Characterization of isoforms of protein 4.1 present in the nucleus

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Although protein 4.1 was originally identified as an element of the erythrocyte membrane skeleton, its presence in most mammalian cell types is now well described. Antibodies raised against erythrocyte protein 4.1 or synthetic peptides corresponding to the spectrin–actin-binding domain of protein 4.1 react with plasma membranes and, unexpectedly, nuclei of different cell types. Nuclear staining was further confirmed in isolated nuclei prepared from rat liver and human leukaemic cell lines. Immunoblot analysis of subcellular fractions derived from these cells revealed three prominent proteins, of 80, 135 and 145 kDa. The structural relationship of the high-molecular-mass proteins with erythrocyte protein 4.1 was demonstrated by peptide mapping. These results indicate that mammalian nucleated cells contain several isoforms of erythrocyte protein 4.1 and that some high-molecular-mass forms may primarily reside in the nucleus.

INTRODUCTION

Erythrocyte membranes are supported by a complex network of proteins that confer stability on the lipid bilayer and regulate the two-dimensional arrangement of transbilayer proteins [1–3]. The elements of this network include two principal proteins, spectrin and actin. Spectrin is an elongated molecule composed of two subunits that behave as dimers in solution but have the capacity to assemble into higher oligomers by a complex head-to-head self-association mechanism [4,5]. Tetramers and larger oligomers of spectrin are also able to bind to short filaments of actin [6–9]. This association is greatly augmented by a third protein, commonly referred to as protein 4.1 [10,11].

Protein 4.1, an 80 kDa thiol-rich phosphoprotein originally identified in non-nucleated erythrocytes, has a number of notable features. In addition to its capacity to enhance the association between spectrin and actin [10], protein 4.1 also binds to myosin [12], tubulin [13] and several transmembrane glycoproteins, including glycophorin A, the anion-exchanger (band 3 protein) and glycophorin C (reviewed in ref. [2]). The connections between spectrin, actin, protein 4.1 and the transmembrane glycoproteins are believed to be one of the ways that the membrane skeleton is attached to the overlying lipid bilayer. These linked proteins complement the attachment of the skeleton to the bilayer that is also provided by ankyrin, which links the β -subunit of spectrin directly to the cytoplasmic segment of the band 3 protein [14].

Many immunochemical studies have indicated that protein 4.1 is not confined to erythrocytes but is present in most cells and tissues [15–23]. Molecular cloning studies have revealed the existence of multiple forms of protein-4.1 cDNAs arising by alternative splicing in erythroid and non-erythroid tissues [24–27]. Some of these non-erythroid cDNAs encode protein 4.1 isoforms of molecular masses higher than 80 kDa [24,27]. The role of the high-molecular-mass isoforms of protein 4.1 is still unknown. Here I present a biochemical characterization of some of these high-molecular-mass isoforms of protein 4.1 and reveal their presence in nuclei of mammalian cells.

MATERIALS AND METHODS

Preparation of antisera

Rabbit polyclonal antibodies to different forms of human erythrocyte protein 4.1 were prepared by using standard tech-

niques of immunization and antibody preparation as described previously [28]. Protein 4.1 was prepared and purified as described previously [29]. Antisera raised against synthetic peptides were those produced for a previous study [28].

Immunofluorescence microscopy

Maden–Darby canine kidney (MDCK) cells were grown in 75 cm flasks (Falcon, Oxnard, CA, U.S.A.) at 37 °C in an atmosphere of 5% CO₂ in air. Eagle's minimal essential medium with Earle's salts was supplemented with 10% (v/v) fetal-calf serum (Gibco, Madison, WI, U.S.A.), 10 mM-Hepes, 1 mM-pyruvate, 1.0 mM-glutamine, 0.1 mM non-essential amino acids and 100 units each of penicillin and streptomycin/ml. MDCK cells grown on glass coverslips were fixed with phosphate-buffered 10% formalin containing 0.2% Trixon X-100 for 20 min on ice. After gentle washing with phosphate-buffered saline (PBS) containing 1% (w/v) BSA, affinity-purified antibodies to protein 4.1 fractions or pre-immune sera were added at concentrations of 10–100 μ g/ml. After 1 h incubation the dishes were washed with PBS/1% BSA, and a secondary rhodamine-labelled goat anti-(rabbit IgG) antibody (Cappel Laboratories, Cochranville, PA, U.S.A.; diluted 1:100) was added. After several PBS washes the coverslips were immersed in 10%, 20% and 30% (v/v) glycerol in succession, mounted on microscope slides, and examined with a Nikon epifluorescence microscope.

