Protein 4.1 is a component of the nuclear matrix of mammalian cells

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Protein 4.1 is a major component of the erythrocyte membrane skeleton that promotes the interaction of spectrin with actin and links the resulting complex network to integral membrane proteins. Here we analyse the distribution of different 4.1 proteins within the nucleus of mammalian cells. Nuclear matrices have been prepared from Madin–Darby canine kidney (MDCK) and HeLa cells and protein fractions isolated at each step of the purifications have been analysed by immunoblotting using characterized polyclonal antibodies against protein 4.1. Two 4.1 polypeptides of $M_r \sim 135000$ and 175000 are extracted after DNase I digestion and 0.25 M ammonium sulphate treatments, suggesting that they may be associated with chromatin.

Interestingly, nuclear matrices isolated after DNase I digestion and sequential treatments with increasing ionic strength contain a third 4.1 polypeptide of $M_r \sim 75000$ (4.1p75), suggesting that it is a component of the nuclear matrix. Immunoblot analyses of nuclear matrices isolated from different cell types and species indicate that 4.1p75 is a common element of the nuclear matrix of mammalian cells. Moreover, 4.1p75 distributes to typical nuclear speckles which are enriched with the spliceosome assembly factor SC35, as revealed by double-label immunofluorescence analyses. Protein 4.1p75 might be an anchoring element of the nucleoskeleton, playing a role similar to that described for the erythroid protein 4.1 in red blood cells.

INTRODUCTION

The nuclear matrix has been biochemically and morphologically defined as the chromatin-free, insoluble framework of the nucleus. Following the original discovery of the nuclear matrix [1,2], methods for separating it from chromatin have been undergoing continuous development [3–6]. A sequential extraction procedure has revealed an intermediate filament-like nuclear network termed 'the core filaments of the nuclear matrix' [6]. These filaments, resembling those described by Jackson and Cook [7], may serve as the core structures around which the matrix is built. The matrix has been associated with a variety of nuclear processes including transcription [8,9], splicing [10] and replication [11,12]. These processes have been localized in discrete nuclear domains rather than diffusely distributed throughout the nucleoplasm, persisting after the removal of chromatin. This suggests that the nuclear matrix plays an important role in maintaining spatial order within the nucleus. Despite many essential functions postulated for the nuclear matrix, few proteins involved in these processes have been characterized so far. Among these are the lamins A, B and C of the nuclear lamina [13–15] and several internal nuclear matrix proteins including topoisomerase II [16], a calmodulin-stimulated protein kinase [17] and the nuclear-mitotic apparatus protein, NuMA [18,19]. A more detailed study of nuclear matrix function will require the characterization of additional nuclear matrix proteins.

Protein 4.1 is a major component of the erythrocyte membrane skeleton that promotes the interaction of spectrin with actin and links the resulting complex network to integral membrane proteins. Although protein 4.1 was originally identified as an element of erythrocytes, its presence in many mammalian cell types is now well documented (reviewed in [20]). The major protein 4.1 isoforms in human erythrocytes are a doublet of $M_r \sim 78000$ and 80000 , but avian erythrocytes and mammalian

non-erythroid tissues present a variety of immunoreactive 4.1 polypeptides of M_r s ranging from 30000 to 210000 [21,22]. Post-translational modifications such as phosphorylation by multiple kinases and glycosylation with O-linked *N*-acetylglucosamine could partly account for the impressive diversity of 4.1 proteins (reviewed in [20]). However, it is clear that multiple structural isoforms of 4.1 can be produced by alternative splicing of the transcript of a single gene both in erythroid and non-erythroid tissues [23–28]. There are at least 10 motifs that can be selectively included or excluded from the mRNA transcript, in various combinations, to produce these isoforms [25,29]. The functional role of non-erythroid 4.1 isoforms remains unknown. Interestingly, isolated erythroid 4.1 binds tubulin [30] and myosin [31], suggesting that certain isoforms of protein 4.1 may play some role in integrating the membrane skeleton with the larger cytoskeleton. It has also been described that antibodies against protein 4.1 stain the mammalian nucleus in diffuse as well as in speckled-like patterns [32] characteristic of antigens located in the nuclear matrix. However, it is not yet known whether one or more 4.1 isoforms are responsible for the different nuclear staining patterns nor if protein 4.1 is indeed an integral component of the nuclear matrix.

