Increase of calcium levels in epithelial cells induces translocation of calcium-binding proteins migration inhibitory factor-related protein 8 (MRP8) and MRP14 to keratin intermediate filaments

Matthias GOEBELER,*†§ Johannes ROTH,* Christian van den BOS,* Gereon ADER† and Clemens SORG*

*Institute of Experimental Dermatology, University of Münster, Münster, †Department of Dermatology, University of Würzburg, Würzburg, and ‡Department of Zoology, University of Münster, Münster, Germany

Migration inhibitory factor-related protein 8 (MRP8) and MRP14, two S-100-like Ca^{2+} -binding proteins, have been described in cells of the epithelial lineage where they are either expressed constitutively (e.g. by mucosal squamous epithelium) or induced during disease (e.g. in keratinocytes during the course of psoriasis). Their biological function, however, is not yet clear. Recent studies have provided evidence that S-100-like proteins may interact with cytoskeletal components; we have therefore studied the biochemical properties and subcellular distribution of MRP8 and MRP14 in epithelial cells. TR146 human squamous carcinoma cells, which were found to express MRP8 and MRP14 in Northern and Western blot studies, were chosen for analysis. Cross-linking experiments using bis(sulphosuccinimidyl)suberate followed by SDS/PAGE and Western blot analysis revealed formation of heteromeric MRP8–MRP14 complexes. On subjecting TR146 cell lysates to two-dimensional gel

electrophoresis and Western blotting, four distinct MRP14 isoforms could be identified resembling those described earlier in macrophages. A differential centrifugation technique revealed a Ca^{2+} -dependent translocation of MRP8–MRP14 from the cytoplasm to the membrane and the Nonidet P40-insoluble cytoskeletal fraction. Double-label immunofluorescence microscopy of Ca^{2+} ionophore A23187-stimulated TR146 cells and cytochalasin B and demecolcine cytoskeleton disruption studies identified these structures as keratin intermediate filaments. Ca^{2+} -dependent binding of MRP8–MRP14 to keratin filaments was additionally confirmed by an *in vitro* binding assay. In conclusion, our data suggest that MRP8 and MRP14 may be involved in Ca^{2+} -dependent reorganization of cytoskeletal filaments in epithelial cells, which could be of importance for events associated with differentiation and inflammatory activation.

INTRODUCTION

In recent years, epithelial cells such as keratinocytes have increasingly been demonstrated to actively participate in immunological events occurring during a variety of inflammatory disorders. For example, induction of MHC class II molecules or up-regulation of cytokines such as interleukin-1, interleukin-6 and tumour necrosis factor- α have been considered to reflect inflammatory activation of keratinocytes during many dermatological disorders (for review, see [1]). Coinciding with these events, the two Ca^{2+} -binding proteins migration inhibitory factor-related protein 8 (MRP8) and MRP14 are induced in keratinocytes [2,3]. Both molecules belong to the S-100 protein family, the members of which are proposed to play a role during differentiation, regulation of the cell cycle and cytoskeletal/membrane interactions [4,5]. MRP8 and MRP14 were initially described as being expressed during differentiation of myelomonocytic cells [6–9]. Both are abundant in infiltrating monocytes and granulocytes during inflammatory disorders such as rheumatoid arthritis, granulomatous diseases, allergic contact dermatitis and renal allograft rejection, but not in resident tissue macrophages and lymphocytes [6, 10–12]. In myeloid cells, MRP8 and MRP14 have been shown to form non-covalently associated heteromeric complexes which are Ca^{2+} -dependently translocated to vimentin filaments [13–15]. Interestingly, epithelial cells in which the intermediate filament network does not contain vimentin express MRP8 and MRP14 during dermatoses involving the epidermis (e.g. psoriasis), whereas they are not

detectable in non-diseased skin or disorders primarily affecting the dermis or subcutis [2,3,16].

So far, little is known about the biochemical properties of MRP8 and MRP14 in epithelial cells. The present study was therefore intended to analyse the occurrence and subcellular distribution of MRP8 and MRP14 in cells of epithelial origin. By employing covalent cross-linking and Western blot analysis we now demonstrate formation of MRP8–MRP14 complexes in epithelial cells. Differential centrifugation studies and double-labelling immunofluorescence microscopy indicate that MRP8 and MRP14 are translocated to cytoskeletal structures, i.e. keratin intermediate filaments, in a Ca^{2+} -dependent manner.

