Identification and characterization of a novel human neutrophil protein related to the S100 family

Florence GUIGNARD*, Jacques MAUEL† and Michèle MARKERT*‡

*Central Laboratory of Clinical Chemistry, CHUV, Lausanne, Switzerland and †the Biochemistry Institute, Epalinges, Switzerland

A rabbit polyclonal antibody raised against myeloid-related protein 8 (MRP-8), a protein of the S100 family, recognized another S100 protein (MRP-14) as well as a protein of 6.5 kDa (p6) in the cytosol of resting neutrophils. p6 was found to be a novel member of the S100 family. It consisted of two isoforms with pI values of 6.2 (the minor form, p6a) and 6.3 (the major form, p6b) and constituted 5% of the total cytosolic proteins. Both isoforms were also demonstrated in the cytosol of monocytes, but not in lymphocytes, as previously shown for MRP-8 and MRP-14. Only the major isoform bound radioactive Ca^{2+} , as also observed for MRP-8, whereas the different variants of MRP-14 were all labelled. On neutrophil activation with

opsonized zymosan, a stimulant known to require extracellular Ca^{2+} , 58% of p6a and 42% of p6b was translocated to the membrane. With phorbol 12-myristate 13-acetate, a Ca^{2+} -independent stimulant, no translocation was detected. This translocation pattern was similar to that observed with MRP-8 and MRP-14. In addition, p6, MRP-8 and MRP-14 were specifically associated with the cytoskeletal fraction of the membrane. The Ca^{2+} -dependent translocation of the novel S100 protein in parallel with MRP-8 and MRP-14 suggests a role for these proteins in regulating the Ca^{2+} signal to the membrane cytoskeleton and thus in regulating neutrophil activation.

INTRODUCTION

Ca^{2+} -binding proteins of the S100 family are a group of low-molecular-mass acidic proteins characterized by cell-type-specific expression and the presence of two Ca^{2+} -binding regions known as EF hands [1]. S100 proteins probably exert their biological effect by modulating the activity of specific effector proteins. This effect results from the binding of Ca^{2+} , inducing exposure of hydrophobic domains which interact with similar hydrophobic domains of the effector protein [2]. The function of these proteins in transducing the Ca^{2+} signal is supposed to be involved in cell-cycle progression, cell-type differentiation, signal transduction and morphological differentiation. Interestingly, some S100 proteins have been shown to alter phosphorylation of specific cytoskeletal-associated proteins [2].

Neutrophils express at least two S100 proteins called myeloid-related protein (MRP)-8 and MRP-14. These proteins are also termed calgranulin A and B, L1 light and heavy chain, p8 and p14, cystic fibrosis antigen and calprotectin respectively [3–6]. They are specific to myeloid cells and are also found in monocytes, but not in normal macrophages, which, however, express MRP-14 during acute and chronic inflammation and MRP-8 in chronic inflammatory tissues only [7,8]. The role of MRP-8 and MRP-14 is not yet known, but several hypotheses have been proposed. MRP-14 is phosphorylated on Thr-113 which is the penultimate residue of the C-terminal region [9]. This amino acid is adjacent to a region similar in sequence to peptides known to have neutrophil immobilizing factor activity. MRP-14 or a complex of MRP-8 and MRP-14 may play a role in immobilizing myeloid cells at endothelial surfaces to allow their exudation into tissues. In human spleen, a complex containing MRP-8 and MRP-14 inhibits casein kinase I and II, two enzymes that mediate RNA polymerase activity [10]. It has been suggested that the MRP-8–MRP-14 complex may modulate myeloid cell maturation and function. Recently, immunogold double-labelling experiments

revealed a co-localization of the MRP-8–MRP-14 complex with intermediate cytoskeletal filaments in monocytes [11]. In addition, we showed that these proteins were translocated from the cytosol to the membrane of human neutrophils on activation with stimuli requiring extracellular Ca^{2+} [12].

Interestingly, a rabbit polyclonal antibody raised against MRP-8 [12] was found to cross-react with MRP-14 and with an unknown 6.5 kDa protein (p6), suggesting structural similarities. In this paper, we describe the identification of p6, by N-terminal amino acid sequencing, as a novel protein of the S100 family that is expressed in neutrophils and monocytes, but not in lymphocytes. Furthermore, characterization and translocation of this protein during neutrophil activation were studied in parallel with MRP-8 and MRP-14.

MATERIALS AND METHODS

Materials

^{45}Ca and enhanced chemiluminescence (ECL) kit were purchased from Amersham (Rahn AG, Zürich, Switzerland); alkaline phosphatase conjugate substrate kit, nitrocellulose and poly(vinylidene difluoride) membranes were obtained from Bio-Rad (Glattbrugg, Switzerland); DNase I from bovine pancreas was purchased from Boehringer-Mannheim (Rotkreuz, Switzerland); Ficoll-Paque, Percoll, Ampholine pH 3.5–9.5, Ampholine pH 4.0–6.5, Immobiline DryStrips pH 3–10 NL 18 cm and Coomassie Blue G250 and R250 were from Pharmacia Biotech (Dübendorf, Switzerland); RPMI 1640 medium and fetal calf serum came from Seromed (Fakola AG, Basel, Switzerland); Mac 387, a monoclonal mouse anti-human antibody, was obtained from Dako (Instrumenten Gesellschaft AG, Zürich, Switzerland); Nonidet P40, CHAPS, leupeptin, pepstatin A, PMSF, phorbol 12-myristate 13-acetate (PMA) and zymosan A from *Saccharomyces cerevisiae* were all from Sigma (Buchs,

Abbreviations used: IEF, isoelectric focusing; MRP, myeloid-related protein or migration inhibitory factor-related protein; OZ, opsonized zymosan; PMA, phorbol 12-myristate 13-acetate; 2-D PAGE, two-dimensional PAGE; ECL, enhanced chemiluminescence; EBV, Epstein–Barr virus; DTE, dithioerythritol.

