Association of hnRNP S1 proteins with vimentin intermediate filaments in migrating cells

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Summary

S1 proteins C2 and D2 are multifunctional hnRNP proteins acting as transcriptional regulators in the nucleus. Immunofluorescence staining of various cells in culture revealed that S1 proteins also occur in the cytoplasm, often in association with vimentin intermediate filaments (VFs). Here, we verified the association of S1 proteins with vimentin using vimentin-deficient cells, crosslinking and immunoprecipitation, and further investigated the biological significance of this association. S1 proteins on VFs, referred to here as S1 fibers, were lost in highly confluent cells, where cell proliferation and cellular metabolic activity greatly decreased owing to cell density-dependent arrest. However, the disappearance of S1 fibers was not related to these reduced activities, but to inhibited cell migration.

Introduction

Vimentin intermediate filaments (VFs) are expressed in tissues of mesenchymal origin. Although mice with knockout mutations in vimentin genes develop and reproduce without an obvious phenotype (Colucci-Guyon et al., 1994), it has been shown that this mutation influences cells and organs in various ways (Henrion et al., 1997; Terzi et al., 1997; Colucci-Guyon et al., 1999). VFs afford mechanical stability of the cells. For example, during cell migration, subcellular structures need to be protected from mechanical lesions. The importance of vimentin cytoskeleton in cell motility has been demonstrated with mouse fibroblasts (Eckes et al., 1998) and human mammary epithelial cells (Gilles et al., 1999). Similarly, it has been also shown to be important during mouse development with neural crest (Cochard and Paulin, 1984), parietal endoderm (Lane et al., 1983; Lehtonen et al., 1983) and mesenchymal cells (Franke et al., 1982). In fact, VFs become prerequisite not only in contraction and reorganization but also in cell motility of connective tissues, and all of these are mandatory events in wound healing (Eckes et al., 1998) (reviewed by Gailit and Clark, 1994).

S1 proteins A-D are ubiquitously found in animal cells (Inoue et al., 1983; Emura et al., 1992; and unpublished results), and extracted as a group of proteins at pH 4.9 from the nuclei treated with either RNase A or DNase I (Inoue et al., 1983; Higashi et al., 1984; Inoue et al., 1986). They are resolved by SDS-PAGE, each as doublets: A1 (an apparent molecular mass of 74.5 kDa), A2 (69.5 kDa); B1 (47.4 kDa), B2 (46.5 kDa); C1 (43.9 kDa), C2 (42.8 kDa); D1 (40.8 kDa) and D2 (39.4 kDa). S1 proteins B-D are multifunctional hnRNP proteins (Inoue et al., 2001; Inoue et al., 2003). Among them, S1 proteins D2 and C2 are identified as CArG-box binding factor-A (CBF-A) (Kamada and Miwa, 1992) and its splicing isoform (GenBank accession number AJ238854) respectively, acting as positive (Bemark et al., 1998; Mikheev et al., 2000) or negative (Kamada and Miwa, 1992) transcriptional regulators. A hybridoma producing monoclonal antibody McAb 351, which is highly specific for S1 proteins C2 and D2, has been isolated. Cell staining with this antibody showed that they occur not only in the nucleus but also in the cytoplasm often in association with VFs in migrating cells (Tsugawa et al., 1997). In this study, we verified the association of S1 proteins with VFs, and investigated its biological significance. We demonstrate that the VF association of S1 proteins is closely related to cell motility.
Materials and Methods

Cell culture

ARL rat liver epithelial cells (ARL J301-3, Japanese Health Science Foundation) were grown in William’s E medium supplemented with 10% fetal calf serum and HaK Syrian hamster kidney cells (CCL15, ATCC) in Eagle’s minimum essential medium supplemented with 10% fetal calf serum. Vimentin-deficient tTA-1 mouse fibroblasts and vimentin (+/+ MFT-6 mouse fibroblasts (Holwell et al., 1997) were cultured in Dulbecco’s modified Eagle medium containing 5% calf serum. Hygromycin (200 µg) was added to the culture of tTA-1 cells. Inhibitors used in cell cultures were aphidicolin (20 µg/ml), hydroxyurea (6 mM), actinomycin D (5.0 µg/ml), okadaic acid (50 nM), genistein (10 µM) or staurosporine (0.2 µM).

