Isolation of the murine S100 protein MRP14 (14 kDa migration-inhibitory-factor-related protein) from activated spleen cells: characterization of post-translational modifications and zinc binding

Mark J. RAFTERY*, Craig A. HARRISON*, Paul ALEWOOD†, Alun JONES† and Carolyn L. GECZY*‡

*Immunology Group, The Heart Research Institute, 145 Missenden Road, Camperdown, New South Wales 2050, Australia, and

†Centre For Drug Design and Development, University of Queensland, St. Lucia, Queensland 4072, Australia

MRP14 (macrophage migration-inhibitory factor-related protein of molecular mass 14 kDa) is an S100 calcium binding protein constitutively expressed in human neutrophils which may be associated with cellular activation/inflammation. Murine MRP14 expression was up-regulated following concanavalin A activation of spleen cells, and the protein was isolated from conditioned medium in high yield (approx. 500 ng/ml). MRP14 had a mass of 12972 ± 2 Da by electrospray ionization MS, whereas the theoretical mass derived from the cDNA sequence, after removal of the initiator Met, was 12918 Da, suggesting that the protein was post-translationally modified. We identified four post-translational modifications of MRP14: removal of the N-terminal Met, N-terminal acetylation, disulphide bond formation between Cys⁷⁹ and Cys⁹⁰, and 1-methylation of His¹⁰⁶; the calculated mass was then 12971.8 Da. Methylation of His¹⁰⁶ was

further characterized after incubation of spleen cells with L-[methyl-³H]Met during concanavalin A stimulation. Sequential analysis of a peptide (obtained by digestion with Lys C) containing methylated His indicated that > 80 % of the label in the cycle corresponded to His¹⁰⁶, suggesting that the methyl residue was transferred from S-adenosyl-L-methionine. Comparison of the C18 reverse-phase HPLC retention times of phenylthiocarbamoyl derivatives of a hydrolysed digest peptide of MRP14 with those of standards confirmed methyl substitution on the 1-position of the imidazole ring. MRP14 bound more ⁶⁵Zn²⁺ than the same amounts of the 10 kDa chemotactic protein (CP10) or S100β. Ca²⁺ decreased Zn²⁺ binding in S100β but it did not influence binding to MRP14, suggesting that the Zn²⁺ binding site was distinct from and independent of the two Ca²⁺ binding domains.

INTRODUCTION

MRP14 (migration-inhibitory-factor-related protein of molecular mass 14 kDa), also known as p14, L1 heavy chain, calgranulin B, and with the recent designation S100A9 [1], is a small acidic protein containing two Ca²⁺ binding EF hands that belongs to the highly conserved S100 protein family [2,3]. These proteins may influence diverse cellular processes, including cell cycle progression, cell differentiation, regulation of protein phosphorylation and cytoskeleton–membrane interactions [4,5]. We previously isolated CP10 (chemotactic protein of 10 kDa), an S100 protein with 59 % amino acid and 64 % nucleotide identity (sequences were compared using the Genetics Computer Group Package, Version 8, Madison, WI, U.S.A.) with human 8 kDa MRP (hMRP8), from supernatants of activated murine spleen cells [6]. CP10 is a potent chemotactic agent for murine and human neutrophils and monocytes/macrophages [7], and causes a sustained inflammatory reaction *in vivo* [7–9]. Macrophages recruited by CP10 can form foam cells *in vivo*, and CP10 may play a major role in the development of atherosclerosis [9].

The two EF hands of S100 proteins are separated by a ‘hinge region’ which has little amino acid sequence identity between members and which may confer functional specificity if exposed

after Ca²⁺ binding [4]. We showed that a synthetic peptide of the hinge region of CP10 [CP10-(42–55)] was chemotactic, whereas a peptide synthesized according to the amino acid sequence of the hinge region of hMRP8 was not [7]. The C-terminal regions of different members of the S100 family are also divergent, but to date only a peptide with sequence identity to the C-terminal domain of human MRP14 (hMRP14) has a potential function [3]. The C-terminal portion is identical to part of the sequence of neutrophil-immobilizing factor (NIF), which inhibits neutrophil chemotaxis in response to C5a and formyl-Met-Leu-Phe [3,10], but the precise role of hMRP14 in leucocyte migration is unclear.

Using a monoclonal antibody to migration inhibitory factor, hMRP14 was isolated from supernatants of activated human peripheral blood mononuclear cells as part of a complex [11]. Matrix-assisted laser desorption ionization MS indicated that hMRP14 consisted of two molecular forms: the full-length protein and another truncated at the N-terminus by four amino acids, attributed to an alternative AUG start codon in the mRNA [12]. The apparent molecular mass, by SDS/PAGE, of hMRP14 was approx 14000 Da, which is unusually high for members of this family and was due to a longer C-terminal domain.

To examine a potential role for murine MRP14 (mMRP14) in

Abbreviations used: MRP14, migration-inhibitory-factor-related protein of molecular mass 14 kDa (the prefixes h and m denote human and murine respectively); CP10, chemotactic protein of molecular mass 10 kDa; NIF, neutrophil immobilizing factor; Con A, concanavalin A; SAM, S-adenosyl-L-methionine; 1-methyl-His, 1-methyl-L-histidine (or π -methyl-L-histidine); 3-methyl-His, 3-methyl-L-histidine (or τ -methyl-L-histidine); PVDF, polyvinylidene difluoride; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; ESI MS, electrospray ionization MS; PTH, phenylthiohydantoin; PTC, phenylthiocarbamoyl; HFBA, heptafluorobutyric acid; TBS, Tris-buffered saline; Ac, acetyl.

‡ To whom correspondence should be addressed.

the regulation of chemotaxis provoked by CP10 and other chemoattractants, it was first necessary to isolate and determine the structural characteristics of this protein. The amino acid sequence of mMRP14 deduced from the cDNA sequence indicates that it comprises 113 amino acids; the regions of greatest identity with the human protein are the Ca^{2+} binding EF hand domains, and those with least similarity occur in the hinge region and the extended C-terminal portion.