‡ To whom correspondence should be addressed.

Switzerland); other chemical products were obtained from Fluka (Buchs, Switzerland) and Merck (Dietikon, Switzerland).

Neutrophil activation and preparation of membranes and cytosol

Human neutrophils were isolated from 450 ml of fresh buffy coats by dextran sedimentation as previously reported [13]. After removal of contaminating erythrocytes by hypo-osmotic lysis, the cell pellet was resuspended in Hanks buffer (pH 7.4) consisting of 137 mM NaCl, 5.3 mM KCl, 0.3 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 0.4 mM MgSO_4 , 0.5 mM MgCl_2 , 1.3 mM CaCl_2 , 4.1 mM NaHCO_3 and 5 mM glucose. Cell suspension (1 ml containing 10^7 – 10^8 neutrophils) was preincubated for 3 min at 37 °C, then activated or not (controls) with PMA (1 $\mu\text{g}/10^7$ cells) for 10 min or opsonized zymosan (OZ; 4 mg/ 10^7 cells) for 7 min. The reaction was stopped by the addition of ice-cold Hanks buffer lacking Ca^{2+} , Mg^{2+} and glucose. The cells were then centrifuged at 150 *g* for 5 min at 4 °C and resuspended in 0.34 M saccharose containing 0.5 mM PMSF, 1 μM pepstatin A and 1 μM leupeptin (antiproteases). The cell suspension was sonicated at full power (350 W) for 3 $\times$ 1 min, 30 s apart, at 0 °C in a Branson Sonifier system (model B-30) fitted with a cup horn. The membrane fraction was purified on a discontinuous sucrose gradient (30%/50%, w/v) as described previously [14] and the cytosolic fraction was carefully removed from the top layer of the sucrose gradient.

Alternatively, neutrophils were isolated from 20 ml of freshly drawn heparinized blood on a discontinuous Percoll gradient [15]. The cells resuspended in Hanks buffer were activated and sonicated as described above, then centrifuged at 100 000 *g* for 30 min at 4 °C. The cytosolic fraction was recovered in the supernatant and the membrane fraction was obtained by resuspension of the pellet in 0.34 M saccharose containing antiproteases.

Preparation of membrane cytoskeletons

Membrane fractions from OZ-activated or resting neutrophils were incubated for 2 h at 4 °C in Triton buffer (pH 7.0) consisting of 21 mM imidazole, 80 mM KCl, 10 mM EGTA, 2 mM NaN_3 and 0.5% Triton X-100 plus antiproteases [16,17]. Triton-insoluble and -soluble fractions were separated by ultracentrifugation at 180 000 *g* for 2 h at 4 °C [17]. The Triton-insoluble pellet was resuspended in Triton buffer without Triton X-100.

To confirm the association with the cytoskeleton, membrane fractions from OZ-activated or resting neutrophils were incubated for 2 h at 4 °C in Triton buffer supplemented with DNase I (2 mg/ml). Control extractions were performed using carbonic anhydrase (2 mg/ml) instead of DNase I. Triton-insoluble and -soluble fractions were then separated as described above.

Purification of monocytes and lymphocytes

Fresh buffy coats were diluted 1:1 in Hanks buffer (pH 7.4) without Ca^{2+} and Mg^{2+} . The cell suspension was layered on Ficoll-Paque and centrifuged at 500 *g* for 30 min at 20 °C. The mononuclear cells removed from the interface were washed three times with ice-cold Hanks buffer (pH 7.4) without Ca^{2+} and Mg^{2+} and resuspended in chilled 0.15 M NaCl. The cell suspension was layered on a 50% Percoll solution and centrifuged at 800 *g* for 60 min at 4 °C. The cell layer removed from the interface was washed three times with ice-cold Hanks buffer (pH 7.4) without Ca^{2+} and Mg^{2+} [18] and resuspended at a density of 0.5×10^6 – 0.75×10^6 cells/ml in RPMI 1640 medium

supplemented with 10% heat-inactivated fetal calf serum. To purify monocytes from lymphocytes, they were allowed to adhere to the plastic of tissue-culture plates for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 . The supernatant was removed and the adherent monocytes were washed twice before being detached in 15 ml of RPMI 1640 medium with a rubber 'policeman'. Monocyte suspensions were centrifuged at 350 *g* for 10 min at 4 °C, resuspended in 0.34 M saccharose plus antiproteases, sonicated and ultracentrifuged at 100 000 *g* for 30 min at 4 °C to separate the membrane fractions from the cytosolic fractions as described above.