EXPERIMENTAL

Cell culture

TR146 epithelial cells, derived from a human buccal squamous cell carcinoma [17], were kindly provided by Dr. M. Osborn, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany. They were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM glutamine and 1 mM sodium pyruvate (all obtained from Biochrom, Berlin, Germany).

Northern blot analysis

Total cellular RNA was isolated according to the guanidine hydrochloride method as described [18]. Briefly, 20 μg of

Abbreviations used: BS³, bis(sulphosuccinimidyl)suberate; MRP, migration inhibitory factor-related protein; NP-40, Nonidet P40; moAb, monoclonal antibody; TBS, 25 mM Tris (pH 7.2) and 0.3 M NaCl.

§ To whom correspondence should be addressed: Department of Dermatology, University of Würzburg, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany.

denatured total RNA was separated on a 1% agarose/formaldehyde gel and vacuum-blotted on Biodyne B membranes (Pall, Dreieich, Germany). Filters were UV-cross-linked and subsequently hybridized with cDNA probes specific for MRP8 (279 nucleotides) or MRP14 (342 nucleotides) [6] which were labelled with ^{32}P by a random primer method (Multiprime DNA labelling system; Amersham, Braunschweig, Germany). Autoradiography was performed using Kodak X-Omat AR films.

Cross-linking experiments

For analysis of MRP8-MRP14 protein complexes, TR146 cells were lysed in PBS containing 1% Nonidet P40 (NP-40) (Sigma, Deisenhofen, Germany) and 2 mM PMSF (Pierce, Cologne, Germany) at 4 °C for 45 min. Cell debris was removed by centrifugation at 13000 *g*. The supernatant was diluted to a protein concentration of 1 mg/ml and cross-linked employing bis(sulphosuccinimidyl)suberate (BS^3) (Pierce) at a final concentration of 1.5 mM [13]. After an incubation period of 45 min at 20 °C the cross-linking reaction was stopped by the addition of 20% (v/v) 50 mM ethanolamine and 20 mM *N*-ethylmaleimide in 50 mM sodium phosphate, pH 7.4. Samples were stored at -80 °C prior to Western blot analysis.

Immunostaining of MRP8-MRP14 on Western blots

TR146 samples obtained after cross-linking or differential centrifugation (see below) were separated by SDS/PAGE on 15% gels under reducing conditions. Proteins were semi-dry-blotted on to polyvinylidene difluoride membranes (Immobilon P, Millipore, Eschborn, Germany). Membranes were then stained with monospecific affinity-purified rabbit antisera (1 $\mu\text{g}/\text{ml}$) against human recombinant MRP8 and MRP14 [6] which were subsequently detected using phosphatase-conjugated second stage antibodies as described previously [9].

Two-dimensional electrophoresis

TR146 cells were analysed by two-dimensional electrophoresis essentially according to the methods described by O'Farrell [19] and Bravo [20]. Cells were lysed as described above, and supernatants were lyophilized and resuspended in isoelectric focusing sample solution containing 1.6% (v/v) Servalys 4-7 (Serva, Heidelberg, Germany), 0.4% (v/v) Ampholytes 3-10 (Pharmacia, Freiburg, Germany), 9.5 M urea and 2% (w/v) CHAPS (Sigma) at a protein concentration of approx. 10 mg/ml. Samples were transferred to isoelectric focusing tube gels (5 mm diameter, 11 cm length; 30% polyacrylamide; 6% Servalys 4-7, 1.2% Ampholytes 3-10, 9 M urea, 2% CHAPS) which were subsequently run for 16.5 h at 400 V plus an additional 1 h at 800 V. After focusing, tube gels were equilibrated with SDS/PAGE sample buffer and applied to SDS/PAGE gels. Proteins were subsequently transferred to Immobilon P membranes and immunostained using anti-MRP14 as described above.

