, they have lower sedimentation coefficients (s) than the latter, indicating more hydrodynamically expanded structures. For example, peaks b and c of NK2 have respective s values of 2.74 and 2.46. As peak b_1 (or b) protein is fully oxidized, a likely explanation for the conformational differences is the presence of mismatched

Figure 4 CD spectra of HGF-derived proteins

(A, B) K1 in 50 mM sodium phosphate, pH 7.5 (spectrum 1) or in buffer plus 5 M Gdn-HCl (A, spectrum 2). (C, D) N-domain (spectrum 3); NK1 (spectrum 2); NK1 minus N difference spectra ($\Delta K1$; spectrum 1). $\Delta K1$ was calculated by subtracting the weighted contribution of N from NK1. (E, F) NK1 (spectrum 1) and the weighted average of the individual N plus K1 spectra (spectrum 2).

disulphide bonds. That disulphide bonds are required for stable folding is also indicated in, for example NK2, where the near-UV CD spectrum of native protein (peak c) treated with dithiothreitol indicates loss of tertiary structure (Figure 4F, spectrum 3).

To study the functional and structural roles of some of the individual K domains in HGF, we expressed in *E. coli* the K1 of HGF. The purified protein appeared folded, exhibiting fairly strong positive CD bands at 278 nm and 282 nm with a shoulder at 288 nm (Figure 4B). This spectrum is similar to that previously reported for the non-recombinant kringle 4 isolated from elastase-treated human PL [48]. The shape of the far-UV CD spectrum for K1 is unusual (Figure 4A, spectrum 1), but similar to other kringle domains previously reported, for example kringle 2 from tissue plasminogen activator (t-PA; [49]), and is derived from predominantly β -sheet ($\approx 23\%$) and β -turn ($\approx 39\%$), based on known kringle structures (e.g. [50]). As mentioned for NK1 and NK2, the broad unresolved peak of positive ellipticity centred at ≈ 230 nm probably originates from aromatic residues. And as was also true for NK1 and NK2, the positive ellipticity in this region was eliminated by incubation with Gdn-HCl (Figure 4A, spectrum 2). A dominant structural feature in kringles is a hydrophobic core formed by a number of aromatic and other hydrophobic side chains [50]. The perpendicular stacking of these aromatic residues in the core region may be responsible for the unusually strong aromatic contribution to the far-UV CD spectra of the kringle and kringle-containing structures.

We compared the conformation of the individual K1 and N-domains with their equivalents covalently associated in the parent NK1 isoform. Firstly, the NK1 minus N-domain difference spectra were calculated (Figures 4C and 4D, spectra 1) and were similar, especially in the near-UV region, to those for K1. Secondly, we calculated the weighted average spectra of the K1 plus N-domain (Figures 4E and 4F, spectra 2), which were almost identical to that of NK1 in both the far- and near-UV regions. These results indicate that the global conformations of the K1 and N-terminal regions of HGF are similar whether they are isolated entities or covalently associated in NK1. By calculating the NK2 minus NK1 difference spectrum (results not shown) we also determined the predicted near-UV CD spectrum for K2. It was similar to that of K1 (Figure 4D, spectrum 1) but contained an additional positive CD band at 295 nm and overall was similar to that of t-PA kringle 2 [49]. As the aromatic residue contents of the K1 and K2 domains of HGF are different, their near-UV CD spectra might be expected to show differences.