In this study, we analyse the distribution of different 4.1 proteins in the nucleus and show that a 4.1 protein of $M_r \sim 75000$ is a component of the nuclear matrix of mammalian cells that distributes in speckle domains enriched with splicing factors.

MATERIALS AND METHODS

Cells and cell culture conditions

The cell lines used in this study were epithelial MDCK cells from canine kidney, epithelial HeLa cells from human cervix, fibroblastic BHK cells from hamster kidney, fibroblastic NIH 3T3

Abbreviations used: FITC, fluorescein isothiocyanate; MDCK, Madin–Darby canine kidney; NLS, nuclear localization signal; RT, room temperature; snRNP, small nuclear ribonucleoprotein particle.

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Figure 1 Scheme of the nuclear matrix isolation methods

In method I chromatin is removed by high-salt extraction followed by nuclease digestion which may induce shrinkage of the nucleus. The sequential extraction steps were carried out with Triton X-100, medium (0.5 M NaCl) and high (2 M NaCl) ionic strength, and DNase I digestion; all solutions were prepared in PEM buffer (see the Materials and methods section). In method II chromatin is removed by nuclease digestion followed by extraction by a gradually increased ionic strength which greatly reduces the nuclear shrinkage [6]. The sequential extraction steps were carried out with Triton X-100, DNase I digestion and a gradual increase in ionic strength; all solutions were prepared in CSK buffer. Both methods leave the nuclear matrix with its attached intermediate filaments. Immunofluorescent analyses were done over the extracted cells after each step of the extractions. Immunoblot analyses carried out in subsequent experiments (Figures 4, 5 and 6) were performed with the extracts (SUP) obtained at each step of method II and with the final pellet corresponding to the nuclear matrix fraction. The asterisks indicate two extraction steps included for the immunoblot analyses and excluded from the immunofluorescence analyses.

cells from mouse embryo, simian virus 40 (SV40)-transformed fibroblastic COS cells from monkey kidney, and A498 cells from human kidney.

Cells were grown on Petri dishes or glass coverslips, in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum (Gibco-BRL, Gaithersburg, MD, U.S.A.), penicillin (50 units/ml) and streptomycin (50 units/ml), at 37 °C in an atmosphere of 5 % CO₂.

Cell extraction

For *in situ* sequential extractions, cells were grown on coverslips to 70–80 % confluency. Coverslips with attached cells were washed twice in PBS and nuclear matrices were prepared by two different methods (see Figure 1).

Method I

Cells were sequentially extracted with (a) 0.5 % Triton X-100 in PEM (100 mM Pipes, pH 6.2, 1 mM EGTA, 1 mM MgCl₂) for 2 min at room temperature (RT); (b) 0.5 M NaCl in PEM for

5 min at RT; (c) 2 M NaCl in PEM for 5 min at RT; (d) RNase-free DNase I (100 µg/ml) in PEM for 30 min at 37 °C and 0.25 M ammonium sulphate in PEM for 5 min at RT.

Method II

Cells on coverslips were lysed for 2 min at RT in cytoskeletal (CSK) buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA) containing 0.5 % Triton X-100, and then digested with 100 µg/ml of RNase-free DNase I in CSK buffer at RT for 30 min and extracted with 0.25 M ammonium sulphate in CSK buffer for 5 min at RT. Finally, the coverslips were immersed in CSK buffer and a similar volume of 4 M NaCl was added dropwise with gentle shaking to a final concentration of 2 M NaCl [6,33]. This process removes the cytosolic soluble proteins, the chromatin and chromatin-associated proteins and the outer nuclear matrix proteins, leaving the nuclear lamina and the internal nuclear matrix [6,34]. After each step, cells on coverslips were fixed and incubated with antibodies as indicated below. All buffers were prepared with a cocktail of protease inhibitors containing 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM PMSF.