We describe here the expression of MRP14 in murine spleen and bone marrow cells and its isolation from concanavalin A (Con A)-activated spleen cell supernatant. The post-translational modifications of mMRP14 were elucidated using a combination of MS, endoproteinase digestion and N-terminal sequencing. Four post-translational modifications were identified: removal of the N-terminal Met, N-terminal acetylation, disulphide bond formation between Cys⁷⁹ and Cys⁹⁰, and 1-methylation of His¹⁰⁶. The modifications described are distinct from those reported for hMRP14 [12, 13], in which a protein kinase C-independent, Ca^{2+} -dependent, phosphorylation of the C-terminal Thr¹¹³ is proposed to play a role in intracellular signalling [13].

MATERIALS AND METHODS

General materials

Reagents and chemicals were analytical grade, and solvents were HPLC grade (Mallinckrodt, Clayton South, Vic., Australia). 1-Methyl-L-histidine (1-methyl-His) and 3-methyl-His were from Sigma (St. Louis, MO, U.S.A.). Activated murine spleen cell supernatants prepared as outlined previously [14] were frozen at -80°C until use.

SDS/PAGE and Western blotting

Murine bone marrow and spleen cells were isolated by standard procedures and frozen at -80°C in lysis buffer until used. Cells (10^7) were lysed for 20 min with Triton X-100 (0.1 %)/Tris (100 mM, pH 8.1, 1 ml) containing the proteinase inhibitors PMSF (5 mM), leupeptin (5 mM) and benzamidine (5 mM). Total protein content was determined using the BCA assay (Pierce, Rockford, IL, U.S.A.). Equivalent amounts of protein (10 μg) loaded on each lane of an SDS/Tricine/15 % polyacrylamide gel [15] and separated using a Mini-Protean II apparatus (Bio-Rad, Hercules, CA, U.S.A.) were silver stained [16]. Western blotting was performed using the Mini Trans-blot cell (Bio-Rad) following the manufacturer's instructions. Briefly, after SDS/PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, U.S.A.) and antigen detected with a rabbit polyclonal anti-MRP14 antibody, prepared as described by Kocher et al. [17]. Bound antibody was visualized using enhanced chemiluminescence (Amersham) with a horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chain; Bio-Rad) antibody.

Liquid chromatography

Liquid chromatographic separations were performed using a non-metallic LC 625 HPLC system (Waters, Bedford, MA, U.S.A.), and UV absorbance was monitored at 214 nm and 280 nm with a Waters 490 UV/visible detector.

The procedure of Ryan and Geczy [14] for anion-exchange chromatography was followed. Briefly, DEAE-Sepharose (Pharmacia, Uppsala, Sweden) was packed according to the manufacturer's instructions into a 150 mm $\times$ 10 mm column (AP-1; Waters). After ultrafiltration, the concentrated activated spleen cell supernatant was dialysed (50 mM Tris, 50 mM NaCl, 0.01 %

Tween 20, pH 7.9; 16 h), clarified by centrifugation (12000 g, 30 min, 4°C) and then loaded on to the DEAE column at a flow rate of 1 ml/min. The flow-through was collected for further separation. Heparin affinity chromatography was essentially as described [14], but employed two heparin EconoPak cartridges (5 ml; Bio-Rad) connected in series and equilibrated with 50 mM Tris, 50 mM NaCl, 0.01 % Tween 20, pH 7.0. The DEAE flow-through, adjusted to pH 7.0 with HCl, was loaded on to the cartridge at 1 ml/min. The cartridge inlet was connected to the LC-625 and the flow rate increased to 2.0 ml/min. When the A_{280} had returned to approx 0 V, bound proteins were eluted with a gradient of 50 mM–1.0 M NaCl over 20 min and fractions were collected at 1 min intervals. Selected fractions were analysed by C4 reverse-phase (RP)-HPLC. A Vydac (Separations Group, Hesperia, CA, U.S.A.) 300 Å (30 nm), 5 μm , 250 mm $\times$ 4.6 mm C4 RP column and a gradient of 25–70 % acetonitrile [0.1 % trifluoroacetic acid (TFA)] at 1 ml/min over 30 min were used. Fractions with major absorbance at 214 nm were collected.

Small amounts of hMRP14 were isolated from human blood neutrophils by the procedure described in [3].

Protein digests

Proteins isolated from C4 RP-HPLC were digested after reducing the volume of eluent (Speedvac; Savant, Farmingdale, NY, U.S.A.) to approx. 250 μl ; ammonium bicarbonate (250 μl , 100 mM, pH 8.0) was added, followed by the endoproteinase. Digests using endoproteinase Lys C, endoproteinase Glu C or trypsin (sequencing grade; Boehringer Mannheim, Castle Hill, NSW, Australia) used an enzyme/substrate ratio of approx. 1:100 at 37°C for 18 h. TFA or heptafluorobutyric acid (HFBA; 1 %) were used to adjust the pH to approx. 2, and digests were applied directly to a C18 RP column (Vydac; 300 Å, 5 μm , 250 mm $\times$ 4.6 mm). Lys C- and Glu C-derived peptides were eluted with a gradient of 5–75 % acetonitrile (0.1 % TFA) at 1 ml/min over 30 min, and tryptic peptides with 5–75 % acetonitrile (0.05 % HFBA) at 1 ml/min over 30 min. Fractions with absorbance at 214 nm were collected manually. Fractionated proteins or digest peptides (typically 250–500 pmol) were N-terminally sequenced using an Applied Biosystems model 473 automated gas phase sequencer (Applied Biosystems, Burwood, Vic., Australia) at the Sydney University Macromolecular Analysis Centre.

Carboxyamidomethylation of MRP14

MRP14 (5 nmol), either native or reduced (dithiothreitol, 100 mM), was isolated by C4 RP-HPLC and treated with iodoacetamide (19 μg ; 100 nmol) in ammonium bicarbonate (100 mM; 250 μl) in the presence or absence of guanidine hydrochloride (6 M) for 30 min at 30°C . After acidification (1 % TFA) the mixture was loaded on to the C4 RP column and protein was eluted with a gradient of 25–70 % acetonitrile (0.1 % TFA) at 1 ml/min over 30 min.

Mass spectrometry

Samples collected from the C4 (proteins) and the C18 (digest peptides) RP columns were concentrated to approx. 20 pmol/ μl (Speedvac). Mass spectra were acquired on a triple quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source (PE-Sciex Instruments, Thornhill, Canada). Samples were injected into a moving solvent [20 μl /min; 1:1 (v/v) water/acetonitrile, 0.05 % TFA] coupled directly to the ionization source via a fused silica capillary interface

(50 $\mu\text{m} \times 50 \text{ cm}$). Sample droplets were ionized at a positive potential of 5 kV. The ions were extracted into the analyser through an interface plate and subsequently through an orifice (100–200 μm) at a potential of 80 V. Full scan mass spectra were acquired over the mass range 600–2000 Da with a scan step of 0.2 Da.