Preparation of white blood cells and chemotaxis

White blood cells were isolated by centrifugation of heparinized peripheral blood from an adult Wistar rat (250 g) at 400 g for 10 minutes at 20°C. The white blood cell layer was collected with a pipette, and the cell suspension diluted 5-15 times with RPMI and incubated at 37°C for 20 minutes between a glass slide and a sheet of Saran wrap. In chemotaxis experiments, the diluted white blood cell suspension (10 µl) and 100 M N-formylmethionyl-leucyl-phenylalanine (fMLP) (10 µl) in RPMI were placed at a 5 mm distance, and covered with a sheet of Saran wrap. After incubation, the Saran wrap and excess fluid were removed, and the specimens were air-dried and stained as described below.

Indirect immunofluorescence staining and antibodies

Cells on slides or coverslips were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, and incubated first with 10% fetal calf serum for 20 minutes, then with primary and secondary antibodies for 1 hour each. Each solution was made in phosphate-buffered saline (PBS) and between each step, cells were washed three or four times with PBS. Primary antibodies were mouse monoclonal antibody against vimentin (V9, 1:150 dilution, IT), rabbit (Cappel or Medak) or goat (RDI or Chemicon) polyclonal antibody against vimentin (1:50-200 dilution), and mouse monoclonal antibody McAb 351 against rat S1 proteins C2 and D2 (1:100 dilution) (Tsugawa et al., 1997). Secondary antibodies (1:30-200 dilution) were FITC-conjugated goat or rabbit anti-mouse IgG antibodies (Biosource International or Zymed), rhodamine-conjugated goat anti-rabbit IgG (AP156R, 1:50 dilution, Chemicon International or Zymed), or TR-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). Images were observed under a fluorescence microscope with a mercury light source (Olympus, model Bx50), or a confocal laser-scanning fluorescence microscope (Carl Zeiss, model LSM510).

Preparation of proteins and immunoblotting

Reference S1 proteins were prepared from rat liver nuclei as described (Inoue et al., 1983; Tsugawa et al., 1997). ARL cells were solubilized in a standard SDS sample buffer (SDS and 2-mercaptoethanol were at 2% and 1% respectively) and the lysates sonicated and heated at 95°C for 5 minutes. Immunoblots of 9.5% SDS-PAGE gels were probed with McAb 351 (1:1000 dilution) and a horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution, Cappel) (Inoue et al., 2001). Bands were visualized on X-ray film (Hyperfilm ECL, Amersham) with an ECL detection kit (RPN 2106, Amersham) and their intensities were measured using an image analyzer (BioRad, Multi-Analyzer). For normalization, DNA in the samples was isolated by a standard method using proteinase K and RNase A dissolved in H2O and determined by measuring absorbance at 260 nm.

Crosslinking

ARL cells grown in 10 cm dishes were incubated at room temperature for 45 minutes with 3 mM dimethyl 3,3′-dithiobispropionimidate (DTBP, Pierce) in PBS (pH 7.4), containing 0.4 mM PMSF and 20 µg/ml leupeptin. The cells were dissolved in an SDS sample buffer without mercaptoethanol, sonicated and heat-treated. After SDS-PAGE on a 6.5% gel, the sample lane was excised with the aid of pre-stained marker proteins (Broad range, BioLabs) run on adjacent lanes. The gel strip was equilibrated in 125 mM Tris-HCl, pH 6.8, incubated with 40 mM DTT in 125 mM Tris-HCl, pH 6.8, 0.1% SDS and 10% glycerol for 5 hours at room temperature, and subjected to the second SDS-PAGE (9% gel with a flat 3% stacking gel). Proteins were immunoblotted with McAb 351, and reprobed with a polyclonal rabbit anti-vimentin antibody (DBS, Fremont, CA).