Immunofluorescence microscopy

Cells grown on glass coverslips were extracted by methods I or II (see Figure 1) and fixed in 10 % formalin (37 % formaldehyde solution, Sigma-F1635) for 15 min at RT. After fixation, cells were washed in PBS and blocked in PBS containing 3 % (w/v) BSA for 20 min. Cells were then incubated with affinity-purified polyclonal antibodies against the 8000-*M_r* domain of protein 4.1 involved in the association of 4.1 with spectrin and actin [32,35,36] for 1 h at RT. Coverslips were rinsed in PBS and then incubated with secondary Texas Red-labelled goat anti-(rabbit IgG) antibodies for 45 min at RT. For double-labelling experiments, monoclonal anti-B1C8 [34] or anti-SC35 [37] antibodies (obtained from Dr. S. Penman and from the American Type Culture Collection, respectively) were added to the coverslips following the incubation step with the secondary anti-(rabbit IgG) antibodies and the reactions were visualized with fluorescein isothiocyanate (FITC)-conjugated goat anti-(mouse IgG) antibodies. After several washes in PBS, preparations were dried, mounted on microscope slides with Mowiol 40.88 and examined with a Zeiss epifluorescence microscope. In some experiments, chromatin was stained with 0.01 µg/ml Hoechst 33258 just before the preparations were mounted.

Analysis of cell fractions by immunoblotting

For electrophoresis and immunoblotting analysis, cells grown on Petri dishes were rinsed twice in PBS and then scraped with a rubber policeman. Method II (see Figure 1) has been followed with slight modifications. After the extraction of soluble proteins with 0.5 % Triton X-100, cells were resuspended in RSB buffer [10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM PMSF, 1 % (v/v) Nonidet P-40, and 0.5 % (v/v) sodium deoxycholate] for 15 min at 0 °C with three periods of vortexing of 30 s each, and centrifuged at 500 *g* for 5 min at 4 °C. This step removes the cytoskeleton, leaving the nuclei with their attached intermediate filaments [6]. Most of the intermediate filament proteins were extracted by homogenizing the pellets in 3 mM imidazole, pH 7.4, [38] by ten passages through a 22G1 1/4-gauge needle fitted on a plastic syringe. The next extraction steps were the same as described in method II for *in situ* sequential extractions. Protein extracts from MDCK, HeLa, NIH 3T3, BHK, COS and A498 cells were precipitated in 7 % tri-

chloroacetic acid, washed in cold ethanol, dried in a Speedvac microfuge, and finally resuspended in Laemmli buffer [39]. Proteins from each fraction were separated by SDS/PAGE in 8% acrylamide minigels. Gels were stained with Coomassie Brilliant Blue R 250 or transferred to Immobilon-PVDF at 100 V for 75 min in 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) buffer, pH 11.

Immobilon membranes with transferred proteins were blocked with 10% (w/v) dry milk containing 0.05% Tween 20 in PBS overnight at 4 °C. After washing with PBS/0.05% Tween 20, Immobilon membranes were incubated with affinity-purified polyclonal antibodies against the 8000- M_r domain of protein 4.1, with gentle shaking for 1 h at RT. Blots were washed again with PBS/0.05% Tween 20 three times for 15 min and then incubated with horseradish peroxidase-conjugated anti-(rabbit IgG), for 1 h at RT. Blots were gently washed again with PBS/0.05% Tween 20 before developing them by chemiluminescence with ECL (Western Blotting Detection Reagents RPN-2106; Amersham Life Science) over Kodak autoradiography films.

RESULTS

Previous studies from our laboratory showed that antibodies against protein 4.1 stained the nucleus of mammalian cells showing a speckle-like pattern, leaving the nucleolus unstained [32]. Since this immunofluorescence staining pattern appears to be characteristic of many nuclear matrix proteins, we have used these characterized antibodies [32,36] to determine whether or not protein 4.1 is a component of the nuclear matrix of mammalian cells.

Localization of protein 4.1 in the nuclear matrix by immunofluorescence microscopy

Two different methods have been used to prepare nuclear matrices *in situ*. In the first one (Figure 1, method I), chromatin is removed by exposure to high salt concentrations (2 M NaCl) followed by nuclease digestion, as most earlier preparations have been performed [1,40–42]. Cells extracted with Triton X-100 and labelled with antibodies to protein 4.1 show diffuse cytoplasmic staining as well as diffuse nuclear staining distributed throughout the nucleoplasm and staining of discrete foci or speckles (Figure 2a). Nucleoli remain unstained, appearing as globular units outlined by the diffusely stained surrounding nucleoplasm. Cells treated with 0.5 M NaCl present the nuclear-speckled staining pattern, more evident because of a reduction in the diffuse nucleoplasmic staining (Figure 2b). This suggests that protein 4.1 diffusely distributed in the nucleoplasm is removed by this ionic strength step, whereas the protein 4.1 responsible for the speckled staining remains after the treatment. Bright speckles are still observed throughout the nuclei following the removal of chromatin by 2 M NaCl treatment (Figure 2c) and DNase I digestion (Figure 2d). Thus, the persistence of the speckled staining at the last step of nuclear matrix preparation suggests that protein 4.1 is a component of the nuclear matrix.