Cultures of Epstein-Barr virus (EBV)-transformed human B-cell lines and Jurkat human T-cell lines were a generous gift from Patricia Escobar (Biochemistry Institute, Epalinges, Switzerland). B- and T- cells were centrifuged at 150 *g* for 10 min at 4 °C, resuspended in 0.34 M saccharose plus antiproteases, sonicated and ultracentrifuged at 100 000 *g* for 30 min at 4 °C to separate the membrane fractions from the cytosolic fractions as described above.

Protein concentration

Protein concentration of the cytosolic and the membrane fractions was measured by using the Bradford reagent (Bio-Rad). Protein in samples containing Triton X-100 was measured by the bicinchoninic acid assay (Pierce).

Two-dimensional PAGE (2-D PAGE)

Isoelectric focusing (IEF) in capillary gels

Membrane and cytosolic fractions were mixed with a final concentration of 2% SDS and 0.03 M 1,4-dithioerythritol (DTE). The samples were boiled for 5 min at 95 °C, then urea and Bromophenol Blue were added to a final concentration of 9 M and 0.0015% respectively. Proteins (15–30 μg) were first separated by IEF in capillary gels (75 mm $\times$ 1.5 mm) consisting of 4% (w/v) acrylamide, 1.7% (w/v) CHAPS, 0.6% (v/v) Nonidet P40, 1.6% (v/v) Ampholine pH 3.5–9.5, 0.4% (v/v) Ampholine pH 4.0–6.5 and 9 M urea [19]. The proteins were focused at 500 V for 10 min, then at 1000 V for 4 h. After IEF, the capillary gels were equilibrated for 3 min in a solution containing 70 mM Tris/HCl (pH 6.8), 2.9% (w/v) SDS and 0.028% Bromophenol Blue [19] before application to the second dimension consisting of SDS/16% polyacrylamide gels [20]. After migration, the gels were blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech). The blots were then incubated with a rabbit polyclonal antibody raised against purified MRP-8 (anti-MRP8) [12] at a dilution of 1:1500 or with Mac 387, a murine monoclonal antibody raised against the MRP-8–MRP-14 complex (Dako; 86 μg of IgG/ml of tissue culture supernatant) [21,22], at a dilution of 1:1500. The primary antibody was revealed with a goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad; dilution 1:3000) when anti-MRP8 was used or a goat anti-mouse IgG conjugated with alkaline phosphatase (Bio-Rad; dilution 1:3000) in the case of Mac 387 as described previously [12]. When the ECL detection system was used, the protocol was performed as described by the manufacturer (Amersham).

IEF in Immobiline Drystrips

Cytosolic proteins (1 mg) from resting neutrophils were prepared for electrophoresis by precipitation in 10% (w/v) trichloroacetic acid in the presence of 0.17% (w/v) sodium deoxycholate followed by extraction of the resulting pellet in 90% (v/v)

acetone/0.1 M HCl at -20°C [23]. After centrifugation at 10000 *g* for 10 min at 4°C , the proteins were resuspended in sample buffer consisting of 35 mM Tris/HCl (pH 6.8), 1% (w/v) DTE, 4% (w/v) CHAPS, 9 M urea and 0.005% Bromophenol Blue [24].

Proteins were first separated in precast gels containing a non-linear pH gradient (Immobiline DryStrips). Voltage was linearly increased from 300 to 3500 V during the first 3 h, then kept at 3500 V to give a total of 300 kVh. After IEF, Immobililine DryStrips were equilibrated for 12 min in a solution containing 50 mM Tris/HCl (pH 6.8), 30% (v/v) glycerol, 6 M urea, 2% (w/v) SDS and 2% (w/v) DTE, then for 5 min in the same solution containing 2.5% (w/v) iodoacetamide and 0.00125% Bromophenol Blue instead of DTE [24] before application to SDS/16% polyacrylamide gels [20].

Electroblotting, staining and microsequencing

Electroblotting on to poly(vinylidene difluoride) membranes was performed as described by Moos et al. [25]. Membranes were stained with 0.1% Amido Black. The spots corresponding to the two isoforms of p6 were excised and applied to a model 473A microsequencer equipped with Problott reaction cartridges (Medical Biochemistry Department, Geneva University, Geneva, Switzerland). Sequence comparison was performed using a combined program from GenBank and EMBL databases.

Calcium overlay

Cytosolic proteins (1 mg) from resting neutrophils were separated by 2-D PAGE in Immobililine Drystrips as described above. The gels were then blotted on to nitrocellulose sheets as described by the manufacturer (Pharmacia Biotech). Labelling with ^{45}Ca was performed as described by Maruyama et al. [26]. Labelled blots were autoradiographed for 3 days with Kodak X-Omat AR films between intensifying screens at -80°C . After autoradiography, nitrocellulose sheets were revealed with anti-MRP8 at a dilution of 1:2000 as described above.

Densitometry

Immunoblots

Immunoblots were scanned with a Molecular Dynamics densitometer (Perkin-Elmer, Le Mont-sur-Lausanne, Switzerland) and analysed using the ImageQuant program (v.3.2).