Immunoprecipitation

ARL cells at about 40% confluence were collected with PBS from six 10-cm culture plates, lysed for 1 hour with occasional mixing with 750 µl lysis buffer (0.15 M NaCl, 1% NP-40, and 50 mM Tris-HCl, pH 8.0) containing leupeptin (10 µg/ml), aprotinin (1000 KIU/ml) and 0.2 mM PMSF, and centrifuged at 10,000 g. All steps were done at 4°C and the washing with lysis buffer. The supernatant extract was pre-cleared by incubation with a 75 µl packed volume of protein A-Sepharose (4 Fast-Flow, Pharmacia), which was previously saturated with non-specific antibodies (100 µl fetal calf serum) and washed. Specific antibody (1 µl, ascites fluid) was incubated for 10 minutes with washed fresh protein A-Sepharose (20 µl packed volume). Then, the pre-cleared cell extract (100 µl) was added and the incubation continued for 30 minutes with occasional mixing. The beads were washed, and bound proteins eluted with SDS sample buffer (60 µl) at 95°C for 5 minutes and collected by centrifugation. Control mouse monoclonal antibodies (ascites fluids) were 1-65 against a toxoplasma membrane protein and 3F7 against RBP-MS (RNA binding protein gene with multiple splicing) protein (Shimamoto et al., 1996).

Determination of cellular RNA and DNA synthesis activities

ARL cells cultured in 5 cm dishes were incubated in fresh medium with [3H]uridine or [3H]thymidine (NEN Life Science Products) for 45 minutes. After rinsing with PBS, cells were solubilized in cold 0.4 N NaOH (3 ml) and sonicated. Half portions were mixed with 3 ml of 10% trichloroacetic acid (TCA), placed on ice for 5 minutes and filtered on glass filters (GF/F, Whatman) under suction. The filters were washed three times with 10% TCA and twice with ethanol and radioactivity was measured. The remaining half portions were neutralized with 0.3 N HCl and 0.1 M Tris-HCl, pH 7.5 and digested with 60 µg/ml RNase A for 4 hours at 37°C. DNA was collected by ethanol precipitation, dissolved in 0.1 N NaOH and determined by reading absorbance at 260 nm.

Ulcerc formation

Gastric ulcers were produced in 8-week-old Wistar male rats (Japan SLC, Hamamatsu, Japan) as described (Tomina, et al., 1997). In brief, rats were fasted for 12 hours and subjected to laparotomy under ether anesthesia. A plastic mold (6 mm in diameter) was tightly placed on the anterior serosal surface of the antral-oxicytic border of the stomach. Acetic acid (60 µl) was poured into the mold and allowed to remain on the gastric wall for 60 seconds. After the solution was removed, the surface of the treated area was wiped with absorbent paper and the abdomen was closed. Control rats received the same laparotomy, without treatment with acetic acid (sham-operated rats). The Animal Care Committee of Osaka City University approved the experimental procedure.
Immunohistochemistry

Under ether anesthesia, ulcerated gastric tissues were excised 5 days after operation and fixed with 10 mM metaperiodate, 75 mM lysine and 2% paraformaldehyde in 40 mM PBS (pH 7.4) for 5 hours. Cryosections were incubated first with non-immunized rabbit serum for 30 minutes, then with a diluted specific antibody for 4 hours at 4°C and 0.3% H$_2$O$_2$/methanol for 15 minutes. They were washed in PBS and stained using a LSAB streptavidin-biotin-peroxidase kit (DAKO, Kyoto, Japan). Incubation with biotinylated rabbit antimouse IgG was for 15 minutes and with peroxidase-labeled streptavidin for 15 minutes. The sections were finally developed in 0.03% 3,3′-diaminobenzidine (DAB) with 0.005% H$_2$O$_2$ and sodium azide (650 μg/ml). Nuclei were counterstained with Methyl Green. Images were obtained using a bright-field microscope (Nikon, Microphoto-FXA).

Northern blotting
cDNAs of vimentin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985) were labeled with $\alpha$cDNAs of vimentin and glyceraldehyde 3-phosphate dehydrogenase cDNAs of vimentin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985) were labeled with $\alpha$ by random primer extension using a nick-translation kit (NEP-103L, NEN). Isolation of total RNA by the guanidium isothiocyanate/phenol/chloroform method, electrophoresis on a 1% agarose gel, blotting to nylon membrane (Gene Screen Plus, NEN) and hybridization in a solution containing 50% formamide were performed essentially as described (Sambrook et al., 1986). The washed membrane was exposed to Kodak XAR-5 film between two intensifying screens at –70°C. For reprobing, the membrane was stripped of the probe in 0.1 M Na$_2$HPO$_4$ solution containing 1% SDS at 100°C and rehybridized. Autoradiographic band intensity was determined on an optical scanner (Seiko, Epson GT-8000). GAPDH mRNA was used as an internal reference.