A more recent method has been developed which preserves the morphology of the nuclear matrix without submitting it to a great degree of shrinkage [6]. In this method (method II, Figure 1), chromatin is removed by digesting nuclei with DNase I followed by elution of chromatin with a low ionic strength (0.25 M ammonium sulphate). This treatment leaves the complete nuclear matrix still connected to the intermediate filaments of the cytoskeleton [6,43]. Further extraction with 2 M NaCl removes most of the outer proteins of the nuclear matrix, leaving the nuclear lamina and the internal nuclear matrix.

Figure 2 Immunofluorescence microscopy of MDCK cells extracted as indicated in method I

The distribution of protein 4.1 after sequential extraction with 0.5% Triton X-100 (a), 0.5 M NaCl (b), 2 M NaCl (c), DNase I + 0.25 M ammonium sulphate (d) in PEM buffer is shown. Notice that cells treated with Triton X-100 show staining in the cytoplasm and in the nucleus. The nuclear staining is diffused as well as aggregated in speckles, leaving the nucleoli unstained (a). After extraction under medium-salt conditions, the speckled pattern is more evident because of the removal of the diffuse staining (b). Extraction with 2 M NaCl and digestion with DNase I do not produce any significant change in the staining patterns (c and d). These data suggest that protein 4.1 may be a component of the nuclear matrix. Bars represent 3 μ m.

Figure 3 Immunofluorescence microscopy of MDCK cells extracted as indicated in method II

Cells sequentially extracted with 0.5% Triton X-100 (**A** and **a**), DNase I + 0.25 M ammonium sulphate (**B** and **b**) and 2 M NaCl (**C**) in CSK were stained with anti-4.1 antibodies (**A**, **B** and **C**) and with Hoechst 33258 (**a** and **b**). Interphase nuclei of 0.5% Triton X-100-extracted cells show the typical 4.1 staining pattern (**A**). After DNase I digestion staining is only present as a speckled pattern in the nucleus (**B**) that remains after 2 M NaCl extraction (**C**). These data suggest the existence of 4.1 isoforms associated with chromatin and present in the nuclear matrix. Staining of chromatin before (**a**) and after (**b**) DNase I digestion is also shown. Bar represents 3 μ m.

When chromatin is removed by DNase I digestion and low-ionic-strength extraction (observe the lack of DNA staining with Hoechst 33258 in Figure 3b and compare with its presence in Figure 3a), a speckled immunofluorescent staining pattern is observed for protein 4.1 in the nuclear matrix preparations (Figure 3B). The diffuse nucleoplasmic immunofluorescent staining observed in cells extracted with Triton X-100 (Figure 3A) was highly reduced (compare Figures 3A and 3B). The speckle-like immunofluorescent staining persists after extensive extractions of cells in solutions of 2 M ionic strength (Figure 3C), indicating that protein 4.1 is a component of the nucleoskeleton.

Immunoblotting analyses of subcellular fractions

Multiple tissue-specific isoforms of protein 4.1, ranging in relative molecular masses from $\sim 30\,000$ to $210\,000$, have been described [21]; however, neither their distribution nor their cellular role is yet known. To learn more about the location of protein 4.1 within the nucleus, we have tried to correlate the observed immunofluorescent staining patterns with immunoblots of equivalent amounts of protein extracts and of nuclear matrices prepared from MDCK cells by method II. To extract the cytoskeletal framework of MDCK cells, two additional steps were included after Triton X-100 treatment. The RSB buffer used for the extraction of cytoskeletal proteins from the majority of cell lines [6,43] is not sufficient to remove the cytoskeletal proteins