Analysis of cell fractions by immunoblotting

JY and Molt-4 cells, B and T leukaemic cell lines respectively, were grown in suspension in RPMI-1640 medium (Gibco, Madison, WI, U.S.A.) supplemented for MDCK cells.

Cells were harvested by centrifugation and washed with fresh growth medium containing 1 mM-EGTA and 0.4 mM-di-isopropyl phosphorofluoridate (buffer A). Subsequently, cells were washed in the presence of 0.25 M-sucrose in Buffer A followed by two washes in 0.025 M-sucrose in the same buffer as above. Cells were disrupted in a Dounce homogenizer and centrifuged in a Sorvall centrifuge at 1000 *g* for 5 min. The nuclear sediment was washed in buffer A, centrifuged at 1000 *g* for 5 min and resuspended in Laemmli solubilizing buffer [30]. The 'nuclei-free' supernatant was layered over a discontinuous sucrose gradient containing 30%, 40% and 50% (w/v) sucrose in 10 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EGTA and 0.4 mM-di-isopropyl phosphorofluoridate and centrifuged at

30000 rev./min for 90 min in a Beckman type 40 Ti rotor. The cytosol fraction at the buffer/sucrose fraction, the fractions at the interfaces between the 40 % and 50 % sucrose and between the 30 % and 40 % sucrose, the fraction at the top of the 30 % sucrose and the high-speed pellet were collected separately, washed in Tris buffer without sucrose, centrifuged and solubilized in Laemmli buffer. The plasma-membrane-enriched fraction is at the interface between the 30 % and 40 % sucrose.

Nuclei were isolated from human Molt-4 leukaemic cells by using the procedure of Warner [31].

Samples were subjected to electrophoresis on SDS/10 % -polyacrylamide slab gels and proteins were transferred on to nitrocellulose according to the procedure described by Towbin *et al.* [32].

NTCB treatment of protein-4.1-immunoreactive bands

Molt-4-cell nuclei preparations were subjected to electrophoresis on SDS/10 % -polyacrylamide gels. Protein 4.1 immunoreactive bands of 80 kDa and of 135/145 kDa were excised

from the gels and electroeluted in 5 mM-NH₄HCO₃/0.5 mM-EDTA/0.05 % SDS/0.1 % 2-mercaptoethanol, pH 8.2, at 150 V for 9 h at room temperature. Samples were freeze-dried and resolubilized in 7.5 M-guanidinium chloride in 50 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA. Finally, 2-nitro-5-thiocyanobenzoic acid (NTCB) was added to some of the samples and the incubation procedure was carried out as described by Leto & Marchesi [29]. Samples were subjected to electrophoresis again on SDS/10 %-polyacrylamide gels, and proteins were transferred on to nitrocellulose and immunoblotted with affinity-purified antibodies. The bands of 135 kDa and 145 kDa were electroeluted and processed together.

The antibodies used in this work have been previously analysed by immunoblotting of NTCB digests of erythrocyte protein 4.1 [22,23]. They recognize the large NTCB-generated fragments of 66, 56 and 51 kDa previously characterized by Leto & Marchesi [29]. None of them recognizes the small NTCB-generated fragments of 14, 24 or 29 kDa derived from the *N*-terminal domain of the protein 4.1 molecule [22,23].