Gels stained with Coomassie Blue

Cytosolic proteins (250 μg) from resting neutrophils were separated in the first dimension in Immobililine DryStrips as described above. The second dimension was 7–16% gradient SDS/polyacrylamide gels which were then stained with Coomassie Blue and scanned with a Molecular Dynamics densitometer (Perkin-Elmer). The proteins were quantified using Melanie, a program for 2-D PAGE image analysis from Geneva University [27].

RESULTS

Characterization of p6, MRP-8 and MRP-14 by 2-D PAGE

Cytosolic fractions (20 μg) from resting neutrophils were separated by 2-D PAGE and transferred to nitrocellulose sheets. The blots were revealed with anti-MRP8 as described in the Materials and methods section. This polyclonal antibody

Figure 1 Immunoblotting profiles of p6, MRP-8 and MRP-14 in the cytosol of resting neutrophils

Cytosolic proteins (20 μg) of resting neutrophils were separated by 2-D PAGE using capillary gels for IEF, electroblotted on to nitrocellulose and revealed with the polyclonal antibody anti-MRP8 (a) as described in the Materials and methods section. Alternatively, the proteins were revealed with the monoclonal antibody Mac 387 (b). The letters a, b, c identify the different variants of p6, MRP-8 and MRP-14 recognized by the antibodies. The arrow indicates a homodimer of MRP-14c at 29 kDa. The results are from one experiment representative of three.

detected MRP-8, and also a protein of molecular mass 14 kDa known as MRP-14 and an unknown protein of 6.5 kDa (p6).

As shown in Figure 1(a), anti-MRP8 revealed two isoforms of p6, a minor one with a pI value of 6.2 (p6a) and a major one with pI 6.3 (p6b). MRP-8 was present as two isoforms of pI 6.8 (MRP-8a) and 7.1 (MRP-8b), and MRP-14 was found to have four different variants. The more acidic form showed a pI of 5.7 (MRP-14a), but was only weakly recognized by anti-MRP8. Two isoforms with slightly different molecular masses appeared at pI 5.9 (MRP-14b) and a last isoform was demonstrated at pI 6.1 (MRP-14c). Anti-MRP8 also reacted with a protein that may be a homodimer of MRP-14c, as it has a similar pI and a molecular mass of 29 kDa (Figure 1a). As it is known that MRP-8 and MRP-14 can form various heteromers [28], the other spots could be such heterocomplexes. To confirm the identity of the 14 kDa protein as MRP-14, the proteins were revealed with Mac 387, a monoclonal antibody raised against the MRP-8-MRP-14 complex [21,22]. As shown in Figure 1(b), the different isoforms and the homodimer of MRP-14c were recognized by Mac 387, confirming the protein as MRP-14. In addition, p6b and MRP-8b were only weakly recognized by this antibody.

The amount of these proteins in the cytosol of resting neutrophils was quantified by densitometry after their separation by 2-D PAGE. Protein p6 (p6a and p6b together) was found to constitute about 5% of the total cytosolic protein whereas MRP-8 and MRP-14 were more abundant, representing about 19 and 30% respectively.

Figure 2 Identification of p6, MRP-8 and MRP-14 in monocytes and B-lymphocytes

Cytosolic proteins (30 μ g) of monocytes (a) and EBV-transformed B-cells (b) were separated by 2-D PAGE using capillary gels for IEF, electroblotted on to nitrocellulose and revealed with the polyclonal antibody anti-MRP8 as described in the Materials and methods section. The results are from one experiment representative of three.

p6a	XXLEEHLEGIIVNIXHQXXXX
p6b	TKLEEHLEGIIVNIFHQYSVR
MRP-14	MTCKMSQLERNIETIINTFHHQYSVK
MRP-8	MLTELEKALNSIIDVYHKYSLI
S100 β	SELEKAMVALIDVFHHQYSGR
S100 α	GSELETAMETLINVFHSHSGK
CABP9K	MSTKKSPEELKRIFEKYAAK
S100D	MPAAWILWAHSHSELHTV.....METPLEKALTTMVTTFHHKYSGR
S100L	MCSSLEQALAVLVTTTFHHKYSQ
S100P	MTELETAMGMIIDVFSRYSGS
S100E	MARPLEQAVAAIVCTFQEYAGR
CAPL	MACPLEKALDVMVSTFHHKYSGR
CACY	MACPLDQAIGLLVAIFFHHKYSGR

Figure 3 N-Terminal amino acid sequences of p6a and p6b in comparison with other human S100 proteins

The isoforms a and b of p6 were purified from the cytosol of human resting neutrophils as described in the Materials and methods section, and their respective N-terminal amino acid sequences were determined. A search for similar sequences in the SWISS-PROT protein database identified the following proteins (accession codes given in parentheses): MRP-14, migration inhibitory factor-related protein 14 (P06702) [8]; MRP-8, migration inhibitory factor-related protein 8 (P05109) [8]; S100 β , S100 protein β -chain (P04271) [29]; S100 α , S100 protein α -chain (P23297) [30]; CABP9K, calbindin D9K (P29377) [31]; S100D, S100D protein (P33763) [32]; S100L, S100L protein (P29034) [33]; S100P, S100P protein (P25815) [34]; S100E, S100E protein (P33764) [32]; CAPL, placental Ca^{2+} -binding protein (P26447) [30]; CACY, calyculin (P06703) [30]. Shaded letters indicate residues common to at least six N-terminal sequences. The bold line shows the beginning of the acidic EF hand. X represents amino acids that could not be identified in the sequence of p6a.