Collision-induced dissociation of the precursor ion was effected by bombardment with ultrapure argon. Bombardment was carried out in quadrupole-2 with a collision cell gas thickness of 3×10^{14} atoms/cm² and a collision energy (Q-0 to Q-2 rod offset voltage) of typically 25–35 eV (relative to the laboratory frame). The resulting collision-induced dissociation spectra were obtained by scanning quadrupole-3 from 50 to 2400 Da in 4 s with a step size of 0.2 Da. Interpretation of the spectra was aided by the software application Peptide Scan (PE-Sciex).

Incorporation of L-[methyl-³H]methionine

RPMI 1640 (400 ml; Gibco-Life Technologies, Grand Island, NY, U.S.A.) was supplemented with L-[methyl-³H]Met (1 mCi; 85 Ci/mmol; Amersham). Spleen cells (2×10^7 /ml) were incubated for 3 h at 37 °C in an atmosphere of 5% CO₂ in air, then Con A-Sepharose (1 $\mu\text{g}/\text{ml}$; Pharmacia) was added and incubation continued for 40 h. A slightly modified procedure was used to isolate radiolabelled MRP14. Briefly, after addition of the appropriate proteinase inhibitors [14], the supernatant was centrifuged at 8000 *g*, dialysed against Tris (50 mM), NaCl (50 mM) and Tween 20 (0.01%), pH 7.0, for 16 h, then loaded directly on to the heparin affinity column; proteins were eluted as described above. Radiolabelled MRP14 was separated by C4 RP-HPLC and treated with endoproteinase Lys C, and digest peptides were isolated as described above. Tritium incorporation into the protein, peptide or phenylthiohydantoin (PTH)-amino acid was determined, after addition of scintillant (0.5 ml; Ultima Gold; PIC, Meriden, CT, U.S.A.), using an LBK 1214 RackBeta (LBK-Wallac, Turku, Finland) liquid scintillation counter.

Amino acid analysis

The peptide GHGHS(H_{Me})GK isolated from the tryptic digest of MRP14 was hydrolysed with HCl (6 M) at 155 °C for 55 min, then HCl was removed (rotary concentrator) and neutralized. Phenylthiocarbamoyl (PTC)-amino acids were prepared by reaction with 10% phenylisothiocyanate at 23 °C for 20 min; excess phenylisothiocyanate was removed by extraction with heptane and PTC-amino acids were analysed using an Exsil GoldPak C18 RP column (250 mm $\times$ 4.6 mm; Activon, Thornleigh, NSW, Australia) at 40 °C and a Waters 600E multisolvent delivery system. The gradient used was [A, sodium acetate (0.14 M, pH 5.75) mixed with B, acetonitrile]: 6% B, 4 min; 6 to 20% B, 5 min; 20 to 35% B, 9.5 min; 35 to 100% B, 25 min; the flow rate was 1.5 ml/min and monitoring was at *A*₂₅₄. The relative retention times of peptide-derived PTC-amino acids were compared with those of a standard mixture of PTC-amino acids (Waters) and synthetic PTC-1- and 3-methyl-His.

⁶⁵Zn binding

Bovine S100 β (500 ng; Sigma), CP10 (500 ng), mMRP14 (650 ng) and hMRP14 (400 μg) separated by SDS/PAGE were transferred to a PVDF membrane. Membranes washed with Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5; $3 \times 7 \text{ ml}$) for 15 min were incubated with agitation with ⁶⁵ZnCl₂ (15 μCi ; 10 mCi/mmol; Amersham) in TBS (7 ml), TBS + ZnCl₂ (3 mM; 7 ml) or TBS + CaCl₂ (3 mM; 7 ml) for 60 min, washed with TBS (7 ml) for $3 \times 10 \text{ min}$, and radioactivity located using a phos-

phorimager (Bas-1000; Fujix). Images were analysed using the MacBas program (Kohshin Graphics Systems).

RESULTS

Identification of MRP14 in cellular extracts and isolation from Con A-activated spleen cell supernatant

Lysates of freshly isolated spleen cells contained negligible monomeric or dimeric MRP14 (Figure 1, lanes 2 and 5), compared with the reactivity of pure MRP14 with a rabbit anti-MRP14 polyclonal antibody which detected both forms (lane 1). In contrast, high levels of monomeric MRP14 were evident in supernatants of activated spleen cells (Figure 1, lanes 3 and 6) and in lysates of bone marrow cells (lanes 4 and 7). The major band had an apparent molecular mass of approx. 14000 Da, which was not altered by reduction. Although disulphide-linked heterodimers of CP10 and MRP14 also reacted with this anti-MRP14 antibody (results not shown), homo- or hetero-dimers were not evident in non-reduced samples of conditioned medium or bone marrow cells (Figure 1, lanes 3 and 4), indicating that MRP14 was predominantly in the monomeric form.

A total of 4 litres of supernatant yielded approx. 2 mg of each protein. The heparin affinity and C4 RP-HPLC chromatograms are shown in Figures 2(A) and 2(B) respectively. MRP14 isolated by C4 RP-HPLC contained less than 5% of the disulphide-linked homodimer, which was not resolved from the monomer and was confirmed by Western blotting (Figure 1, lane 1). A single band at 14000 Da was present after reduction (results not shown). Low levels of disulphide-linked CP10 homodimer were eluted from C4 RP-HPLC immediately after CP10 (Figure 2B).

Figure 1 Identification of MRP14 in lysed spleen cells (lanes 2 and 5), supernatants of activated spleen cells (lanes 3 and 6) and lysed bone marrow cells (lanes 4 and 7)

Samples were analysed by SDS/PAGE (15% gels) in the absence (lanes 2–4) or presence (lanes 5–7) of dithiothreitol (100 mM). Equal amounts of total protein (10 μg) were loaded. Fractionated native MRP14 (250 ng) was used as standard (lane 1). Detection with an anti-MRP14 antibody is described in the Materials and methods section. Non-specific reactivity of the second antibody was assessed using a sample of activated spleen cell supernatant and omitting the anti-MRP14 antibody (lane 8). Molecular masses (kDa) are indicated on the left. Bands at 25, 50 and 150 kDa correspond to light chain, heavy chain and IgG respectively known to be present in these cell types.