Biological activity: mitogenicity and cell scattering

NK1 exhibited mitogenic activity after refolding, as determined by [3 H]thymidine incorporation in B5/589 human mammary epithelial cells (Figure 5). The properly folded bacterially expressed protein (peak c_1 in Figure 1) at a concentration of 5 nM stimulated $\approx 35\%$ of the maximum mitogenic stimulation observed for full-length HGF at 0.5 nM (Figure 5, top panel). This level of mitogenicity is comparable with the results obtained using eukaryotically expressed NK1, as described previously [24]. Folded, prokaryotically derived NK1 produced a maximum stimulation of $\approx 60\%$ of the HGF-stimulated maximum at a 10-fold higher concentration (50 nM). NK1 protein obtained in peak b_1 (Figure 1) was less potent (25% of the maximum HGF-stimulated response at 5 nM, 40% of the maximum HGF-stimulated response at 50 nM) than properly folded protein (Figure 5, top panel). When cells were treated with a combination of HGF and folded (peak c_1) NK1 in 30-fold molar excess, the HGF-stimulated DNA synthesis was reduced to the maximum induced by NK1 (Figure 5, top panel). Thus bacterially expressed NK1 exhibits the same partial agonist and antagonist properties displayed by its eukaryotically expressed counterpart (Figure 5; [24]). Misfolded NK1 (peak b_1) also inhibited HGF-stimulated DNA synthesis, but with lower potency (Figure 5, top panel). This partial activity was not due to contamination with the correctly folded protein, as the analyses were performed on selected fractions (usually the peak apex) from the gel-filtration separations (Figure 1) that showed no evidence of this protein (peaks c or c_1) on re-chromatography.

NK2 purified from cultured mammalian cells lacks the partial agonist activity displayed by NK1 on B5/589 cells; in this setting NK2 acts only as an antagonist of HGF-stimulated DNA synthesis [23]. Prokaryotically expressed, properly folded NK2 (peak c) also lacked mitogenic activity on these cells (Figure 5, middle panel). When added together with HGF, a 10-fold molar excess of NK2 measurably decreased HGF-induced DNA synthesis, whereas 100-fold excess decreased HGF-stimulated mitogenesis by about 80% (Figure 5, middle panel). Misfolded (peak b) NK2 protein was substantially less effective in blocking HGF-stimulated DNA synthesis (Figure 5, middle panel). As mentioned above, the NK2 mutant C214A was constructed to prevent the formation of a glutathione-cysteine adduct at position 214 that occurred as a consequence of the refolding protocol. A comparison of NK2 C214A and wild-type NK2 revealed that they were identical both in their abilities to block HGF-stimulated DNA synthesis, and in their lack of mitogenic activity when

Figure 5 Effects of bacterially expressed HGF isoforms on DNA synthesis by B5/589 cells

DNA synthesis is expressed as a percentage of the maximum value observed following stimulation with HGF (0.5 nM). Concentrations (nM) of the truncated HGF isoforms tested are indicated on the abscissa. Top, NK1 peak c₁ + HGF (■); NK1 peak b₁ + HGF (●); NK1 peak c₁ (□); NK1 peak b₁ (○). Middle, NK2 peak c + EGF (■); NK2 peak b + HGF (●); NK2 peak b + EGF (▲); NK2 peak c (□); NK2 peak b (○); NK2 peak c + HGF (△). Bottom, NK2 C214A peak b + HGF (■); NK2 C214A peak c + HGF (●); NK2 peak c + HGF (▲); NK2 C214A peak b (□); NK2 C214A peak c (○); NK2 peak c (△).

tested alone (Figure 5, bottom panel). As reported previously, NK1, NK2 and HGF compete for binding to the same cell-surface receptor, the Met protein [23,24]. Like their eukaryotically expressed counterparts, the competitive antagonism of HGF-stimulated DNA synthesis displayed by the bacterially expressed truncated proteins was HGF specific: no effect on EGF-stimulated [³H]thymidine incorporation by B5/589 cells was observed (Figure 5, middle and bottom panels; results not shown).