Subcellular fractionation

TR146 cells were fractionated as described previously [15]. Briefly, cells were washed in PBS (pH 7.5) and resuspended in 20 mM Hepes (pH 7.5) and 2 mM PMSF (buffer 1) containing either 100 μM Ca^{2+} or 1 mM EGTA. These concentrations of Ca^{2+} or EGTA were maintained throughout the fractionation

procedure. Cells were subsequently homogenized by sonification (Branson sonifier 250; Branson Ultrasonics, Danbury, CT, U.S.A.). To obtain the cytoskeletal fraction, homogenates were centrifuged at 1000 *g* for 10 min. Pellets were washed three times in buffer 1 supplemented with 1% NP-40, sonicated, lyophilized and stored at -80 °C prior to Western blot analysis. The 1000 *g* supernatant was centrifuged at 10000 *g* for 30 min to remove cellular organelles or aggregated membrane vesicles. The supernatant obtained was then centrifuged at 100000 *g* for 90 min. The resulting supernatant represented the cytoplasmic fraction. The membrane fraction (100000 *g* pellets) was washed three times in 5 mM Hepes containing either 100 μM Ca^{2+} or 1 mM EGTA in order to release cytoplasmic proteins still present in membrane vesicles by osmotic shock. After employing such a procedure, the contaminating cytoplasmic proteins in both membrane and cytoskeletal fractions was less than 0.1%, as reflected by the activity of lactate dehydrogenase in the final washing step. Samples obtained from cytoplasmic and cytoskeletal fractions were finally studied by Western blot analysis as described above.

Immunofluorescence microscopy

To study cytoskeletal filaments, TR146 cells were grown to confluency in Lab-Tek tissue culture eight-chamber slides (Nunc, Wiesbaden, Germany). They were either left untreated or incubated with the Ca^{2+} ionophore A23187 for 1 h at a final concentration of 10 μM in order to increase intracellular Ca^{2+} levels. Cells were washed with PBS and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. After two rinses in PBS they were permeabilized with 0.5% Triton X-100 in PBS for another 10 min, washed again with PBS and subsequently incubated in blocking solution containing 1% BSA and 1% normal goat serum in PBS. For single- and double-label experiments, all antibody incubation periods lasted 60 min at room temperature and were followed by three rinses in PBS. Keratin filaments were labelled with mouse monoclonal antibody (moAb) Lu5 (dilution 1:2) (Boehringer Mannheim, Mannheim, Germany) which detects cytokeratins 1-19 [21]. Cells were then incubated with species-specific Texas red-labelled goat anti-[mouse IgG F(ab)₂] (dilution 1:50) (Dianova, Hamburg, Germany). For visualization of MRP8-MRP14 complexes, 5 $\mu\text{g}/\text{ml}$ biotinylated mouse moAb 27E10 (kindly donated by BMA Biomedicals, Augst, Switzerland) was employed, which has been described to solely recognize MRP8-MRP14 heteromers [22,23]. Cells were finally treated with dichlorotriazinyl amino-fluorescein-conjugated streptavidin (dilution 1:50; Dianova). After rinsing in PBS and mounting in Glycergel (Dako, Hamburg, Germany), cells were examined using a Zeiss photomicroscope and photographed (Kodak Ektachrome 320T). No cross-reactivity or spillover was detected in control experiments after omitting specific antibodies or replacing them with isotype-matched control antibodies of irrelevant specificity.

In cytoskeleton disruption experiments, TR146 cells were treated as described above and subsequently incubated with either 10 μM cytochalasin B (Sigma) or 1 μM demecolcine (Sigma) for 1 h. Cells were then fixed and processed according to the double-label procedure described above or stained for single-label immunofluorescence microscopy of actin microfilaments or microtubules. Briefly, cells were incubated with 0.165 μM fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR, U.S.A.) or a mouse moAb against β -tubulin (dilution 1:1000; Amersham). The latter was visualized using a fluorescein-labelled goat anti-[mouse IgG F(ab)₂] second stage antibody (dilution 1:50; Dianova).

Cytoskeletal filament binding assay

For analysis of Ca^{2+} -dependent binding of MRP8–MRP14 to keratin intermediate filaments, an ELISA-based assay system was employed. Briefly, 96-well flat-bottomed plates were coated for 1 h at 37 °C with 0.5 μg of bovine cytokeratin 8 per well (Boehringer Mannheim) in 25 mM Tris (pH 7.2) and 0.3 M NaCl (TBS), and subsequently blocked with 5% (w/v) BSA in TBS for 45 min at 37 °C or overnight at 4 °C. Then 8, 4, 2, 0.5, 0.25, 0.125 or 0.0625 μg /well MRP8–MRP14 in 25 mM Tris (pH 7.2), 154 mM NaCl, 0.05% (v/v) Tween and 1% (w/v) BSA was added to the wells and incubated in the presence of either 2 mM EGTA or 0.1 mM CaCl_2 for 45 min at 37 °C. MRP8–MRP14 had been isolated previously from human buffy coat granulocytes by FPLC Mono Q HR 10/30 (Pharmacia, Freiburg, Germany) purification of supernatants obtained after ammonium sulphate precipitation of granulocyte lysates, as described previously [24]. After several washes with TBS in the presence of 2 mM EGTA or 0.1 mM CaCl_2 , the cytokeratin-bound MRP8–MRP14 fraction was determined by an ELISA technique employing anti-MRP8–MRP14 rabbit antiserum and peroxidase-coupled second stage antibodies. After staining with *o*-phenylenediamine as peroxidase substrate, binding was quantified using an MRW Dynatech ELISA reader at 492 nm. The ELISA was calibrated with purified MRP8–MRP14 in concentrations from 1 to 125 ng/well. Binding is expressed as ng of MRP8–MRP14 per μg of cytokeratin 8.