Electrospray ionization (ESI) MS of native and carboxyamidomethylated MRP14

The ESI mass spectrum of MRP14 produced one series of multiply charged ions with a reconstructed mass of $12972 \pm 2 \text{ Da}$ (Figure 2C). The average mass calculated from the derived cDNA sequence, after removal of the initiator Met, was 12918 Da [18], some 54 Da less than the experimentally determined value. In contrast, the ESI mass of CP10 was $10164 \pm 2 \text{ Da}$, in good

Figure 2 Fractionation of MRP14 and CP10 from Con A-activated spleen cell supernatant

(A) Elution of MRP14 and CP10 from the heparin affinity cartridge. MRP14 and CP10 were eluted at approx 0.25–0.3 M NaCl (bar). (B) C4 RP-HPLC chromatogram [25–70% acetonitrile (0.1% TFA); 30 min] of the heparin fraction eluted as indicated in (A). (C) ESI mass spectrum of native MRP14 eluted from C4 RP-HPLC with a retention time of 17.4 min. A series of multiply charged ions corresponds to an average mass of 12972 ± 2 Da (inset).

agreement with the theoretical mass of 10163.4 Da [7]. Because the error in average mass of proteins can be as low as 0.005% using this technique [19], the mass indicated either an incorrect sequence or potential post-translational modifications.

The cDNA sequence of MRP14 indicated three cysteines in the protein. A single peak was eluted from C4 RP-HPLC after reaction with iodoacetamide in the presence or absence of guanidine hydrochloride; the retention time of the carboxyamido-methylated protein was approx. 25 s less than that of native MRP14. MS of carboxyamido-methylated MRP14 produced one series of multiply charged ions with a reconstructed mass of 13029 ± 2 Da (i.e. +57 Da), corresponding to the addition of a single carboxyamido-methyl group. Reduction of native or de-

natured MRP14 with dithiothreitol produced a single peak by C4 RP-HPLC which had a retention time 65 s greater than that of native MRP14; alkylation of reduced MRP14 yielded a mass of 13143 Da (+171 Da), corresponding to the addition of three carboxyamido-methyl groups to the native protein.

Characterization of the post-translational modifications of MRP14

To locate post-translational modifications, peptides derived from MRP14 treated with endoproteinase Glu C were separated by C18 RP-HPLC; their retention times and relative intensities are shown in Table 1. With the exception of peptide V2 (mass 1017 Da), digest peptides were assigned probable structures by comparing their experimentally determined mass with calculated masses of peptides from the hypothetical digestion of the protein derived from the cDNA sequence (Table 1). Sequencing indicated that the N-terminus of V2 was blocked and was therefore assumed to correspond to the N-terminal digestion product. The predicted mass of this peptide (MANKAPSQME) was 1106 Da, whereas the experimental mass was 89 Da less. Acetylation of the N-terminal alanine, after removal of the initiator Met, would yield a peptide of calculated mass 1017.1 Da.

The product ion spectrum of V2 generated after collisional activation of the single charged parent ion (m/z 1018 Da) enabled partial sequencing of V2 by MS. A series of N-terminal b-type ions were observed: b_1 , 114 Da; b_2 , 226 Da; b_3 , 356 Da; b_4 , 427 Da; these corresponded to the acylium ions $[N\text{-AcA}]^+$, $[N\text{-AcAN}]^+$, $[N\text{-AcANK}]^+$ and $[N\text{-AcANKA}]^+$ respectively (where Ac is acetyl). Another ion at 905 Da was attributed to a y-type fragmentation (y_8) which corresponded to the loss of the N-terminal amino acid (N-AcA) from the parent ion. These data confirm that peptide V2 was derived from the N-terminus of MRP14, the Met was removed and the N-terminal Ala was N-acetylated to yield the sequence N-AcANKAPSQME.

Based on their theoretical masses, peptides V8 and V9 (Table 1) were attributed to fragments containing residues 79–96 and 79–92 respectively; each would contain Cys at positions 79 and 90. Their experimental masses suggested the presence of a disulphide bond between these residues (Table 1). After treating V9 with endoproteinase Lys C the retention time of the single product from C18 RP-HPLC decreased from 20.8 min (Table 1, V9) to 17.02 min. The mass of this peptide increased from 1639 Da (V9) to 1657 Da, corresponding to the addition of water (18 Da). This peptide, produced after internal cleavage of the Lys–Leu bond, confirmed a disulphide linkage between Cys⁷⁹ and Cys⁹⁰. N-terminal sequence analysis of the first five amino acids of the Lys C-treated V9 peptide detected two free N-termini, consistent with the sequences CMMLMAK and LIFACHE and a disulphide bond between the Cys⁷⁹ and Cys⁹⁰.

Additional peptides were produced by Lys C digestion (which overlapped with the Glu C digest) of MRP14, and their retention times and relative intensities are outlined in Table 2. Except for L2 (1691 Da), all were assigned probable structures based on the digestion pattern of the protein predicted from the cDNA sequence. The likely sequence, by Edman degradation, of this anomalous peptide was LHENNPRGHGHSAGK, although the calculated mass of the peptide containing Ala is 1611 Da, 80 Da lower than the experimental mass. The peptide corresponded to a C-terminal portion of MRP14 [MRP14-(94–108)] and the cDNA sequence prediction is LHENNPRGHGHSAGK, of calculated mass 1677 Da, 14 Da lower than the experimental mass. Figure 3 compares the C18 RP-HPLC trace of a standard PTH-amino acid mixture with the PTH-amino acid obtained after cycle 15 (residue 13) of peptide L2. The retention time was 10.75 min, somewhat greater than that of PTH-Ala (10.48 min)

Table 1 Comparison of the observed ESI MS mass of peptides obtained from MRP14 following Glu C digestion with the theoretical sequence and mass of peptides derived from Glu C digestion of the protein obtained from the cDNA sequence

Retention times were obtained from C18 RP-HPLC. Values in parentheses are relative intensities (%) at 214 nm. The predicted mass is the average calculated molecular mass. ** indicates the presence of an internal disulphide bond between the two Cys residues.