Results

Localization of S1 proteins on VFs

Indirect immunofluorescence staining with antibody McAb 351, specific for S1 proteins C2 and D2, shows localization of these proteins on vimentin filaments (VFs) as well as in the nuclei of VF-containing cells such as HaK cells and HeLa cells (Tsugawa et al., 1997). S1 proteins on VFs (S1 fibers) were similarly observed in ARL rat liver epithelial cells (Fig. 1, A1). Localization of S1 proteins on VFs was determined by double staining with McAb 351 (Fig. 1, A2) and a polyclonal anti-vimentin antibody (Fig. 1, A3).

Association of S1 proteins with VFs

To confirm the association of S1 proteins C2 and D2 with VFs, we first examined S1 fibers in vimentin-deficient mouse fibroblasts. As expected, S1 fibers were absent in these cells, in contrast their presence in wild-type cells (Fig. 2).

The association of S1 proteins with VFs was also examined by a crosslinking reaction. Proteins in ARL cells were first reacted with DTBS, a cleavable bifunctional crosslinker, and subjected to the first SDS-PAGE without prior reduction of disulfide bonds. Then, crosslinked proteins in an excised sample lane were separated in gel with dithiothreitol, which cleaves DTBS in the middle of the molecule. The second dimensional PAGE showed that proteins not crosslinked in the crosslinking reaction run forming a curved diagonal (Fig. 3A). On the other hand, vimentin lay on a horizontal line at its monomer size (Fig. 3C), indicating that it was crosslinked to larger products of progressively increasing sizes. The results are exactly those expected for its occurrence as polymer filaments. S1 proteins C2 and D2 were also crosslinked in a similar manner (Fig. 3B). Their crosslinking patterns, similar to that of vimentin, support the fact that S1 proteins are associated with polymerized structures. However, the non-crosslinked population was significantly high with S1 proteins, probably because they occur in the nucleus as well as in the cytoplasm.

To further examine the S1 protein association with vimentin, ARL cell extract was immunoprecipitated with McAb 351. As expected, the antibody against S1 proteins precipitated vimentin (Fig. 4). No vimentin was precipitated without the antibody or with control antibodies irrelevant to S1 proteins. As McAb 351 does not recognize vimentin (Tsugawa et al., 1997), the results verified that S1 proteins form complexes with vimentin.

Relationship of S1 fibers and cell confluency

ARL cells in culture can be maintained in a confluent monolayer for up to 3 weeks without a change of the medium. In such confluent ARL cells, S1 fibers were found to disappear and S1 proteins were detected only in the nuclei (Fig. 1, B1).
The results suggested dependency of S1 fibers on cell confluency. On the other hand, VFs remained, although their networks had dwindled according to the decrease in cell size (Fig. 1, B2). Their nuclei had also reduced in size, and were stained less intensely with McAb 351 when compared with growing cells at lower cell densities (Fig. 1, B1 compared to A1). In fact, after 2 weeks in such a confluent state, S1 proteins C2 and D2 decreased to 57 and 63% of the levels observed in growing cells (Fig. 1C, lane 1 compared to lane 2).

The correlation of S1 fibers with cell confluency was confirmed. First, a small volume of trypsinized cell suspension was placed at a corner of a chamber slide. After the cells had attached to the substratum, enough medium was added and the cell culture was continued for 2.5 weeks at a tilted position (tilted culture experiment). Although hardly detected inside the high cell-density area, prominent S1 fibers were observed in the cells on the margin and in those moving ahead of the dense area (not shown). These cells had large nuclei with a strong S1 protein staining intensity as well as well-developed VFs. Likewise, ARL cells plated at ~10% confluency and cultured for 3 days exhibited prominent S1 fibers in contrast to the cells plated at full confluency, which showed few S1 fibers (not shown). These results thus confirmed that S1 fibers become absent from confluent cells or cells at high cell densities.

S1 fiber formation is dissociated from cell proliferation and cellular metabolic activity

Biosynthetic activities of ARL cells at 50-60% confluency and those cultured for 1 and 3 weeks at full confluency were assayed by incubation of the cells with [3H]thymidine or [3H]uridine (Fig. 5A). The RNA synthesis activity of the 3-week confluent cells was reduced by 70% compared to that measured in the low-density cells. DNA synthesis was also drastically reduced in the 3-week confluent cells, with the activity reduced by more than 99%. These results thus indicate that under these conditions, cellular metabolic activity greatly decreased, and cell proliferation ceased almost completely.