of MDCK cells, which are mostly removed, however, in 3 mM imidazole. This is illustrated in Figure 4(a), which shows the Coomassie Blue staining patterns of protein extracts obtained at different steps of the purifications as well as that in the final pellet corresponding to the nuclear matrix. The lamins are observed in the pellet fraction. Figure 4(b) shows the immunoblot analysis of the fractions analysed in Figure 4(a). Total homogenates of MDCK contain several 4.1-immunoreactive bands, the major ones of $M_r \sim 50\,000$, $80\,000$, $100\,000$ and $135\,000$ (designated 4.1p50, 4.1p80, 4.1p100 and 4.1p135 respectively). Proteins of similar relative molecular masses have been detected in other cell lines [21,27,28,32]. Several 4.1-immunoreactive bands are extracted at different steps of the nuclear matrix isolation procedure. Triton X-100 treatment, which removes soluble proteins leaving the cytoskeleton framework and associated proteins attached to the remnants, resulted in the extraction of some 4.1-immunoreactive proteins (4.1p50, 4.1p80 and 4.1p100). The cytoskeleton extraction buffer, RSB, removes a small amount of 4.1p80 and some of 4.1p50, whereas imidazole extracts are enriched in 4.1p50. These results suggest that 4.1p80 and 4.1p50 may be associated with the cytoskeletal network. Indeed, the association of protein 4.1 with spectrin and actin [44] through its $8000\text{-}M_r$ domain [35,36] and with tubulin [30] has already been described. When the remaining nuclear fraction is digested with DNase I, two major 4.1-immunoreactive polypeptides of $M_r \sim 135\,000$ and $175\,000$ are extracted, suggesting that these proteins

Figure 4 Immunoblot analyses of MDCK fractions prepared by method II

Proteins were separated by SDS/PAGE (8% acrylamide) and the resulting gels were either stained by Coomassie Blue (a) or transferred to Immobilon-PVDF and incubated with affinity-purified polyclonal antibodies to 4.1. (b) Key to lanes: porcine red blood cell ghost proteins (GHOST), MDCK total extract proteins (TOTAL), soluble proteins extracted with 0.5% Triton X-100 (TX100), cytoskeletal proteins extracted with RSB buffer containing 1% Nonidet P-40 and 0.5% sodium deoxycholate (RSB), cytoskeletal proteins, enriched in intermediate filaments, extracted with 3 mM imidazole (3 mM IMIDAZ), chromatin and chromatin-associated proteins extracted with 100 μ g/ml RNase-free DNase I (DNase I) and with 0.25 M ammonium sulphate (0.25 M A.S.), outer nuclear matrix proteins extracted with 2 M NaCl (2 M NaCl), and nuclear matrix fraction that remains after the complete extraction in CSK buffer (PELLET). Arrow in lane PELLET of the immunoblot shows the 4.1 protein (4.1p75) present in the nuclear matrix fraction. Arrows on the left of the Figures point to the erythrocyte 4.1 protein present in red blood cell ghosts. Numbers on the left-hand side indicate relative molecular masses ($\times 10^{-3}$) of protein standards (M.W.).

may be associated with the chromatin. Protein 4.1p135 could be the same as that present in the nucleus of Molt-4 cells, which appears to be a moderately bound element of the nuclear matrix [45]. Only faint immunoreactive bands are observed with the 0.25 M ammonium sulphate extraction step. The 2 M NaCl treatment does not extract any 4.1-immunoreactive band, indicating that protein 4.1 is not an outer protein of the nuclear matrix. Finally, a band of $M_r \sim 75000$ (4.1p75) is retained in the nuclear matrix fraction. Protein 4.1p75 is also detected with other polyclonal antibodies to protein 4.1 (results not shown). These results further confirm the nuclear matrix localization of protein 4.1 revealed by immunofluorescence analyses.