HPLC peak no.	Retention time (min)	Observed mass (Da)	Predicted residue nos.	Peptide structure	Predicted mass (Da)
V0	9.38 (8)	525	53–56	KLHE	525.6
V1	10.1 (68)	1111	27–36	GHPDTLSKKE	1111.2
V2	10.6 (44)	1017	1–9*	N-AcANKAPSQME*	1017.1*
V3	12.9 (42)	809	37–42	FRQMVE	809.0
V4	14.0 (36)	1693	43–56	AQLATFMKKEKRNE	1694.0
V5	14.5 (11)	1166	43–52	AQLATFMKKE	1166.4
V6	16.9 (100)	2095	10–26	RSITTIIDTFHQYSRKE	2095.3
V7	20.3 (32)	†			
V8	19.5 (10)	2146	79–96	**CMMLMAKLIFA**CHEKLHE	2146.7
V9	20.8 (22)	1639	79–92	**CMMLMAKLIFA**CHE	1639.1

* Peptide V2 was sequenced by MS; see text for details.

† No ions were observed in the mass spectrum of V7.

Table 2 Comparison of the observed ESI MS mass of peptides obtained from MRP14 following Lys C digestion with the theoretical sequence and mass of peptides derived from Lys C digestion of the protein obtained from the cDNA sequence

Retention times were obtained from C18 RP-HPLC. Values in parentheses are relative intensities (%) at 214 nm. The predicted mass is the average calculated molecular mass.

HPLC peak no.	Retention time (min)	Observed mass (Da)	Predicted residue nos.	Peptide structure	Predicted mass (Da)
L1	9.5 (5)	819	4–10	APSQMER	817.9
L2	9.7 (100)	1691*	94–108*	LHENNPRGHGHS(H _{Me})GK*	1690.8*
L3	9.9 (70)	1111	26–35	EGHPDTLSKK	1111.2
L4	10.2 (4)	983	26–34	EGHPDTLSK	983.1
L5	13.4 (7)	†			
L6	14.9 (6)	1810	11–25	SITTIIDTFHQYSRK	1810.1
L7	18.1 (70)	1959	35–51; 37–52	(K)EFRQMVEAQLATFMK(K)	1957.3‡
L8	18.7 (65)	1830	37–51	EFRQMVEAQLATFMK	1829.2
L9	19.1 (15)	2610	4–25	APSQMERSITTIIDTFHQYSRK	2610.0

* The N-terminal sequence obtained was LHENNPRGHGHS_{Me}GK (see text for details).

† No ions were observed in the mass spectrum of L5.

‡ Two possible digest peptides with a molecular mass of 1957 Da are given.

Figure 3 Partial N-terminal sequence analysis of peptide L2

(A) PTH derivatives of an unmodified amino acid standard mixture separated on C18 RP-HPLC. (B) The retention time of the PTH derivative of an *N*-methyl-His residue obtained after N-terminal sequencing of the peptide LHENNPRGHGHS(H_{Me})GK (cycle 15, corresponding to residue 13) is indicated. Major peaks with retention times of 8.9 min and 18 min corresponded to dimethylphenylthiourea and diphenylthiourea respectively, which are by-products of the derivatization process.

and it eluted as a broad peak (Figure 3B). Reproducible results from three separate experiments strongly indicated that residue 13 of L2 was not Ala. If the predicted His at residue 13 were methylated, the calculated mass would be 1690.8 Da. Furthermore, the retention time of the PTH derivative of methyl-His from C18 RP-HPLC is almost identical to that of PTH-Ala, except that PTH-methyl-His elutes as a broader peak [20]. Synthetic 1-methyl-His and 3-methyl-His were converted into their PTH derivatives and analysed by C18 RP-HPLC using the same conditions as for sequencing peptide L2. The retention times of PTH-1-methyl-His and -3-methyl-His were identical (10.7 min) and the same as that observed for residue 13 of peptide L2 (10.75 min).

Further characterization of methylated His¹⁰⁶

To investigate whether methylation of His¹⁰⁶ was specific, and to identify the source of the transferred group, L-[methyl-³H]Met was added to the culture medium before stimulation of spleen cells with Con A, allowing incorporation of L-[methyl-³H]Met into *S*-adenosyl-L-methionine (SAM) [21] and subsequent in-

Figure 4 Sequential analysis of ^3H incorporated into each amino acid in the peptide LHENNPRGHGS(H_{Me})GK

Analysis was performed after Edman degradation as outlined in the Materials and methods section.

corporation of radiolabelled methyl into newly synthesized MRP14. Lys C digest peptides L2, L7, L8 and L9 were labelled; radioactivity in peptides L7, L8 and L9 could be explained by the incorporation of L-[methyl- ^3H]Met. Peptide L2 did not contain Met (Table 2); this peptide was N-terminally sequenced and PTH-amino acids derived from each cycle of Edman degradation were sequentially isolated and analysed for radioactivity. PTH derivatives corresponding to amino acids 1, 13 and 14 incorporated the majority of the radioactivity, with most in amino acid 13 (Figure 4). This corresponded to the predicted position of the methylated His residue in peptide L2. The low amount of

Figure 5 C18 RP-HPLC of PTC derivatives

PTC derivatives are of (A) synthetic 1-methyl-His, (B) synthetic 3-methyl-His, and (C) amino acids obtained after hydrolysis of the peptide GHGHS(H_{Me})GK obtained from a tryptic digest of MRP14. Retention times (RTs) of the derivatives are indicated.

Figure 6 $^{65}\text{Zn}^{2+}$ binding to S100 β (lanes 1, 5 and 9), CP10 (lanes 2, 6 and 10), mMRP14 (lanes 3, 7 and 11) and hMRP14 (lanes 4, 8 and 12)

The proteins were separated by SDS/PAGE and transferred to PVDF membranes as described in the Materials and methods section. The membranes were incubated with $^{65}\text{Zn}^{2+}$ in TBS (lanes 1–4), TBS + 3 mM ZnCl_2 (lanes 5–8) or TBS + 3 mM CaCl_2 (lanes 9–12) and washed (TBS); radioactivity was located using a phosphorimager. Molecular mass markers are shown (lane 13).

label in the fraction corresponding to amino acid 1 may have been due to incomplete binding of the peptide in the polybrene used in the automated sequencing or to degradation of the peptide after isolation or storage, and that in amino acid 14 was probably due to carry-over from residue 13.