RESULTS

Expression of MRP8 and MRP14 in TR146 epithelial cells

First of all, TR146 cells cultured in RPMI medium were processed for Northern blot analysis. Hybridization of total RNA with specific cDNA probes MRP8 (279 bp) and MRP14 (342 bp) revealed a specific band for each of these molecules (Figure 1a). Then complex formation of MRP8–MRP14 at the protein level was studied. After covalent cross-linking of cell lysates with BS^3 and subsequent SDS/PAGE and Western blot analysis under reducing conditions, protein bands of 25, 35 and 48 kDa became apparent on immunostaining of membranes with monospecific

Figure 1 Northern and Western blot analysis of MRP8 and MRP14 in TR146 human squamous epithelial cells

(a) RNA was isolated from TR146 cells as described in the Experimental section. Samples of 20 μg of total RNA were separated on an agarose/formaldehyde gel and membranes were subsequently hybridized with MRP8 and MRP14 cDNAs. (b) Lysates of TR146 cells were subjected to SDS/PAGE (15% acrylamide) under reducing conditions, semi-dry-blotted and immunostained for MRP8 and MRP14 as indicated. (c) Lysates of TR146 cells were cross-linked by BS^3 as outlined in the Experimental section. Samples were subsequently separated on SDS/PAGE (15% acrylamide) under reducing conditions, semi-dry-blotted and immunostained with anti-MRP8 and anti-MRP14 as indicated. Bands representing the 25, 35 and 48 kDa heteromers as well as the MRP8 (10 kDa) and MRP14 (14 kDa) monomers are observed.

Figure 2 Analysis of MRP14 isoforms in TR146 lysates

Lysates of TR146 cells were subjected to two-dimensional gel electrophoresis as described in the Experimental section. Subsequent Western blotting and immunostaining with anti-MRP14 revealed four distinct MRP14 isoforms. IEF, isoelectric focusing.

antisera against either MRP8 or MRP14. In addition, a protein band of 10 kDa could be detected after staining with anti-MRP8 and a 14 kDa band after labelling with anti-MRP14 (Figure 1b). The pattern of MRP8–MRP14 complex formation in epithelial cells (Figure 1c) resembles that observed previously in monocytes. Detailed studies have revealed that the 25 kDa band represents the (MRP8)₂(MRP14) heterodimer, the 35 kDa band the (MRP8)₂(MRP14) trimer, and the 48 kDa band the (MRP8)₂(MRP14)₂ tetramer [13].

In myeloid cells, different isoforms of MRP14 have been detected which show minor differences in molecular mass and isoelectric point [25]. In order to study MRP14 isoforms in epithelial cells, lysates of TR146 cells were subjected to two-dimensional electrophoresis. Subsequent Western blot analysis revealed four MRP14 isoforms with small differences in mobility: (i) two major isoforms with lower ($\text{pI} = 6.2$) and higher ($\text{pI} = 5.9$) mobility in the second dimension, and (ii) two minor isoforms with lower ($\text{pI} = 5.9$) and higher mobility ($\text{pI} = 5.7$) (Figure 2). The isoform pattern observed in TR146 cells thus resembled that reported for myeloid cells [25]. In the latter the two isoforms with lower molecular mass, designated MRP14', have been reported to have a molecular mass that is 410 Da lower than that of MRP14. This is due to the lack of the first four amino acids. Both MRP14 and MRP14' exist in a phosphorylated (i.e. more acidic) and non-phosphorylated (i.e. more basic) isoform. Only one MRP8 isoform could be detected (results not shown).