MDCK cells express the HGF receptor, and in this particular cell line HGF potently stimulates cell movement and the concomitant loss of adhesion between neighbouring cells, a phenomenon known as 'cell scatter' [4]. Our early attempts to examine the effects of NK2 on MDCK cell scattering were limited by the amount of purified protein then available. NK1 and NK2 clearly stimulate cell scatter, although with less potency than HGF itself (Figure 6). The results indicate that the potencies of bacterially produced NK1 and NK2, folded and oxidized *in vitro* from denatured protein, are comparable with that of NK1 expressed as a soluble protein in Sf9 insect cells by a baculovirus vector [24], i.e. ≈ 25 –50-fold lower than for eukaryotically expressed

Figure 6 Comparison of bacterially expressed and baculovirus-expressed HGF isoforms on MDCK cell scatter

MDCK cells were seeded into 24-well tissue-culture plates, and left untreated (A), or treated with 0.1 nM HGF (B), 5 nM NK1 (C), 2.5 nM NK1 (D), 2.4 nM NK2 (E) or 1.7 nM NK2 (F) for 16 h, and then fixed and stained with Methylene Blue. Fields were chosen from duplicate wells that were representative of results obtained from three separate experiments. Magnification: 100 $\times$.

full-length HGF. In contrast, the individually prepared N and K1 domains of HGF had little or no activity in both the scatter and mitogenic assays (results not shown). However, recent work has demonstrated that the N-domain retains both the heparin-binding and ligand-oligomerization properties of HGF [51].

Ligand–receptor interactions: covalent affinity cross-linking and receptor autophosphorylation

Both truncated HGF isoforms were labelled with Na¹²⁵I using chloramine-T as described previously [24]. Conditions for NK1 iodination were devised that preserved biological activity, as determined in the [³H]thymidine incorporation assay. Radiolabelled protein had at least 50% of the specific mitogenic activity of the native protein, assuming 100% recovery of radiolabelled protein after iodination and heparin–Sepharose affinity chromatography (to remove unbound Na¹²⁵I; results not shown). NK2 was radiolabelled using the same protocol as that developed for NK1.

Covalent affinity cross-linking experiments using radiolabelled NK1 and NK2 demonstrated that both truncated isoforms interacted directly and specifically with the HGF receptor Met (Figure 7). A radiolabelled band with a molecular mass consistent with that expected of a ¹²⁵I-labelled NK1–Met β -subunit complex (assuming 1:1 binding stoichiometry) was observed, and was effectively displaced when 25-fold molar excess of unlabelled NK1 was included in the binding reaction (Figure 7A, left panel). The specificity of this displacement was demonstrated by the failure of an equivalent excess of unlabelled IGF-1 to displace

Figure 7 Ligand–receptor covalent affinity cross-linking

Covalent affinity cross-linking of ¹²⁵I-labelled NK1 (**A**) or ¹²⁵I-labelled NK2 (**B**) to Balb/MK cells. Samples for SDS/PAGE and autoradiography were prepared from cell lysates (left panels) or anti-Met immunoprecipitates (right panels). Estimated molecular masses of radiolabelled complexes are shown at the left. Cells were incubated with ¹²⁵I-labelled proteins alone (—), or in the presence of unlabelled ligands at 100-fold molar excess as indicated above each lane. To demonstrate the specificity of anti-Met recognition of the cross-linked protein complex, the sample in the extreme right lane in **A** was immunoprecipitated in the presence of the anti-Met peptide antigen (pep) at 100-fold molar excess.

¹²⁵I-labelled NK1 from this complex (Figure 7A, left panel). The same ¹²⁵I-labelled NK1 complex was immunoprecipitated with peptide antisera against Met, as shown in Figure 7(A) (right panel). Immunoprecipitation of the ¹²⁵I-labelled complex was inhibited by 100-fold molar excess of the peptide antigen, suggesting that immunological recognition by anti-Met sera was specific (Figure 7A, right panel).