The isomers of synthetic PTH-1-methyl-His and PTH-3-methyl-His were not resolved by C18 RP-HPLC using a variety of conditions (results not shown), whereas those of the PTC derivatives can be resolved by RP-HPLC [22]. The C18 RP-HPLC retention times were 10.77 min for PTC-1-methyl-His (Figure 5A) and 11.4 min for PTC-3-methyl-His (Figure 5B). To simplify the PTC RP-HPLC chromatogram, a small hydrophilic peptide [GHGHS(H_{Me})GK] produced by tryptic digestion of MRP14 was isolated by C18 RP-HPLC using HFBA (0.05%) as co-solvent. ESI MS gave a mass of 830 Da, and N-terminal analysis indicated the sequence GHGHS(H_{Me})GK, confirming homogeneity and methylation of His¹⁰⁶. C18 RP-HPLC analysis of the PTC-derivatized amino acids produced five peaks following hydrolysis: PTC-Ser, -Gly, -His, -1-methyl-His and -Lys, in relative proportions of approx. 1:3:2:1:1 (Figure 5C). Peaks at 12.86, 17.25, 17.76, 19.29 and 20.6 min were by-products of the derivatization reaction. The retention time of the peak corresponding to PTC-1-methyl-His was 10.66 min, which compared well with that of synthetic PTC-1-methyl-His (10.77 min; Figure 5A). Synthetic PTC-3-methyl-His had a retention time of 11.4 min (Figure 5B). Co-injection of PTC-1-methyl-His and the PTC derivative of the hydrolysed peptide mixture indicated co-elution of the PTC-1-methyl-His peaks (results not shown).

Comparison of ^{65}Zn binding to MRP14 and other S100 proteins

hMRP14 and mMRP14 both bound more $^{65}\text{Zn}^{2+}$ (Figure 6, lanes 3 and 4) than similar quantities of CP10 or S100 β (lanes 1 and 2). All $^{65}\text{Zn}^{2+}$ binding was inhibited by excess ZnCl_2 (lanes 5–8). Excess CaCl_2 partially inhibited Zn^{2+} binding to S100 β and CP10 (lanes 9 and 10), whereas binding to hMRP14 and mMRP14 was not affected (lanes 11 and 12).

DISCUSSION

The amino acid sequence of mMRP14 was deduced from the cDNA sequence [18], a technique that does not identify post-translationally modified amino acids. Here we describe the isolation and characterization of the post-translational modifications of MRP14, including an unusual 1-methylation of His¹⁰⁶.

Figure 7 Comparison of the structures of mMRP14 (top) and hMRP14 (bottom)

Two gaps were incorporated into the sequences to allow optimal alignment. Post-translational modifications of mMRP14 were deletion of the N-terminal Met, acetylation of the N-terminal Ala, a disulphide bond between Cys⁷⁹ and Cys⁹⁰, and 1-methylation of His¹⁰⁶. Putative Zn²⁺ binding domains are shown. Post-translational modifications of hMRP14 were deletion of the N-terminal Met, acetylation of the N-terminal Thr, and phosphorylation of Thr¹¹². The hinge region and Ca²⁺ binding domains are shown, and the truncated protein start site is indicated by an arrow.

mMRP14 shares 59% amino acid identity and 64% nucleotide identity with hMRP14 [18]. A comparison of the reported post-translational modifications of hMRP14, which include a blocked N-terminus and phosphorylated forms [12,13], with those deduced by us for the murine protein is shown in Figure 7.

mMRP14 has 31% amino acid sequence identity (60% similarity) to CP10 (also called mMRP8 [18]), a potent chemo-attractant for leucocytes isolated from conditioned medium of activated murine spleen cells by our group [6,7]. S100 proteins are commonly purified as complexes [2,3], and the extracellular neurite outgrowth activity of S100b is dependent on disulphide-linked dimer formation [23]. No significant amounts of disulphide-linked homo- or hetero-dimers of MRP14 and CP10 in bone marrow cells or conditioned medium were observed using non-reducing conditions for SDS/PAGE and Western blotting (Figure 1). CP10 has greatest amino acid identity with hMRP8 (59%) [7], an S100 protein co-isolated with hMRP14 [11], with which it occurs predominantly as a non-covalently linked complex [12,24]. mMRP14 co-eluted with CP10 following ion-exchange chromatography; both had moderate affinity for heparin and were subsequently separated by C4 RP-HPLC (Figures 2A and 2B). Others have found high levels of expression of MRP14 in murine bone marrow cells and in 8% of spleen cells (with the characteristics of neutrophils) by immunohistochemical staining with a polyclonal antibody to hMRP14 [18]. The hMRP8-hMRP14 complex was isolated from supernatants of activated peripheral blood mononuclear cells by immunoaffinity chromatography [11]. Similarly, and although we have not identified the cell types responsible, experiments reported here indicate increased expression of MRP14 following Con A stimulation of spleen cells, as indicated by Western blotting (Figure 1), release and isolation in high yield (approx. 500 µg/l) from conditioned medium, and incorporation of L-[methyl-³H]Met into MRP14, strongly suggesting that MRP14 is a product of cellular activation.

ESI MS of MRP14 indicated a mass of 12972 ± 2 Da (Figure 2C), 54 Da more than the theoretical mass of the protein derived from the cDNA sequence [18]. This difference did not correspond to known post-translational modifications and suggested either several modifications or that the cDNA sequence was incorrect. No protein with characteristics of a truncated form of MRP14 was isolated. Analysis of the tandem (MS/MS) spectrum of a Glu C-derived peptide of MRP14 (V2; Table 1) indicated specific fragmentations attributed to an N-terminal acetylated alanine residue and confirmed that the N-terminal Met was removed (Figure 7). In contrast to hMRP14, which has a single Cys at position 3 of the full-length protein (Figure 7), and based on the reactivity of MRP14 (native and reduced) with iodoacetamide and analysis of a Glu C digest peptide which contained two Cys

residues (V9; Table 1) in addition to Cys¹¹⁰, MRP14 contained one internal disulphide-bonded loop between Cys⁷⁹ and Cys⁹⁰. The retention times of the non-reduced and reduced proteins on C4 RP-HPLC were different (17.05 and 18.1 min respectively), indicating that disulphide bond formation had a marked influence on the tertiary structure, although its relevance to the function of MRP14 is unknown. Analysis by Western blotting of the proteins from conditioned medium which eluted from C4 RP-HPLC in the region of fully reduced MRP14 indicated less than 5% of the reduced form (results not shown). We propose that MRP14 normally contains a disulphide-bonded loop which is probably not formed by fortuitous oxidation during isolation. A C-terminal peptide from the Lys C digestion, identified by MS and N-terminal sequencing, had a mass of 1691 Da, 14 Da greater than the expected value (L2; Table 2), and contained a methylated His (Figure 7). Recalculation of the mass after considering all the post-translational modifications identified, i.e. loss of Met (−131 Da), N-acetylation (+42 Da), an internal disulphide bond (−2 Da) and methylation of His¹⁰⁶ (+14 Da), gave a mass for MRP14 of 12971.8 Da, which compared well with the experimentally determined value of 12972 ± 2 Da.