HeLa cells are more widely used to study nuclear matrix proteins than MDCK cells; therefore we have analysed whether the removal of chromatin in HeLa cells is also accompanied by the extraction of polypeptides of $M_r \sim 135000$ and 175000 and

Figure 5 Immunoblot analyses of HeLa fractions prepared by method II

Proteins were separated by SDS/PAGE (8% acrylamide), transferred to Immobilon-PVDF membranes and incubated with affinity-purified polyclonal antibodies to protein 4.1. Key to lanes: porcine red blood cell ghosts (GHOST), HeLa total extract proteins (TOTAL), soluble proteins extracted with 0.5% Triton X-100 (TX100), cytoskeletal proteins extracted with RSB buffer containing 1% Nonidet P-40 and 0.5% sodium deoxycholate (RSB), cytoskeletal and intermediate filament proteins extracted with 3 mM imidazole (3 mM IMIDAZ), chromatin and chromatin-associated proteins extracted with 100 μ g/ml RNase-free DNase I + 0.25 M ammonium sulphate (DNase I), outer nuclear matrix proteins extracted with 2 M NaCl (2 M NaCl), and nuclear matrix fraction that remains after complete extraction (PELLET). Arrow in lane PELLET shows that 4.1p75 is a component of HeLa nuclear matrices. Arrow on the left points to erythrocyte protein 4.1. Numbers to the left indicate $M_r \times 10^{-3}$ of protein standards.

whether the final pellet contains 4.1p75 as in the case of MDCK cells. Figure 5 shows the immunoblot analyses of nuclear matrix preparations from HeLa cells. Indeed, extraction of chromatin is accompanied by the removal of 4.1p175 and 4.1p135 proteins, whereas a 4.1p75 protein remains in the nuclear matrix pellet. These results suggest that 4.1p135 and 4.1p175 may be associated with chromatin and that 4.1p75 may be a component of the nuclear matrix common to different mammalian cells.

Protein 4.1p75 is a common component of the nuclear matrix of mammalian cells

Electrophoretic analyses have shown that the nuclear matrix is composed of a complex set of proteins, some of which are cell-type specific whereas others appear to be ubiquitous [46,47]. To determine whether 4.1p75 is cell-type specific or not, we have prepared nuclear matrices from different cell lines (Figure 6). A protein of $M_r \sim 75000$ is present in nuclear matrices of 3T3, BHK, COS and A498 cells; at longer exposure times a polypeptide of $M_r \sim 50000$ is also observed. These results suggest that 4.1p75 is a common element of the nuclear matrix of mammalian cells.

Protein 4.1p75 is concentrated in nuclear speckles enriched with splicing proteins

B1C8 is a nuclear matrix protein of $M_r \sim 180000$ which gives a punctate intranuclear staining by immunofluorescence microscopy [34]. This antigen was originally identified in nuclear matrices prepared by the sequential extraction procedure referred as to method II in this work. To compare the distribution patterns of 4.1p75 and B1C8 antigens in nuclear matrix preparations of MDCK cells, we have performed double-label immunofluorescence analyses. Protein 4.1p75 is concentrated in

Figure 6 Immunoblot analyses of nuclear matrices prepared from several cell lines

Nuclear matrices from NIH 3T3 (3T3), BHK (BHK), COS (COS), and A498 (A498) cells were separated by SDS/PAGE (8% acrylamide), transferred to Immobilon-PVDF membranes and incubated with affinity-purified polyclonal antibodies to protein 4.1. Numbers to the left indicate $M_r \times 10^{-3}$ of protein standards. The arrow points to erythrocyte 4.1 present in red blood cell ghosts (GHOST).

Figure 7 Protein 4.1p75 co-localizes with the nuclear matrix protein B1C8 and with the splicing factor SC35

MDCK cells sequentially extracted by method II to produce nuclear matrices were double-immunostained with affinity-purified polyclonal antibodies to 4.1 (a and c) and with anti-B1C8 monoclonal antibodies (b) or with anti-SC35 monoclonal antibodies (d). These data suggest that 4.1p75 concentrates to nuclear speckles enriched with splicing proteins. Bar represents 3 μ m.

nuclear speckles that also contain the nuclear matrix antigen B1C8 (Figures 7a and 7b, respectively).

Splicing proteins accumulate in typical nuclear speckle domains (reviewed in [48]). To analyse whether or not the

nuclear speckles containing 4.1p75 are enriched with splicing proteins, we have carried out double-label immunofluorescence studies. A monoclonal antibody against the non-small nuclear ribonucleoprotein particle (snRNP) splicing factor SC35 [37] has been used to detect nuclear speckle domains enriched with splicing factors. Figure 7(c) shows the distribution of 4.1p75 in nuclear matrices prepared from MDCK cells and Figure 7(d) shows that of protein SC35. The results indicate that 4.1p75 is concentrated in nuclear speckles enriched with splicing proteins.