As S100 proteins are cell-type-specific, the presence of p6 in monocytes and lymphocytes was investigated. The two isoforms of p6 were demonstrated in the cytosol of monocytes (Figure 2a), but were not found in EBV-transformed B-cell lines (Figure 2b) or Jurkat T-cells (results not shown). To confirm this observation, 300 μ g of membrane and cytosolic proteins from EBV-transformed B-cells and Jurkat T-cells were separated by 2-D PAGE using Immobiline Drystrips for IEF, transferred to nitrocellulose and revealed with anti-MRP8. Despite the use of the very sensitive ECL detection system, the two isoforms of p6 were not observed in lymphocytes at all (results not shown). Blotting efficiency was controlled by staining the gels with Coomassie Blue after the transfer. This result suggests that p6 is specific for the myeloid cell lineage, as also shown for MRP-8 and MRP-14.

Amino acid sequence analysis of p6a and p6b

A search using the 20 N-terminal amino acids of p6b in databases identified p6 as a novel protein of the S100 family. Figure 3 shows the N-terminal amino acid sequences of both acidic (p6a)

Figure 4 Identification of Ca^{2+} -binding activity of the different isoforms of p6, MRP-8 and MRP-14

Cytosolic proteins (1 mg) of resting neutrophils were separated by 2-D PAGE using Immobiline Drystrips for IEF, blotted on to nitrocellulose sheets and incubated with ^{45}Ca . Autoradiography is shown in (a). To identify the different Ca^{2+} -binding proteins, the same nitrocellulose sheets were incubated with anti-MRP8 after autoradiography (b). The results are from one experiment representative of at least three.

Figure 5 Immunoblotting profiles of membrane proteins during neutrophil activation

Membrane proteins (20 μ g) of resting (a), O_2 -activated (b) and PMA-stimulated (c) neutrophils were separated by 2-D PAGE using capillary gels for IEF, blotted on to nitrocellulose sheets and revealed with anti-MRP8 antibody as described in the Materials and methods section. The results are from one experiment representative of three.

and basic (p6b) variants of the p6 protein and compares them with the other human S100 proteins. The best homologies were found with MRP-14 (55 % identity) and MRP-8 (42 % identity). The complete identity of p6a and p6b N-terminal sequences suggests that they might be isoforms of the same protein.

Identification of Ca^{2+} -binding sites

To identify potential Ca^{2+} -binding sites on p6, proteins from the cytosol of resting neutrophils were separated by 2-D PAGE and transferred to nitrocellulose sheets. On incubation of the membranes with ^{45}Ca , p6b was labelled as seen in Figure 4(a). In

Table 1 Levels of p6, MRP-8 and MRP-14 isoforms in the membrane and cytosol of resting and activated neutrophils

Neutrophils (1.8×10^7 cells/ml) were activated with OZ or PMA or left untreated (resting neutrophils). Membranes and cytosolic fractions were separated as described in the Materials and methods section, separated by 2-D PAGE using capillary gels for IEF and blotted on to nitrocellulose sheets. The blots were revealed with anti-MRP8, and the different variants of p6, MRP-8 and MRP-14 were quantified in the membrane and cytosolic fractions by densitometry. Values are percentages and are means $\pm$ S.D. of three experiments from different cell preparations. ND, not detectable.

Cells	p6		MRP-8		MRP-14		
	pl 6.2	pl 6.3	pl 6.8	pl 7.1	pl 5.7	pl 5.9	pl 6.1
Resting neutrophils							
Membrane	ND	8 $\pm$ 7	ND	4 $\pm$ 2	ND	4 $\pm$ 5	24 $\pm$ 16
Cytosol	100	92 $\pm$ 7	100	96 $\pm$ 2	100	96 $\pm$ 5	76 $\pm$ 16
OZ-activated cells							
Membrane	58 $\pm$ 23	50 $\pm$ 17	44 $\pm$ 14	59 $\pm$ 14	66 $\pm$ 6	70 $\pm$ 12	68 $\pm$ 4
Cytosol	41 $\pm$ 23	50 $\pm$ 17	56 $\pm$ 14	41 $\pm$ 14	34 $\pm$ 6	30 $\pm$ 12	32 $\pm$ 4
Translocation	58 $\pm$ 13	42 $\pm$ 11	44 $\pm$ 8	55 $\pm$ 8	66 $\pm$ 3	66 $\pm$ 8	44 $\pm$ 10
PMA-activated cells							
Membrane	ND	9 $\pm$ 4	ND	26 $\pm$ 8	ND	13 $\pm$ 5	32 $\pm$ 5
Cytosol	100	91 $\pm$ 4	100	74 $\pm$ 8	100	87 $\pm$ 5	68 $\pm$ 5
Translocation	0	1 $\pm$ 5	0	22 $\pm$ 5	0	9 $\pm$ 8	8 $\pm$ 10

addition, isoform b of MRP-8 and the three forms a, b and c of MRP-14 also bound ^{45}Ca . The minor acidic forms of p6 and MRP-8 did not appear to be labelled although they reacted with anti-MRP8 on the blot after its use for autoradiography (Figure 4b).