The interaction of ¹²⁵I-labelled NK2 with Met is shown in Figure 7(B). Interestingly, covalent cross-linking of ¹²⁵I-labelled NK2 to its receptor on the surface of Balb/MK cells yielded three distinct radiolabelled complexes. The band migrating fastest in SDS/PAGE corresponded to the expected molecular mass of ¹²⁵I-labelled NK2–Met β -subunit complex, assuming 1:1 binding stoichiometry (Figure 7B, left panel). The estimated molecular mass of the next-slower migrating band could arise from several possible protein complexes: interaction with an immature, un-cleaved form of Met or with an alternatively spliced Met isoform [52], or cross-linking to the Met β -subunit with a ligand/receptor stoichiometry of 2:1. The most slowly migrating ¹²⁵I-labelled NK2 complex has the mass expected of a complex in which both α - and β -subunits of Met were cross-linked, a complex also observed previously [13]. ¹²⁵I-labelled NK2 in all three bands was displaced by excess unlabelled ligand, unaffected by excess unlabelled IGF-1, and immunoprecipitable with antisera to Met (Figure 7B). These results are comparable with previous observations made using eukaryotically expressed proteins ([13,24]; P. T. Wingfield, unpublished work).

Finally, we examined the ability of the prokaryotically expressed and refolded proteins to stimulate Met autophosphoryl-

Figure 8 HGF receptor phosphorylation

Autophosphorylation of the 145 kDa Met β -subunit in response to treatment of B5/589 cells with bacterially expressed NK1 or NK2, or baculovirus-expressed HGF. Cultures were left untreated (—), or incubated for 20 min with NK1 (3 nM), NK2 (10 nM) or HGF (1 nM) as indicated above each lane, and then processed for immunoprecipitation (IP) and immunoblotting using antibodies as indicated below each panel.

ation in intact B5/589 cells. To demonstrate receptor phosphorylation on tyrosine, cell lysates prepared after brief exposure to growth factor were immunoprecipitated with antisera to Met or anti-pY, followed by SDS/PAGE, transfer to PVDF, and immunoblotting using the antibody not used in immunoprecipitation (Figure 8). By these criteria, both truncated HGF isoforms were capable of stimulating Met autophosphorylation to levels previously observed for full-length HGF [24]. Thus the prokaryotically expressed, truncated HGF isoforms behave indistinguishably from their eukaryotically expressed counterparts with respect to high-affinity receptor binding and activation.

DISCUSSION

Protein purification

The HGF isoforms NK1 and NK2 were originally purified from mammalian and baculovirus expression systems by heparin affinity and size-exclusion chromatography [23,24]. Although neither of the smaller isoforms displayed the potent mitogenic activity characteristic of full-length HGF, both bound to the high-affinity HGF receptor Met, stimulated HGF receptor autophosphorylation [24,25], and exhibited agonist or antagonist activity in mitogenic and motility bioassays [23,24]. Further biological characterization of NK1 and NK2, and especially analysis of their structure, was impractical in light of the paucity of eukaryotically expressed protein. These circumstances provided a strong impetus to design an efficient prokaryotic expression system for the production of the large quantities of biologically active protein required for these studies.

Using the expression and fermentation protocols described, the HGF-derived proteins accumulate as aggregates in inclusion bodies. Due to the enrichment of the protein in these aggregates, we were able to purify solubilized protein by gel filtration under denaturing conditions. Following protein folding and oxidation, one additional gel-filtration step was required to resolve folded from misfolded protein (Figure 1). This simple purification procedure was particularly helpful because lysine affinity chromatography, used to purify other plasminogen-related proteins, could not be used, as all of the kringle-containing proteins described here had a low affinity for lysine (P. T. Wingfield, unpublished work). Moreover, the method was very efficient, enabling the purification of large amounts of the various HGF domains. For example, starting with 40–50 g wet weight of bacterial cells (from a 1 litre fermentation), about 200 mg of NK1 or 100 mg of NK2 was recovered. This represents a

significant improvement over previous methods; for example, Lokker and Godowski [53] reported a recovery of about 500 μg of NK1 from 100 g of *E. coli* cells, and baculovirus expression of NK1 yielded only 40 μg from 1 litre of conditioned medium [24].