Methylation of His is a rare post-translational modification of proteins [21], in which the methyl group is believed to be derived from Met via SAM, then transferred to His enzymically [21,22]. Enzymes that directly methylate His have not been identified. After isolation of MRP14 from conditioned media of spleen cells activated in the presence of L-[methyl-³H]Met, the incorporated radioactivity in a Lys C-derived peptide (L2; Table 2) was analysed and His¹⁰⁶ was found to have incorporated the majority of the radioactivity (Figure 4), indicating that the methyl group was transferred from Met to MRP14 via SAM and an unknown methyltransferase.

Methylation of His at either the 1 or the 3 position of the imidazole ring is relatively rare. A few proteins containing 3-methyl-His, including actin, myosin and rhodopsin, have been characterized [21,25], whereas the only reports of 1-methyl-His are in the dipeptide carnosine [26] and rabbit skeletal muscle myosin light chain kinase, which is partially 1-methylated on His¹⁵⁷ [27]. The simplest procedure to determine the position of methylation on the imidazole ring of His would be to compare the relative C18 RP-HPLC retention times of the PTH derivatives of synthetic 1-methyl-His and 3-methyl-His using N-terminal sequence analysis with the retention time of the PTH derivative of Me-His¹⁰⁶. Despite a previous report describing this procedure [27] we were unable to resolve the two synthetic isomers. Identification and analysis of 3-methyl-His by C18 RP-HPLC following pre- or post-column derivatization [28] is a qualitative index of muscle protein catabolism [29], whereas identification of 1-methyl-His is scant. Analysis of carnosine methylating enzymes

[22] used PTC derivatization of 1-methyl-His and 3-methyl-His and C18 RP-HPLC to characterize the two forms; although they were not fully resolved, the order of elution was PTC-1-methyl-His followed by PTC-3-methyl-His [22]. We analysed the PTC derivatives of hydrolysis products of a tryptic peptide, GHGHS(H_{Me})GK (MRP14¹⁰¹⁻¹⁰⁸), by C18 RP-HPLC which showed peaks corresponding to PTC-Ser, -Gly, -His, -1-methyl-His and -Lys with approximate proportions of 1:3:2:1:1. The retention time compared well with that of the synthetic PTC-1-methyl-His standard (Figure 5) and excluded 3-methylation, which had a retention time 45 s later. These experiments strongly indicate that His¹⁰⁶ of MRP14 is 1-methylated.

No dimethylated derivative of MRP14 was observed by ESI MS (Figure 2C) even though it has seven His residues, two of which occur within five amino acids of His¹⁰⁶. Figure 4 shows low levels (< 10%) of methylation of His¹⁰⁴, indicating that methylation was not totally specific. Formation of an N-substituted amine, as in His¹⁰⁶ of MRP14, is generally an irreversible reaction resulting in an amino acid with altered physico-chemical properties [21]. The precise reason for this unusual methylation and its effects on MRP14 structure and function are unknown. Phosphorylation of Thr¹¹³ in the extended C-terminal domain of hMRP14 in neutrophils and monocytes is up-regulated by ionomycin [13]. This region is identical to part of the NIF sequence and phosphorylation is postulated to regulate generation of NIF [13]. Comparison of the C-terminal regions of mMRP14 and hMRP14 shows only 46% identity (cf. 59% identity for the full-length proteins); also, the Thr residue is absent from the murine protein (Figure 7) and there was no evidence of phosphorylation. Whether the C-terminal region of mMRP14 has NIF activity is unknown, but our preliminary evidence indicates that the full-length protein has no direct effect on chemotactic responses of neutrophils provoked by formyl-Met-Leu-Phe, interleukin 8 or CP10 (results not shown). Furthermore, hMRP14 inhibits the growth of *Candida albicans* *in vitro* [30], but we were unable to demonstrate anti-microbial activity with up to 20 µg/ml mMRP14 [17] and the precise function of the murine protein, particularly in the extracellular milieu, is unclear.

S100 proteins commonly bind Ca²⁺ and Zn²⁺ [31,32]. S100b binds eight Zn²⁺ ions, four with high affinity and four with low affinity, probably via His residues, but the precise binding sites are uncharacterized [31]. Ca²⁺ antagonizes the low-affinity Zn²⁺ binding due to a conformational change [31]. These properties may explain the multiple interactions and different extracellular and intracellular targets of S100b in the brain [5]. Our blotting experiments suggest that both mMRP14 and hMRP14 have substantially higher affinity for Zn²⁺ than do S100β or CP10 (Figure 6). We confirmed an earlier report [31] that Ca²⁺ partially antagonizes Zn²⁺ binding to S100β (the monomer of S100b) whereas, as with calyculin [32], excess Ca²⁺ did not appear to antagonize Zn²⁺ binding to MRP14, indicating that the binding site was distinct and independent from the two Ca²⁺ binding domains. Analysis of the amino acid sequence of mMRP14 and hMRP14 does not indicate putative 'zinc fingers' [33] or clusters (4–12) of acidic amino acids [34] which potentially may bind Zn²⁺, but mMRP14 contains seven His residues and an unmodified Cys residue, some of which may adopt an appropriate conformation for binding [35]. For example, His residues in the sequence HXXXXH bind Zn²⁺ if they are located within an α-helix [36]. mMRP14 has two such motifs (Figure 7), one of which contains 1-methyl-His¹⁰⁶ which may potentially modify its Zn²⁺ binding characteristics. This domain (HXXXXH) is not present in bovine S100β, murine calyculin or hMRP14, whereas the pig S100 protein calgranulin C, postulated to bind Zn²⁺,

contains this motif [37]. Additional studies required to further characterize Zn²⁺ binding to mMRP14 include defining the Zn²⁺ binding constant, identifying the precise location of the Zn²⁺ binding ligands and determining the potential significance of the methylation of His¹⁰⁶.