In confluent states, ARL cells are restricted from migration, and they are in the resting G0 state owing to cell density-dependent arrest. Therefore, the disappearance of S1 fibers in confluent cells may have arisen from the suppressed cell proliferation, lowered metabolic activity of G0 state or inhibited cell movement. These possibilities were examined. First, ARL cells doubling every 18.5 hours at a low cell density...
were incubated for 3 days in the presence of high concentrations of aphidicolin (20 µg/ml) or hydroxyurea (6 mM). These chemicals block DNA synthesis by inhibiting DNA polymerase α and deoxyribonucleotide-producing ribonucleotide reductases. Neither drug exerted appreciable effects on the S1 fiber formation (Fig. 5B), which indicated that S1 fiber formation is dissociated from cell proliferation.

To examine the effect of lowered metabolic activity in the G0 state, ARL cells growing at a low cell density were brought to G0 by serum starvation (Zetterberg and Skold, 1969; Zetterberg and Larsson, 1991). S1 fibers, observed 3 days later, appeared similar in the absence or presence of serum (Fig. 5C). The results indicated that S1 fibers are not correlated with resting G0 state or reduced cellular metabolic activity.

S1 fibers and cell motility

To examine the relationship of S1 fibers with cell migration, scratch-wound experiments were performed on confluent cells, where cells were allowed to migrate towards the cleared space. A central area of a 3-week monolayer sheet of ARL cells was removed by scratching and observed after 30 hours. The ARL cells exposed to open substratum detached from the high cell-density area and migrated into the cleared space exhibiting well-developed S1 fibers (Fig. 6A,B). Essentially the same results were obtained with HaK cells (a hamster kidney cell line): S1 fibers appeared in the cells on the margin of dense regions as early as 8 hours after scratching (data not shown). Furthermore, even under the conditions where aphidicolin (20 µg/ml) was added to scratched monolayers, cell migration and reappearance of S1 fibers were still observed (Fig. 6C), thereby confirming that S1 fibers are unrelated to cell proliferation. With hydroxyurea, the same conclusion was obtained (not shown).

In addition, when 3-week confluent ARL cells were trypsinized and replated at a lower cell-density, S1 fibers appeared within 10 hours. Similar results were obtained with confluent HaK cells. All of these results indicated that the S1 fibers that had disappeared in confluent monolayer appeared again in a reversible manner when the cells were placed under non-restricted conditions for cell movement.

Further evidence for the correlation of S1 fibers and cell migration

Different types of cells have different migratory potentials. Accordingly, we analyzed S1 proteins by immunohistochemistry in various cells of the rat tissues. It was evident that S1 proteins were commonly detected only in the nuclei in all non-migratory cells examined (K.T. and N. Ikeda, unpublished data). The results also confirmed that different metabolic activities between the various cell types had no correlation with the presence of S1 fibers. In the cerebellum (Fig. 7A) for example, the large nuclei of Purkinje cells were strongly stained with McAb 351 in contrast to the granule cell nuclei, probably because of high RNA synthesis activity in the
former cells. The S1 proteins were confined in the nuclei in both Purkinje cells and granule cells (Fig. 7Ab) and did not merge with cytoplasmic VFs in theVF-containing Purkinje cells (Fig. 7Aa).

On the other hand, among the various cell types examined, only migratory cells revealed S1 proteins in the cytoplasm, as expected. White blood cells were isolated from the blood of the rat, and incubated for 20 minutes between a slide glass and Saran wrap. Monocytes with kidney-shaped nuclei, which represent typical migratory cells (Alberts et al., 2002), contained many S1 fibers in the cytoplasm (Fig. 7B). The neutrophil is another typical migratory cell that has characteristic polymorphonuclear structures and these cells similarly exhibited large amounts of S1 fibers. When stimulated with the chemoattractant, N-formylmethionyl-leucyl-phenylalanine (fMLP), neutrophils showed distinct S1 fibers (Fig. 7B, panels 2-4); without fMLP, S1 fibers were less distinct, even though cytoplasmic S1 proteins were as abundant as in stimulated cells (Fig. 7, panel 5).