Influence of Ca^{2+} on the binding of MRP8 and MRP14 to cytoskeletal and membrane subcellular fractions

TR146 epithelial cells were sonicated and processed for differential centrifugation as described in the Experimental section. Subsequently, proteins were separated by SDS/PAGE under reducing conditions and MRP8 and MRP14 were detected by Western blot analysis. In the presence of the Ca^{2+} chelator EGTA (1 mmol) the majority of MRP8 and MRP14 was found in the cytoplasmic fraction, i.e. the 100000 g supernatant. In contrast, when EGTA was replaced by 0.1 mmol of Ca^{2+} , both proteins were mainly detected in the membrane fraction, i.e. the 100000 g pellet, as well as in the cytoskeletal portion, which is defined as the NP40-insoluble 1000 g pellet (Figure 3).

Binding of MRP8–MRP14 to cytoskeletal filaments

To further analyse the influence of Ca^{2+} on the subcellular distribution of MRP8–MRP14 complexes as detected by moAb

Figure 3 Ca^{2+} -dependent subcellular localization of MRP8 and MRP14 in TR146 cells

Subcellular fractionation of TR146 cells was performed in the presence of 0.1 mM CaCl_2 (lanes a) or 1 mM EGTA (lanes b) as described in the Experimental section. A 100 μg sample of protein from each fraction [cytoplasm (CP), 100 000 g supernatant; membrane (MB), 100 000 g pellet; cytoskeleton (CS), 1000 g pellet] were separated on SDS/PAGE (15% acrylamide), blotted and immunostained with anti-MRP8 or anti-MRP14 as indicated.

Figure 4 Ca^{2+} -dependent (co)localization of MRP8-MRP14 and keratin in TR146 cells

TR146 cells were studied by indirect immunofluorescence microscopy either under control conditions or after exposure to 10 μM Ca^{2+} ionophore A23187 for 1 h as described in the Experimental section. (a) Staining of non-stimulated cells with moAb 27E10 against the MRP8-MRP14 heterodimer revealed a diffuse cytoplasmic staining pattern. (b) In A23187-treated TR146 cells a filamentous pattern was to be observed after immunolabelling with moAb 27E10. (c) Double-labelling with moAb Lu5 against cytokeratins showed an identical staining pattern. Bar represents 5 μm .

Figure 5 Effect of cytochalasin B on the organization of the MRP8-MRP14 filamentous network in A23187-stimulated TR146 cells

TR146 cells pre-exposed to A23187 were incubated for 1 h in the absence (a) or presence (b-d) of 10 μM cytochalasin B, and labelled with fluorescein isothiocyanate-conjugated phalloidin for actin microfilaments (a, b) or double-labelled with moAbs for the MRP8-MRP14 heterodimer (c) and cytokeratins (d). Treatment with cytochalasin B destroyed the actin filament network (b), whereas the filamentous pattern stained by moAb 27E10 and the keratin filaments were not affected (c, d). Bar represents 5 μm .

27E10 [22,23] at a morphological level, immunofluorescence microscopy was employed. In non-stimulated TR146 cells a diffuse staining pattern for MRP8-MRP14 complexes was observed (Figure 4a). In contrast, after exposure of TR146 cells to the Ca^{2+} ionophore A23187 for 1 h, a filamentous pattern was obtained after staining with moAb 27E10 (Figure 4b). To study the co-localization of MRP8-MRP14 with the three major cytoskeletal components of epithelial cells, double-labelling experiments were performed. The pattern obtained after staining of A23187-treated cells with moAb Lu5 against cytokeratins 1-19 was identical to that seen after labelling with moAb 27E10 (Figures 4b and 4c). Cytochalasin B and demecolcine studies were performed to exclude the co-localization of MRP8-MRP14 with microfilaments and microtubules. The actin network in Ca^{2+} -ionophore-treated cells as visualized by labelling with fluorescein-conjugated phalloidin (Figure 5a) is morphologically different from the 27E10-stained filamentous structures. Addition of cytochalasin B (final concentration 10 μM) for 1 h to the culture medium led to disruption of the microfilament

Figure 6 Effect of demecolcine on the organization of the MRP8-MRP14 filamentous network in A23187-stimulated TR146 cells

A23187-stimulated TR146 cells were either left untreated (a) or exposed to 1 μ M demecolcine for 1 h (b-d). Following drug exposure, cells were immunostained with a moAb against β -tubulin (a, b) or double-labelled for the MRP8-MRP14 heterodimer (c) and cytokeratin (d) as described in the Experimental section. Demecolcine treatment destroyed microtubules (b) but did not affect the organization of the MRP8-MRP14 filamentous network, or the keratin intermediate filaments (c, d). Bar represents 5 μ m.