RESULTS

Identification of isoforms of protein 4.1 relies on the use of antibodies prepared against different forms of the erythrocyte protein 4.1 isolated from human erythrocyte membranes. For purposes of discussion, erythrocyte protein 4.1 can be defined as having four domains identified on the basis of their resistance to mild proteolytic cleavage [29]; these are defined in Fig. 1. Antibodies have been prepared by immunizing animals with the antigens listed in Fig. 1. Antibodies prepared in rabbits against intact protein 4.1 are found to react primarily with 16 kDa and 24 kDa domains, on the basis of immunoblot analysis of controlled-proteolytic-digestion studies [23]. Antibodies against the 8 kDa domain were prepared by immunizing animals with two synthetic peptides, prepared according to the previously reported amino acid sequence [28]. All antibody preparations were ultimately purified by their immunoaffinity to native erythrocyte protein 4.1 to ensure that they all recognize deter-

Fig. 1. (a) Domains of erythrocyte protein 4.1 defined by restricted proteolytic cleavage [29] and (b) forms of protein 4.1 used for immunization

Numbers in parentheses refer to numbering of amino acid residues in the principal erythrocyte form of protein 4.1 [26].

Fig. 2. Immunofluorescence staining of MDCK cells

Cells were fixed with Triton/formalin and stained with (a) anti-(8 kDa domain) serum and (b) anti-(intact protein 4.1) serum.

minants present on the native protein 4.1 molecule, as was shown previously [22,23].

Nuclear localization of protein 4.1 isoforms

Antiserum that was generated against intact erythrocyte protein 4.1 [anti-(intact protein 4.1) serum] mainly stains the plasma membrane in cultured MDCK cells (Fig. 2*b*), a finding similar to that reported for other cells [2,3]. However, these antibodies also stain the nucleus in a very diffuse granular manner (Fig. 2*b*). Antibodies raised against the synthetic peptides [anti-(8 kDa domain) serum] mainly show a diffuse granular localization throughout the nucleoplasm (Fig. 2*a*). They show much less reactivity with the plasma membrane. All antibodies leave nucleoli unstained, these appearing as globular units outlined by the diffusely stained surrounding nucleoplasm.

Nuclear staining by the different anti-(protein-4.1) antibody preparations is also seen when the antisera are applied to isolated nuclei free of contaminating cytoplasmic elements. The staining patterns of isolated nuclei prepared from human leukaemic cells with anti-(8 kDa domain) and anti-(intact protein 4.1) sera are shown in Figs. 3(*a*) and 3(*b*) respectively. Although these nuclei are derived from a different species and cell type, both antibodies reacted, giving a microgranular pattern similar to that seen with the cultured MDCK cells.

Immunoblotting analysis of subcellular fractions

To explore further the apparent nuclear localization of antigens labelled by these anti-(protein 4.1) sera, large-scale cultures of human leukaemic cells were subjected to standard subcellular fractionation procedures. Samples of intact cells, nuclear pellets and different fractions of microsomal elements separated by sucrose gradients were analysed by standard SDS/PAGE immunoblotting as described above. One such experiment is shown in Fig. 4. Antibodies raised against the 8 kDa domain of protein 4.1 react with three prominent bands on these gels, which correspond to proteins of 80, 135 and 145 kDa. In addition to these prominent bands, weaker staining bands of lower molecular mass are also seen in some of the fractions. The nuclear fraction (Fig. 4*a*, lane 2) contains the same three prominent bands seen in the whole-cell preparations and in the membrane fractions. In contrast, relatively small amounts of these bands are seen in the fraction that corresponds to the cytoplasm of the cells (Fig. 4*a*, lane 4). The 80 kDa components found in both nuclei and membrane fractions of leukaemic cells have the same apparent molecular mass as the human erythrocyte form of protein 4.1. When antibodies raised against native protein 4.1 were used, bands of molecular masses 80 and 135 kDa were also recognized. These two bands were present in the nuclear fraction (Fig. 4*b*, lane 2). Some other bands of lower molecular mass were weakly stained by these antibodies in some of the fractions (Fig. 4*b*). Both sets of immunoblots further confirm the nuclear localization revealed by immunofluorescence.

To determine the generality of this nuclear localization of protein 4.1 isoforms I analysed nuclear preparations from HeLa cells and adult rat liver. The patterns obtained by immunofluorescence of these preparations were similar to those presented in Fig. 3. Immunoblots of purified adult rat liver nuclei showed three prominent bands of molecular masses 80, 115 and 135 kDa that reacted with both the anti-(8 kDa domain) and the anti-(intact protein 4.1) sera (results not shown). The existence of a complex pattern of tissue-specific protein 4.1 isoforms in whole-tissue homogenates has been previously reported [23].