Translocation of the different isoforms of p6, MRP-8 and MRP-14

To examine whether p6 was translocated to the membrane on cell activation, as previously shown for MRP-8 and MRP-14 [12], neutrophils were activated with either OZ, a stimulant known to require extracellular Ca^{2+} for activity and to induce translocation of MRP-8 and MRP-14, or PMA, a Ca^{2+} -independent stimulant which failed to induce translocation of MRP-8 and MRP-14 [12]. Figure 5(a) and Table 1 show that only a very small amount of the two p6 isoforms was detected in the membrane of resting cells. On OZ-induced activation, 58% of p6a and 42% of p6b were translocated to the membrane (Figure 5b and Table 1). The different isoforms of MRP-8 and MRP-14 translocated to the membrane in comparable amounts: 44% of MRP-8a and 55% of MRP-8b, 66% of MRP-14a and MRP-14b and 44% of MRP-14c (Figure 5b and Table 1). Activation with other stimuli known to require extracellular Ca^{2+} for activity and to induce translocation of MRP-8 and MRP-14, such as formylmethionyl-leucylphenylalanine, the Ca^{2+} ionophore A23187 and arachidonic acid [12], also caused p6 to move to the membrane (results not shown). When the cells were stimulated with PMA, no significant translocation of the different isoforms of p6 and MRP-14 was observed (Figure 5c and Table 1). Interestingly, some isoform b of MRP-8 was translocated to the membrane of PMA-activated cells, but no translocation of MRP-8a was observed (Figure 5c and Table 1).

Association of p6, MRP-8 and MRP-14 with membrane cytoskeleton

As some members of the S100 family are involved in membrane-cytoskeletal interactions, association of p6, MRP-8 and MRP-14 with submembranous cytoskeleton was examined after extraction of the membrane from OZ-activated neutrophils and resting cells with Triton X-100. This detergent separates membranes into a Triton-insoluble fraction containing the membrane cytoskeleton and a Triton-soluble fraction consisting of

Figure 6 Association of p6, MRP-8 and MRP-14 with the membrane cytoskeleton on OZ stimulation

Triton-insoluble and -soluble fractions were prepared from membranes of OZ-activated neutrophils as described in the Materials and methods section using cytoskeleton buffer alone (a) or cytoskeleton buffer supplemented with DNase I (b) or with carbonic anhydrase (c). The proteins (15 μg) of the different fractions were separated by 2-D PAGE using capillary gels for IEF, electroblotted on to nitrocellulose sheets and revealed with anti-MRP8. The results are from one experiment representative of three.

non-cytoskeletal membrane proteins [17,35]. Analysis of these two fractions by immunoblotting after 2-D PAGE showed that the isoforms of p6, MRP-8 and MRP-14 that had moved to the membrane were almost completely associated with the cytoskeleton (Figure 6a). The very small percentage of proteins associated with the membrane in resting neutrophils was also essentially recovered in the cytoskeletal fraction (results not shown).

To distinguish between physiological association with the cytoskeleton and insolubility in Triton buffer, the cytoskeleton was dissolved with DNase I [17,35], which should solubilize proteins associated with the cytoskeleton but not affect proteins that are intrinsically insoluble in Triton buffer. The different isoforms of p6, MRP-8 and MRP-14 were solubilized by DNase I (Figure 6b), but not by carbonic anhydrase, a protein similar in size to DNase I (Figure 6c). These results suggest that p6, MRP-

8 and MRP-14 are associated with the membrane cytoskeleton in activated neutrophils.

DISCUSSION

A rabbit polyclonal antibody raised against purified MRP-8, a protein of the Ca^{2+} -binding S100 family, recognized another S100 protein identified as MRP-14 and a protein of 6.5 kDa (p6). N-Terminal amino acid sequencing of p6 isolated from the cytosol of resting neutrophils allowed its identification as a novel member of the S100 family, which showed greatest sequence identity with MRP-8 and MRP-14 (42 and 55% respectively). On high-resolution 2-D PAGE, p6 was revealed by anti-MRP8 at two different isoelectric points, suggesting post-translational modifications which are neither phosphorylation nor glycosylation (results not shown). Heterogeneity of MRP-8 and MRP-14 was also demonstrated by the presence of several isoforms, probably arising from phosphorylation processes in the case of MRP-14 [9].

The S100 proteins known to date have molecular masses ranging from 9 to 12 kDa. The unusually large molecular mass of MRP-14 is due to a C-terminal segment approximately 20 amino acids longer than the other members of the S100 family [36,37]. This sequence is identical with that of the polypeptide known as neutrophil-immobilizing factor, suggesting that the C-terminus of MRP-14 has neutrophil-immobilizing factor activity [36,37]. Interestingly, the particularly low molecular mass of p6 makes this protein unusual compared with the other members of the family and may indicate a particular function. Moreover, p6 was found to constitute about 5% of total cytosolic proteins in resting neutrophils, much less than MRP-8 and MRP-14 which together constitute 45–49% (the present work; [5]). During the preparation of this manuscript, a similar protein was identified in pig neutrophils and was found to constitute about 8% of pig cell extracts [38]. Other Ca^{2+} -binding proteins such as calbindin and parvalbumin are also present at similar high concentrations, suggesting that one of their functions may be to act as Ca^{2+} buffers protecting cells against excess intracellular Ca^{2+} concentration [39].