DISCUSSION

Protein 4.1 was originally identified as a membrane skeleton element of erythrocytes, where its functional role as a linker of the spectrin-actin network to the plasma membrane through interactions with integral proteins has been established (reviewed in [20]). Molecular cloning studies have shown the existence of multiple forms of protein 4.1 cDNAs arising by alternative splicing in erythroid and non-erythroid tissues [24,26–28]. At this time little is known about protein 4.1 function in non-erythroid cells, although 4.1 localization is not strictly confined to the cell membrane.

This study deals specifically with the localization and possible role of protein 4.1 in the interphase nucleus. Using previously characterized affinity-purified antibodies to 4.1 [32,36] we show for the first time that protein 4.1 is a component of the nuclear matrix of mammalian cells. This behaviour was best illustrated either by immunofluorescence studies or by immunoblotting using the sequential extraction procedure introduced by Penman and co-workers [3,6,33]. A 4.1 polypeptide of $M_r \sim 75000$ (4.1p75) is present in nuclear matrices isolated from cell lines derived from different tissues from man, monkey, dog, baby hamster and mouse, suggesting that 4.1p75 is not species- nor cell type-specific. Two other 4.1 proteins, 4.1p135 and 4.1p175, are present in cellular fractions extracted by combined DNase I and 0.25 M ammonium sulphate treatments, suggesting that they may be associated with chromatin. Protein 4.1p135 could be analogous to that described in Molt-4 nuclei [27,32] which appeared to be a moderately bound element of the nuclear matrix [45]. Bands of lower M_r that share antigenic determinants with erythrocyte protein 4.1 are also observed. Some of the lower- M_r bands could be proteolytic artifacts despite the use of protease inhibitors at all stages of sample preparation, but it is also possible that they exist as such within the cell, since 4.1 polypeptides of M_r s ranging from 30000 to 210000 have been described [21].

Most nuclear proteins contain short amino acid sequences, known as nuclear localization signals (NLSs), which mediate their import to the nucleus [49–51]. Protein 4.1 sequence contains two putative NLSs that fit the NLS consensus sequence, K-K/R-X-K/R, proposed by Chelsky and co-workers [52]. One putative NLS sequence, KRPK, would be encoded by exon 2; the second one, KKRR, would be generated by joining exon 13 and 16 sequences as a result of eliminating exons 14 and 15 by alternative splicing. Interestingly, a similar alternative splicing event has been described to generate the NLS sequence, KKRRK, that targets multifunctional Ca^{2+} /calmodulin-dependent protein kinase to the nucleus [53]. The amino acid sequence responsible for the import of protein 4.1 to the nucleus remains to be established.

Pre-mRNA splicing factors are enriched in at least two distinct types of nuclear structures: 'speckles', which exist at the level of 20–50 per mammalian cell nucleus (reviewed in [48]) and 'coiled bodies', which exist at the level of one to five (reviewed in [54]). These structures are retained in nuclear matrix preparations

[55–58] and there is evidence that splicing occurs in association with the nuclear matrix [10,59–61], thus indicating that some elements of the nucleoskeleton are anchoring splicing factors either directly or indirectly. Our results show that 4.1p75 distributes in irregularly shaped speckles also containing B1C8 antigen, a nuclear matrix protein involved in pre-mRNA processing [34,62] and the non-snRNP splicing factor SC35 [37]. Interestingly, the distribution of 4.1p75 in irregularly shaped speckle domains in transcriptionally active cells is modified during states of transcriptional inhibition (results not shown) in a similar way as for the splicing factor SC35 [63].

The role that 4.1p75 may be playing as an element of the nuclear matrix is intriguing. The presence of both actin [64,65] and spectrin [40] in the nucleus has been reported, therefore it is possible that a protein meshwork similar to that found in the membrane skeleton could constitute part of the scaffolding to which splicing factors and/or other nuclear matrix proteins may bind. Whether 4.1p75 is playing a structural role as protein 4.1 does in the red blood cell, or is directly involved in pre-mRNA processing as are other nuclear matrix proteins, remains to be elucidated.

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