organization (Figure 5b), whereas the identical staining pattern of 27E10- and Lu5-labelled filaments was not altered during this procedure (Figures 5c and 5d). The staining pattern of microtubules in A23187-exposed TR146 cells was also clearly distinguishable from the 27E10-labelled network (Figure 6a). Exposure of these cells to 1 μ M demecolcine destroyed the organization of the microtubule network (Figure 6b), whereas the filament structure labelled by moAbs 27E10 and Lu5 remained intact (Figures 6c and 6d). To control the specificity of the immunolabelling procedures, antibodies against MRP8-MRP14 or cytoskeletal components were omitted or replaced by isotype-matched antibodies of non-relevant specificity at corresponding concentrations in all experiments; no cross-reactivity or spillover was detected (results not shown).

To study Ca^{2+} -dependent binding of MRP8-MRP14 to keratin filaments *in vitro*, an ELISA-based assay system was employed. In the presence of 0.1 mM Ca^{2+} the portion of MRP8-MRP14 observed to be bound to cytokeratin 8 was significantly higher than that detectable in the presence of 2 mM EGTA (Figure 7).

Figure 7 Ca^{2+} -dependent binding of MRP8-MRP14 to keratin intermediate filaments *in vitro*

Cytokeratin 8 coated to 96-well plates was exposed to 0–8 μ g of native purified MRP8-MRP14 in the presence of either 0.1 mM CaCl_2 (—) or 2 mM EGTA (---) for 45 min. After several washes with TBS, bound MRP8-MRP14 was detected by an ELISA method as described in the Experimental section. Essentially similar results were obtained in several independent experiments.

Ca^{2+} -dependent binding of MRP8-MRP14 to keratin was found to be roughly four times higher compared with binding to actin or myosin (results not shown).

DISCUSSION

MRP8 and MRP14 are two S-100-like Ca^{2+} -binding proteins which were initially described in myelomonocytic cells [6]. In the past few years a number of reports have appeared describing the expression of both molecules by epithelial cells [2,3,16]. In these studies predominantly histological aspects of MRP8 and MRP14 expression have been investigated; however, analysis of their biochemical properties has been neglected. We therefore studied the epithelial cell line TR146 which, like activated human keratinocytes [26,27], has been found to express MRP8 and MRP14 at the mRNA and protein level. On employing a cross-linking technique, both proteins were shown to assemble in complexes of 25, 35 and 48 kDa in epithelial cells. These protein bands are thus similar to those characterized in detail previously in cells of myeloid origin [13], indicating that MRP8-MRP14 complex formation is a prerequisite for function, e.g. inhibition of protein kinase activity [28]. MRP8-MRP14 complex formation thus appears to be a common property for MRP8 and MRP14 which is independent of an epithelial or myeloid pathway of differentiation. Similarly, the MRP14 isoforms demonstrated by two-dimensional electrophoresis are similar in TR146 and myeloid cells [25].

Other members of the S-100 family, e.g. p11/annexin II and S-100 α/β , have been demonstrated to be associated with cytoskeletal structures [29–32]. Corresponding to these results, we recently demonstrated a Ca^{2+} -dependent co-localization of MRP8-MRP14 to cytoskeletal structures in activated monocytes. By employing a double-labelling immunogold electron microscopy technique these structures could be identified as vimentin intermediate filaments [15]. To elucidate the site of MRP8-MRP14 action in epithelial cells, which do not contain vimentin, differential centrifugation with subsequent SDS/PAGE and Western blot analysis was employed. Our data provide evidence that MRP8-MRP14 binds to the membrane and cytoskeletal fractions of epithelial cells in a Ca^{2+} -dependent

manner. The latter structures were identified by a double immunofluorescence labelling technique as keratin intermediate filaments. Accordingly, destruction of microfilaments and microtubules by cytochalasin B and demecolcine did not interfere with Ca^{2+} -dependent co-localization of MRP8-MRP14 to intermediate filaments. The question arises as to whether the MRP8-MRP14 complex binds directly to distinct conserved motifs of keratin and vimentin intermediate filaments or is associated with these proteins via a third, so far unidentified, molecule. Studies on MRP8-MRP14 binding to cytoskeletal filaments *in vitro* show that a direct Ca^{2+} -dependent binding to keratin appears to occur. However, involvement of an additional ligand in interactions of MRP8-MRP14 with the cytoskeleton cannot be excluded, since the *in vitro* assay system employed does not necessarily reflect the conditions *in vivo*.