A conserved feature of the S100 proteins is the presence of two Ca^{2+} -binding sites called EF hands and characterized by different Ca^{2+} affinities [1,2]. The EF hand located in the C-terminal domain contains 12 amino acids and is similar to that found in the calmodulin protein family. However, the N-terminal EF hand in each S100 protein contains 14 amino acid residues instead of 12. This structural difference produces a significant decrease in the Ca^{2+} -binding affinity of the N-terminal EF hand compared with the C-terminal EF hand. In spite of its low Ca^{2+} -binding affinity, the N-terminal EF hand is conserved in all S100 proteins, suggesting that it has an important function [2]. The presence of Ca^{2+} -binding site(s) was detected on p6, but only on the major isoform of the protein (p6b). Similarly to p6, only the major form of MRP-8 (MRP-8b) bound Ca^{2+} , whereas Ca^{2+} -binding site(s) could be demonstrated on each of the MRP-14 isoforms. Defective Ca^{2+} binding by certain isoforms could be explained by denaturation of the EF hands during electrophoresis. An alternative explanation is the presence of defective Ca^{2+} -binding loops in some of the isoforms as shown for another S100 protein, the calpactin light chain [2]. The differential ability of the isoforms of p6, MRP-8 and MRP-14 to bind Ca^{2+} could also indicate a more subtle regulation by Ca^{2+} .

Expression of S100 proteins is cell-type-specific, suggesting that specific cellular functions depend on the phenotype of the cell that expresses them. Therefore S100 proteins may be involved in transducing the signal of an increase in intracellular Ca^{2+} in a

cell-type-specific fashion [1]. Interestingly, the p6 protein has been observed in human neutrophils and monocytes, but not lymphocytes, indicating a specificity of this protein for the myeloid cell lineage, as shown for MRP-8 and MRP-14 [7,8].

With the use of one-dimensional electrophoresis, MRP-8 and MRP-14 have been found to be translocated to the membrane during stimulation of neutrophils by OZ, formylmethionyl-leucylphenylalanine, the Ca^{2+} ionophore A23187 and arachidonic acid, i.e. agents that are known to require extracellular Ca^{2+} for activity [12,40]. Under Ca^{2+} -depleted conditions, translocation of the two proteins to the membrane was almost completely abolished. Moreover, the two proteins were not translocated when the cells were activated with PMA, a stimulus known to be independent of Ca^{2+} [12,40]. The use of 2-D PAGE allowed us to characterize this migration pattern further. The similar translocation of the different isoforms of p6, MRP-8 and MRP-14 on OZ activation supports the idea of a common regulation. It is also striking that the different isoforms of p6 and MRP-14 remained in the cytosol of PMA-stimulated cells. Interestingly, the minor form of MRP-8 was also found in the cytosol whereas a small percentage of its major isoform was translocated to the membrane. These results suggest a different regulation of, or a different function for, the two isoforms of MRP-8 in PMA-activated cells.

In addition, it was observed that all of the isoforms of p6, MRP-8 and MRP-14 that had migrated to the membrane were specifically associated with the cytoskeletal fraction. Roth and co-workers [11] also found co-localization of MRP-8 and MRP-14 with intermediate filaments in monocytes. Other S100 proteins such as S100 α , S100 β and calpactin light chain have also been shown to interact with cytoskeletal structures [2]. As several proteins known to be involved in signal transduction are located in the cytoskeletal fraction, it is tempting to speculate that the three S100 proteins may play a role in the control of Ca^{2+} flux to this region of importance for many neutrophil functional events, including migration, chemotaxis, degranulation, phagocytosis and respiratory burst. S100 proteins are known to form dimers, and the dimeric form has been assumed to represent the active form of these proteins [5]. Whether p6, MRP-8 and MRP-14 act separately or in complex forms and how they are involved in modulating their target protein(s) will require further investigation.

The expert technical assistance of Martine Vaglio is greatly appreciated. We are grateful to the Lausanne Blood Transfusion Center for provision of blood and buffy coats. This work was supported by a grant from the Swiss National Science Foundation (31-37590.93).