In summary, we have demonstrated a number of modifications that would potentially alter the physico-chemical properties of mMRP14 to yield significant differences from the human homologue. The S100 family is highly conserved among vertebrates [4] and potential alterations in their structural characteristics by post-translational processes may represent mechanisms of functional diversification. Because these proteins are generally located intracellularly, although some also have important extracellular functions, comparison of the structures of the constitutive forms of MRP14 and CP10 with those produced by cell activation [6,38] may yield important information regarding their function and the post-translational mechanisms involved in their regulation.

This work was supported in part by grants (to C. L. G. and P. A.) from the National Health and Medical Research Council of Australia. Members of the Immunology group who prepared the spleen cell supernatants are gratefully acknowledged.

REFERENCES

- Schäfer, B. W., Wicki, R., Engelkamp, D., Mattei, M.-G. and Heizmann, C. W. (1995) *Genomics* **25**, 638–643.
- Zimmer, D. B., Cornwall, E. H., Landar, A. and Song, W. (1995) *Brain Res. Bull.* **37**, 417–429.
- Hessian, P. A., Edgeworth, J. and Hogg, N. (1993) *J. Leukocyte Biol.* **53**, 197–204.
- Kligman, D. and Hilt, D. C. (1988) *Trends Biochem. Sci.* **13**, 437–443.
- Fanó, G., Biocca, S., Fulle, S., Mariggió, M. A., Belia, S. and Calissano, P. (1995) *Prog. Neurobiol.* **46**, 71–82.
- Lackmann, M., Cornish, C. J., Simpson, R. J., Moritz, R. L. and Geczy, C. L. (1992) *J. Biol. Chem.* **267**, 7499–7504.
- Lackmann, M., Rajasekariah, P., Iismaa, S. E., Jones, G., Cornish, C. J., Hu, S., Simpson, R. J., Moritz, R. L. and Geczy, C. L. (1993) *J. Immunol.* **150**, 2981–2991.
- Devery, J. M., King, N. J. C. and Geczy, C. L. (1994) *J. Immunol.* **152**, 1888–1897.
- Lau, W., Devery, J. M. and Geczy, C. L. (1995) *J. Clin. Invest.* **95**, 1957–1965.
- Watt, K. W. K., Brightman, I. L. and Goetzl, E. J. (1983) *Immunology* **48**, 79–86.
- Odink, K., Cerletti, N., Brügggen, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R. and Sorg, C. (1987) *Nature (London)* **330**, 80–82.
- Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hagar, G., Karas, M. and Sorg, C. (1991) *J. Biol. Chem.* **266**, 13462–13467.
- Edgeworth, J., Freemont, P. and Hogg, N. (1989) *Nature (London)* **342**, 189–192.
- Ryan, J. and Geczy, C. L. (1986) *J. Immunol.* **137**, 2864–2870.
- Schagger, H. and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
- Henkeshoven, J. and Dernick, R. (1988) *Electrophoresis* **9**, 28–32.
- Kocher, M., Kenny, P. A., Farram, E., Abdul Majid, K. B., Finlay-Jones, J. J. and Geczy, C. L. (1996) *Infect. Immun.*, in the press.
- Lagasse, E. and Weissman, I. L. (1992) *Blood* **79**, 1907–1915.
- Ashton, D. S., Beddell, C. R., Green, B. N. and Oliver, R. W. A. (1994) *FEBS Lett.* **342**, 1–6.
- Crankshaw, M. W. and Grant, G. A. (1993) *Applied Biosystems Technical Note: Identification of Modified PTH-Amino Acids in Protein Sequence Analysis, The Association of Biomolecular Resource Facilities*.
- Clarke, S. (1993) *Curr. Opin. Cell Biol.* **5**, 977–983.
- Raghavan, M., Lindberg, U. and Schutt, C. (1992) *Eur. J. Biochem.* **210**, 311–318.
- Kligman, D. and Marshak, D. R. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7136–7139.
- Edgeworth, J., Gorman, M., Bennett, R., Freemont, P. and Hogg, N. (1991) *J. Biol. Chem.* **266**, 7706–7713.
- Paik, W. K. and Kim, S. (1971) *Science* **174**, 114–119.
- McManus, I. R. (1962) *J. Biol. Chem.* **237**, 1207–1211.
- Meyer, H. E. and Mayr, G. W. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 1607–1611.
- Teerlink, T. and De Boer, E. (1989) *J. Chromatogr.* **491**, 418–423.
- Young, V. R., Alexis, S. D., Baliga, B. S., Munro, H. N. and Muecke, W. (1972) *J. Biol. Chem.* **247**, 3592–3600.
- Murthy, A. R. K., Lehrer, R. I., Harwig, S. S. L. and Miyasaki, K. T. (1993) *J. Immunol.* **151**, 6291–6301.

-
- 31 Baudier, J., Glasser, N. and Gerard, D. (1986) *J. Biol. Chem.* **261**, 8192–8203
- 32 Filipek, A., Heizmann, C. W. and Kuznicki, J. (1990) *FEBS Lett.* **264**, 263–266
- 33 Evans, R. M. and Hollenberg, S. M., (1988) *Cell* **52**, 1–3
- 34 Brand, I. A., Heinickel, A., Kratzin, H. and Söling, H.-D. (1988) *Eur. J. Biochem.* **177**, 561–568
- 35 Vallee, B. L. and Auld, D. S. (1990) *Biochemistry* **29**, 5647–5659
- 36 Higaki, J. N., Fletterick, R. J. and Craik, C. S. (1992) *Trends Biochem. Sci.* **17**, 100–104
- 37 Dell'Angelica, E. C., Schleicher, C. H. and Santomé, J. A. (1994) *J. Biol. Chem.* **269**, 28929–28936
- 38 Hu, S.-P., Harrison, C., Xu, K., Cornish, C. J. and Geczy, C. L. (1996) *Blood*, in the press
-

Received 22 November 1995/18 January 1996; accepted 26 January 1996