The physiological relevance of MRP8-MRP14 co-localization with intermediate filaments is not yet clear. For a long time the intermediate filament network was considered as a more or less static cytoskeletal system. However, factors regulating intermediate filament assembly are now being increasingly investigated. Recently, Bianchi et al. [29] described the binding of S-100a and S-100b to glial fibrillary acidic protein, another member of the intermediate filament family [29]. Evidence was provided that S-100 is involved in the Ca^{2+} -dependent regulation of the state of assembly of these intermediate filaments by binding to and sequestering non-polymerized glial fibrillary acidic protein. Bearing in mind the comparable biochemical properties of MRP8-MRP14 and S-100, it would be an attractive hypothesis to speculate that the MRP8-MRP14 complex might also act on the assembly or disassembly of keratin and vimentin filaments. The dynamic properties of the intermediate filament network has been shown to be at least partially under the control of post-translational regulatory mechanisms which involve, for example, phosphorylation reactions (for reviews, see [33–36]). Binding of a protein kinase C isoform to keratins has been reported which resulted in phosphorylation of the latter [37]. Murao and colleagues demonstrated that complexes of MRP8 and MRP14, but not the single monomers, modulated protein kinase activity [28]. MRP8-MRP14 complex formation itself is a process regulated by Ca^{2+} , indicating a role in intracellular signalling [13]. In accordance with this, elevation of intracellular Ca^{2+} levels leads, as previously demonstrated, to reorganization of the epithelial cytofilament network [38]. In hyperproliferative dermatoses (e.g. psoriasis), reorganization of the keratin filament network has also been observed [39,40]. MRP8 and MRP14 are expressed by keratinocytes in such hyperproliferative dermatoses but not in the epidermis of non-lesional skin [3,16]. Morphological evidence has been provided that MRP8 and MRP14 are associated with cytoskeletal components in various skin disorders [41]. The data presented for TR146 cells in the present study thus appear to account for keratinocytes *in situ* as well. Interestingly, the genes for MRP8 and MRP14 have been found to form a cluster on chromosome 1q21 together with genes encoding other S-100-like Ca^{2+} -binding proteins as well as profilaggrin, trichohyalin, loricrin and involucrin, the expression of which has been proposed to be co-regulated [42]. Since these proteins are in part specifically expressed during terminal differentiation of epithelial cells and, like profilaggrin and trichohyalin, are associated with intermediate filaments, a functional role in the reorganization of the cytoskeleton during hyperproliferative skin disease may also be assumed for MRP8 and MRP14.

We thank K. Fischer, D. Kortevoss and C. Wulff for excellent technical assistance. This work was supported by a grant from the Bundesministerium für Forschung und Technologie, Bonn, Germany.