Protein conformation and structure

Whereas there is a wealth of structural information on single kringle domains (e.g. [50]) and more recently on multikringle structures (e.g. [54]), there is little on any of the functional and structural domains of HGF. We have shown here that K1 of HGF appears to be structurally related to K4 of human PL based on the similarity of their respective near-UV CD spectra, a sensitive indicator of overall tertiary structure. These results are consistent with the approach taken by Lokker et al. [55], who modelled the structure of HGF K1 using the atomic co-ordinates from the X-ray structure of human PL K4. The N-terminal domain of HGF is proposed to contain a hairpin loop, based on the predicted disulphide bonding pattern (C70–C96, C74–C84), and our spectroscopic data indicate that it consists predominantly of β -sheet and β -turn, similar in this respect to kringle domains, with a small ($\approx 5\%$) helical contribution. It is worth mentioning that for the protein domains studied here by CD, all of which contain substantial β -sheet structure, more reliable estimates of secondary structure might be expected using Fourier transform IR spectroscopy [56].

Although the individual N and K1 domains of HGF have limited biological activity, they appear folded in a physiologically relevant manner, based on the spectroscopic evidence summarized in Figure 4. Here it was shown that the weighted average CD spectra of the individual domains almost perfectly match, especially in the near-UV region, that of biologically active NK1. This result also suggests that in NK1 there are unlikely to be extensive protein–protein interactions between the N-terminal and K1 domains. Such interactions might perturb local and global conformation, resulting in potential spectroscopic change. However, molecular details of the HGF domain structure and its organization will require high-resolution structure determinations.

The spectral and physical properties of the misfolded NK1 and NK2 proteins purified as a by-product (Figure 1) are of interest. First, they illustrate that conclusions on protein conformation based on CD measurements from one spectral region only, usually the far-UV region, can be misleading. For example, proteins such as elastase, which contain little or no α -helix and a high portion of β -sheet, often in short irregular strands (classified as β -II proteins), have far-UV spectra that can easily be mistaken for those of denatured protein [46]. Similarly, the NK1 and NK2 variants also appear fully denatured in the far-UV region, yet, according to their more conformationally sensitive near-UV spectra, appear to be partially folded. The latter is confirmed by their biological activity, albeit at a reduced level. In contrast, protein-folding intermediates, termed molten globules, often have native-like far-UV CD spectra but appear denatured in the near-UV region [57]. Secondly, the misfolded proteins, unlike most partially or fully denatured proteins, are soluble monomers under physiological conditions. Further structural investigation and determination of the disulphide linkage pattern in these variants may provide some insight into structure–function relationships of the growing family of plasminogen-like proteins.

Biological activity

The results of our biological and biochemical analyses provide further evidence that the bacterially expressed proteins attain

native conformations. In mitogenic and motility assays, as well as in cross-linking and tyrosine-phosphorylation experiments that directly examined receptor binding and activation, the recombinant proteins described here were indistinguishable from eukaryotically expressed proteins. In addition, the mutant form of NK2 (C214A), which was prepared to prevent glutathionylation of the unpaired cysteine-214, displayed similar bioactivity as wild-type NK2, supported by similar spectroscopic properties (CD results not shown). NK1 produced using methods described herein appears more potent in stimulating DNA synthesis and tyrosine phosphorylation than the bacterially expressed NK1 preparation described by others [53,58]. It is possible that the latter material, which was generated with a protocol lacking a specific step to optimize disulphide formation, contained misfolded protein similar to the product we isolated in side fractions following gel filtration.

Conclusions

We have described methods that enable the production of large quantities of well characterized subdomains of HGF. The proteins have similar physicochemical and biological activities to the corresponding eukaryotically expressed proteins, with the advantage of a much greater yield of protein. The fidelity of our NK1 and NK2 preparations in multiple assays also suggests that these proteins will be a reliable and valuable resource for detailed structural analysis by NMR and X-ray crystallography.

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