Peptide mapping of nuclear protein 4.1 isoforms

Cleavage of erythrocyte protein 4.1 at cysteine residues, which

Fig. 3. Immunofluorescence staining of isolated nuclei

Nuclei were prepared from Molt-4 cells as described in the Materials and methods section, attached to poly-L-lysine-coated slides, fixed in Triton/formalin and stained with (*a*) anti-(8 kDa domain) serum and (*b*) anti-(intact protein 4.1) serum.

Fig. 4. Immunoblot analysis of subcellular fractions of Molt-4 cells

Homogenates of intact cells (lanes 1), nuclear pellet (lanes 2), crude cytosol (lanes 3) and five fractions obtained by separating crude microsomes on a sucrose step gradient and sampling the cytoplasm-containing supernatant (lanes 4), each interface (lanes 5, 6 and 7) and the pellet (lanes 8), all stained with (*a*) anti-(8 kDa domain) serum or (*b*) anti-(intact protein 4.1) serum.

are all located within the 30 kDa domain, produces a characteristic peptide mapping of the protein [29]. To analyse to what extent the nuclear immunoreactive forms of protein 4.1 are

Fig. 5. NTCB cleavage of nuclear immunoreactive 4.1 proteins

Immunoreactive forms of protein 4.1 that are present in Molt-4-cell nuclei or human erythrocyte protein 4.1 were cleaved by the cysteine-specific reagent NTCB as described in the Materials and methods section. Samples in lanes 2, 3 and 5 were incubated in the presence of NTCB. Samples in lanes 1 and 4 were incubated in the absence of NTCB, under the same conditions. Human erythrocyte protein 4.1 (lanes 1 and 2), Molt-4-cell 80 kDa protein (lanes 3), and Molt-4-cell 135/145 kDa proteins (lanes 4 and 5) were immunoblotted with (a) anti-(8 kDa domain) serum or (b) anti-(intact protein 4.1) serum. Positions of molecular-mass markers are indicated on the right.

structurally related to the erythroid protein, I treated them with NTCB, a chemical reagent that specifically cleaves at cysteine residues. The 80 kDa immunoreactive form of protein 4.1 that is present in human leukaemic-cell nuclei and the 80 kDa erythroid protein are not only similar in size but they also share analogous NTCB-cleavage peptide maps, as detected with the anti-(8 kDa domain) serum (Fig. 5a, lanes 2 and 3) and the anti-(intact protein 4.1) serum (Fig. 5b, lanes 2 and 3). The high-molecular-mass forms of 135 and 145 kDa give peptide maps identical with those of both 80 kDa erythrocyte and lymphocyte proteins 4.1 when tested with either the anti-(8 kDa domain) serum (Fig. 5a, lane 5) or the anti-(intact protein 4.1) serum (Fig. 5b, lane 5). This could be explained by addition of protein sequence at the *N*-terminal end of the 80 kDa species. Erythrocyte protein 4.1 contains three cysteine residues located within the first 31 amino acid residues at the *N*-terminal end of the molecule; the remaining four cysteine residues are all located within the 30 kDa domain of the 80 kDa molecule (see Fig. 1). Thus additional sequence at the *N*-terminal end would lead to NTCB cleavage patterns detected by both the anti-(8 kDa domain) and the anti-(intact protein 4.1) sera similar to that obtained for the 80 kDa protein 4.1.

DISCUSSION

Protein 4.1 is a phosphoprotein whose role has been mainly studied in erythroid cells, where it was originally identified, and little is known about its function in non-erythroid cells.