REFERENCES

- 1 Kligman, D. and Hilt, D. C. (1988) *Trends Biochem. Sci.* **13**, 437–443
- 2 Hilt, D. C. and Kligman, D. (1991) in *Novel Calcium-Binding Proteins: The S100 Protein Family: A Biochemical and Functional Overview* (Heizmann, C. W., ed.), pp. 65–103, Springer-Verlag, Berlin
- 3 Wilkinson, M. M., Busuttill, A. B., Hayward, C., Brock, D. J. H., Dorin, J. R. and Van Heyningen, V. (1988) *J. Cell Sci.* **91**, 221–230
- 4 Bernzén, H. B. and Fagerhol, M. K. (1988) *Scand. J. Clin. Lab. Invest.* **48**, 647–652
- 5 Edgeworth, J., Gorman, M., Benett, R., Freemont, P. and Hogg, N. (1991) *J. Biol. Chem.* **266**, 7706–7713
- 6 Murthy, A. R. K., Lehrer, R. I., Harwig, S. S. L. and Miyasaki, K. T. (1993) *J. Immunol.* **151**, 6291–6301
- 7 Zwadlo, G., Brüggen, J., Gerhards, G., Schlegel, R. and Sorg, C. (1988) *Clin. Exp. Immunol.* **72**, 510–515
- 8 Odink, K., Cerletti, N., Brüggen, J. et al. (1987) *Nature (London)* **330**, 80–82
- 9 Edgeworth, J., Freemont, P. and Hogg, N. (1989) *Nature (London)* **342**, 189–192
- 10 Murao, S., Collart, F. R. and Huberman, E. (1989) *J. Biol. Chem.* **264**, 8356–8360
- 11 Roth, J., Burwinkel, F., Van den Bos, C., Goebeler, M., Vollmer, E. and Sorg, C. (1993) *Blood* **82**, 1875–1883

- 12 Lemarchand, P., Vaglio, M., Mauël, J. and Markert, M. (1992) *J. Biol. Chem.* **267**, 19379–19382
- 13 Markert, M., Andrews, P. C. and Babior, B. M. (1984) *Methods Enzymol.* **105**, 358–365
- 14 Markert, M., Glass, G. A. and Babior, B. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3144–3148
- 15 Heierli, C., Markert, M., Lambert, P. H., Kuwahara, T. and Wauters, J. P. (1988) *Nephrol. Dial. Transplant.* **3**, 773–783
- 16 Wallace, P. J., Packman, C. H., Wersto, R. P. and Lichtman, M. A. (1987) *J. Cell. Physiol.* **132**, 325–330
- 17 El Benna, J., Ruedi, J. M. and Babior, B. M. (1994) *J. Biol. Chem.* **269**, 6729–6734
- 18 Pertoft, H., Johnsson, A., Wärnegård, B. and Seljelid, R. (1980) *J. Immunol. Methods* **33**, 221–229
- 19 Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, M. J. and Merrill, C. R. (1988) *Anal. Biochem.* **173**, 424–435
- 20 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 21 Flavell, D. J., Jones, D. B. and Wright, D. H. (1987) *J. Histochem. Cytochem.* **35**, 1217–1226
- 22 Goebeler, M., Roth, J., Teigelkamp, S. and Sorg, C. (1994) *J. Leukoc. Biol.* **55**, 259–261
- 23 Hubbard, A. L., Bartles, J. R. and Braiterman, L. T. (1985) *J. Cell Biol.* **100**, 1115–1125
- 24 Bjellqvist, B., Pasquali, C., Ravier, F., Sanchez, J. C. and Hochstrasser, D. (1993) *Electrophoresis* **14**, 1357–1365
- 25 Moos, M., Nguyen, N. Y. and Liu, T. Y. (1988) *J. Biol. Chem.* **263**, 6005–6008
- 26 Maruyama, K., Mikawa, T. and Ebashi, S. (1984) *J. Biochem. (Tokyo)* **95**, 511–519
- 27 Appel, R. D., Hochstrasser, D. F., Funk, M. et al. (1991) *Electrophoresis* **12**, 722–735
- 28 Teigelkamp, S., Bhardwaj, R. S., Roth, J., Meinardus-Hager, G., Karas, M. and Sorg, C. (1991) *J. Biol. Chem.* **266**, 13462–13467
- 29 Jensen, R., Marshak, D. R., Anderson, C., Lukas, T. J. and Watterson, D. M. (1985) *J. Neurochem.* **45**, 700–705
- 30 Engelkamp, D., Schäfer, B. W., Erne, P. and Heizmann, C. W. (1992) *Biochemistry* **31**, 10258–10264
- 31 Howard, A., Legon, S., Spurr, N. K. and Walters, J. R. I. (1992) *Biochem. Biophys. Res. Commun.* **185**, 663–669
- 32 Engelkamp, D., Schäfer, B. W., Mattei, M. G., Erne, P. and Heizmann, C. W. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6547–6551
- 33 Lee, S. W., Tomasetto, C., Swisshelm, K. and Sager, R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2504–2508
- 34 Becker, T., Gerke, V., Kube, E. and Weber, K. (1992) *Eur. J. Biochem.* **207**, 541–547
- 35 Fox, J. E. B. (1985) *J. Biol. Chem.* **260**, 11970–11977
- 36 Freemont, P., Hogg, N. and Edgeworth, J. (1989) *Nature (London)* **339**, 516 (letter)
- 37 Watt, K. W. K., Brightman, I. L. and Goetzl, E. J. (1983) *Immunology* **48**, 79–86
- 38 Dell'Angelica, E. C., Schleicher, C. H. and Santomé, J. A. (1994) *J. Biol. Chem.* **269**, 28929–28936
- 39 Rogers, J. (1989) *Nature (London)* **339**, 661–662
- 40 Rossi, F. (1986) *Biochim. Biophys. Acta* **853**, 65–89