REFERENCES

- Nickoloff, B. J. and Turka, L. A. (1993) *Am. J. Pathol.* **143**, 325–331
- Brandtzaeg, P., Dale, I. and Fagerhol, M. K. (1987) *Am. J. Clin. Pathol.* **87**, 700–707
- Kelley, S. E., Jones, D. B. and Fleming, S. (1989) *J. Pathol.* **159**, 17–21
- 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
- Odink, K., Cerletti, N., Brüggem, J., Clerc, R. G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R. and Sorg, C. (1987) *Nature (London)* **330**, 80–82
- Zwadlo, G., Brüggem, J., Gerhards, G., Schlegel, R. and Sorg, C. (1988) *Clin. Exp. Immunol.* **77**, 510–515
- Roth, J., Goebeler, M., van den Bos, C. and Sorg, C. (1993) *Biochem. Biophys. Res. Commun.* **191**, 565–570
- Goebeler, M., Roth, J., Henseleit, U., Sunderkötter, C. and Sorg, C. (1993) *J. Leukocyte Biol.* **53**, 11–18
- Delabie, J., de Wolf-Peters, C., van den Oord, J. J. and Desmet, V. J. (1990) *Clin. Exp. Immunol.* **81**, 123–126
- Roth, J., Sunderkötter, C., Goebeler, M., Gutwald, J. and Sorg, C. (1992) *Int. Arch. Allergy Immunol.* **98**, 140–145
- Goebeler, M., Roth, J., Burwinkel, F., Vollmer, E., Böcker, W. and Sorg, C. (1994) *Transplantation* **58**, 355–361
- Teigelkamp, S., Bhardwaj, R., Roth, J., Meinardus-Hager, G., Karas, M. and Sorg, C. (1991) *J. Biol. Chem.* **266**, 13462–13467
- Edgeworth, J., Gorman, M., Bennett, R., Freemont, P. and Hogg, N. (1991) *J. Biol. Chem.* **266**, 7706–7713
- Roth, J., Burwinkel, F., van den Bos, C., Goebeler, M., Vollmer, E. and Sorg, C. (1993) *Blood* **82**, 1875–1883
- Kunz, M., Roth, J., Sorg, C. and Kolde, G. (1992) *Arch. Dermatol. Res.* **284**, 386–390
- Rupniak, H. T., Rowlatt, C., Lane, E. B., Steele, J. G., Trejdosiewicz, L. K., Laskiewicz, B., Povey, S. and Hill, B. T. (1985) *J. Natl. Cancer Inst.* **75**, 621–635
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Bravo, R. (1984) in *Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications* (Celis, J. E. and Bravo, R., eds.), pp. 3–36. Academic Press, New York
- von Overbeck, J., Stähli, C., Gudat, F. et al. (1985) *Vichows Arch. A Pathol. Anat.* **407**, 1–12
- Zwadlo, G., Schlegel, R. and Sorg, C. (1986) *J. Immunol.* **137**, 512–518
- Bhardwaj, R., Zolt, C., Zwadlo-Klarwasser, G., Roth, J., Goebeler, M., Mahne, K., Falk, M., Meinardus-Hager, G. and Sorg, C. (1992) *Eur. J. Immunol.* **22**, 1891–1897
- van den Bos, C. (1993) Ph.D. Thesis, University of Münster, Germany
- Edgeworth, J., Freemont, P. and Hogg, N. (1989) *Nature (London)* **342**, 189–192
- Saintigny, G., Schmidt, R., Shroot, B., Juhlin, L., Reichert, U. and Michel, S. (1992) *J. Invest. Dermatol.* **99**, 639–644
- Madsen, P., Rasmussen, H. H., Leffers, H., Honoré, B., Dejgaard, K., Olsen, E., Kiil, J., Walbum, E., Andersen, A. H., Basse, B., Lauridsen, J. B., Ratz, G. P., Celis, A., Vandekerckhove, J. and Celis, J. E. (1991) *J. Invest. Dermatol.* **97**, 701–712
- Murao, S., Collart, F. R. and Huberman, E. (1989) *J. Biol. Chem.* **264**, 8356–8360
- Bianchi, R., Giambanco, I. and Donato, R. (1993) *J. Biol. Chem.* **268**, 12669–12674
- Baudier, J. and Cole, R. D. (1987) *Biochemistry* **27**, 2728–2736
- Baudier, J. and Cole, R. D. (1988) *J. Biol. Chem.* **263**, 5876–5883
- Zokas, L. and Glenney, J. R. (1987) *J. Biol. Chem.* **105**, 2111–2121
- Steinert, P. M. and Roop, D. R. (1988) *Annu. Rev. Biochem.* **57**, 593–625
- Steinert, P. M. (1993) *J. Invest. Dermatol.* **100**, 729–734
- Coulombe, P. A. (1993) *Curr. Opin. Cell Biol.* **5**, 17–29
- Blumenberg, M. (1993) in *Molecular Biology of the Skin: The Keratinocyte* (Darmon, M. and Blumenberg, M., eds.), pp. 1–32. Academic Press, San Diego
- Omary, M. B., Baxter, G. T., Chou, C. F., Riopel, C. F., Lin, W. Y. and Strulovici, B. (1991) *J. Biol. Chem.* **117**, 583–593
- Zamansky, G. B., Nguyen, U. and Chou, I. N. (1991) *J. Invest. Dermatol.* **97**, 985–994
- Weiss, R. A., Eichner, R. and Sun, T. T. (1984) *J. Biol. Chem.* **98**, 1397–1406
- Thewes, M., Stadler, R., Korge, B. and Mischke, D. (1991) *Arch. Dermatol. Res.* **283**, 465–471
- Kelly, S. E., Hunter, J. A. A., Jones, D. B., Clark, B. R. and Fleming, S. (1991) *Br. J. Dermatol.* **124**, 403–409
- Korge, B. P., Volz, A., Ziegler, A. and Mischke, D. (1994) *J. Invest. Dermatol.* **103**, 445 (Abstr.)