Here I report that antibodies raised against native human erythrocyte protein 4.1 or peptides derived from it react with nuclei of a number of different cells, a localization that was confirmed by immunoblot analysis of subcellular fractions. Immunoblots also show that several polypeptide chains react with the same antisera used to stain the nuclear structures. An 80 kDa band, similar in size to erythrocyte protein 4.1, is one of the reactive components, and it could be identical with the erythrocyte protein. Indeed, besides the immunological relationship of those two proteins, they share analogous NTCB-cleavage peptide maps, which confirms their structural relationship. Bands of higher or lower molecular mass that share antigenic determinants with erythrocyte protein 4.1 are also observed. Some of the lower-molecular-mass bands could be proteolytic artifacts despite the use of proteinase inhibitors at all stages of sample

preparation, but it is also possible that they exist as such within the cell. I have focused on the high-molecular-mass forms of 135 and 145 kDa. NTCB-cleavage peptide mapping analyses indicate that these proteins are structurally related to both the 80 kDa erythroid and lymphoid proteins. Moreover, the fact that these high-molecular-mass forms have an NTCB-cleavage pattern like that of the 80 kDa species could be explained if they contain additional sequences at their *N*-terminal regions. Otherwise, peptides different from those obtained for the 80 kDa erythroid protein should also be recognized by these antisera. Recently the existence has been described of several isoforms of protein 4.1 produced by alternative splicing in human erythroid and non-erythroid cells [24–27]. One of these isoforms is a 135 kDa protein that has been identified in Molt-4 cells, the same cell line used in this study. The novel 135 kDa protein 4.1 is extended at the *N*-terminal end of the 80 kDa lymphoid protein [27]. These results strongly suggest that the 135 kDa protein described in the present paper and that cloned by Tang *et al.* [27] are probably the same one.

Recently, the sequencing of ezrin cDNA has revealed a high degree of similarity within the *N*-terminal domain of the protein that it encodes and that of erythrocyte protein 4.1 [33]. Sequences downstream of the *N*-terminal domain in protein 4.1 bear no similarity to those in ezrin [33]. Thus it is very unlikely that the antibodies used in the present work recognize ezrin since none of them reacts with the *N*-terminal domain of protein 4.1 [22,23,28].

It is noteworthy that the anti-(8 kDa domain) antibodies were raised against synthetic peptides whose amino acid sequence corresponds to part of a 67-amino acid-residue segment of erythrocyte protein 4.1 that is involved in promoting spectrin-actin interactions [28,34]. These antibodies are also able to interfere with the capacity of native erythrocyte protein 4.1 to link spectrin and actin together [28]. If the determinants they recognize on the nuclear proteins described here also share the capacity to promote interactions between actin and spectrin, their nuclear localization is especially interesting, since substantial amounts of actin exist in nuclei [35,36], and the presence of spectrin in the nucleus has recently been described [37]. Since protein 4.1 was first described in erythrocytes and is found bound to the cytoplasmic surface of their membrane, we have been accustomed to the idea that protein 4.1 is primarily a membrane protein. Antibodies raised against erythrocyte protein 4.1 also stain filamentous structures in non-erythroid cells [15] as well as

parts of the nucleus as described here. Interestingly, the intra-nuclear dot-like staining pattern seen with antibodies to protein 4.1 resembles that of other proteins that are associated with the nucleoskeleton [38–40]. Thus it is conceivable that some 4.1 proteins may carry out important functions within the nucleus as part of the nucleoskeleton. The presence of actin [35,36], spectrin [37] and, as described here, protein 4.1 within the nucleus suggests that a protein meshwork similar to that found in the membrane skeleton may constitute an important part of the scaffolding to which ribonucleoprotein particles and specific chromatin regions may bind. Some differences have been described between the nucleoskeleton of proliferating and quiescent cells [37]. This suggests that the changes of nuclear function during the cell cycle may partly depend on modifications of the nucleoskeletal structure. Phosphorylation and dephosphorylation of protein 4.1 might constitute one of the mechanisms for the modification of the nucleoskeleton through the modulation of the formation and dissociation of the spectrin–protein 4.1–actin ternary complex (for a review see ref. [41]). Thus protein 4.1 phosphorylation would result in the loosening of the nucleoskeleton lattice, by decreasing formation of the ternary complex, and, conversely, protein 4.1 dephosphorylation would favour a stiffening of the nucleoskeleton.

One must also consider the possibility that different isoforms of protein 4.1 relocate from one compartment to another depending upon factors that regulate their capacity to bind to specific sites. A subset of protein 4.1 molecules capable of binding to surface membrane under one set of conditions might be able to shift to other cytoskeletal structures or even migrate into the nucleus if an appropriate stimulus is provided. However, whether the same or different protein 4.1 isoforms are found in each compartment remains to be established.

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