To examine other migratory cells, we chose fibroblasts engaging in tissue remodeling (Alberts et al., 2002). In the healing stage of a gastric ulcer, fibroblasts on the edge of the ulcer migrate actively towards the center of ulcerated area, forming granulation tissue (Tominaga et al., 1997). The spindle-shaped fibroblasts exhibited S1 proteins in the cytoplasm as well as in the nuclei (Fig. 8A,C). In contrast, the fibroblasts in normal gastric tissues showed S1 proteins only in the nuclei (Fig. 8B,D). VFs were present in the cytoplasm of both the ulcerated (Fig. 8E,G) and intact tissue cells (Fig. 8F,H). However, the vimentin staining intensity was several fold stronger in the ulcerated gastric tissues. In accordance with this, the expression of vimentin mRNA measured by Northern blotting on day 5 increased 5.6-fold in the ulcerated tissues compared with expression levels in sham-operated tissue (Fig. 8I, mean value of six determinations; $P<0.05$).

**Fig. 6.** Scratch-wound experiments. The center areas of 3-week confluent monolayers of ARL cells grown on coverslips were removed by scratching with a pipette tip, and the incubation was continued in fresh medium for 30 hours. Cells were stained with McAb 351 (A), or double-stained with McAb 351 (B1) and an anti-vimentin antibody (B2). (C) After scratching, ARL cells were further incubated in the presence or absence of aphidicolin (20 µg/ml) for 30 hours. Cells were stained with McAb 351. In all panels, right-hand areas correspond to the marginal regions of cleared areas made by scratching. Bar, 50 µm.

**Fig. 7.** S1 fibers in animal tissues. (A) A section of the cerebellum from an adult rat was double-stained with an anti-vimentin antibody (a, red) and McAb 351 (b, green). Purkinje cells are seen on the diagonal, and the granule cells on the right-hand side. Bar, 50 µm. (B) White blood cells from an adult rat were incubated at 37°C for 20 minutes between a slide glass and a sheet of Saran wrap in the presence (2-4) or absence (1, 5) of fMLP. The cells were stained with McAb 351 (a), DAPI (1b-3b), and an anti-vimentin antibody (4b, 5b). Panels 1, monocytes; panels 2-5, neutrophils. Bar, 20 µm.
Possible involvement of S1 fibers in the formation/reorganization of VFs

When RNA synthesis of ARL cells was inhibited with actinomycin D (5 µg/ml), well-developed S1 fibers diminished relatively rapidly in intensity and fiber length. The decrease continued over 9 hours, with a reciprocal increase in the nuclear S1 protein staining intensity (Fig. 9A). The results suggest the dependence of S1 fibers on RNA synthesis and relocalization of S1 proteins from VFs to the nucleus upon inhibition of RNA synthesis.

Effects of S1 protein phosphorylation on S1 fibers were examined. When ARL cells after 2 weeks of confluent culture were treated with staurosporine and genistein, inhibitors of PKC and tyrosine kinases respectively, no effects on the S1 fibers were observed. In contrast, the phosphatase inhibitor okadaic acid, which induces reorganization of VFs through hyper-phosphorylation of vimentin molecules (Inagaki et al., 1987; Lee et al., 1992), exerted a strong effect: in accord with the alteration of VF networks, S1 fibers appeared as thick bundles in the confluent cells (Fig. 9B).

Discussion

We have shown previously by cell staining using McAb 351 that in addition to their presence in the nucleus, hnRNP S1 proteins C2 and D2 are found in the cytoplasm in association with VFs and not with microtubules, microfilaments, cytokeratin filaments or desmin filaments (Tsugawa et al., 1997). In the present study, we verified the VF association of S1 proteins by three different approaches. First, vimentin-deficient fibroblasts were shown not to form S1 fibers, as expected. Second, crosslinking experiments showed that S1 proteins were indeed associated with polymer filament structures. Third, immunoprecipitation with McAb 351 coprecipitated vimentin.

In this study, the biological significance of the association of the S1 proteins with VFs was also studied. Prominent S1 fibers formed in ARL cells growing at low cell densities, and when the cells became confluent and entered into cell density-dependent arrest, S1 fibers disappeared. The disappearance of S1 fibers was not related to inhibited cell proliferation nor to a reduced cellular metabolic activity, but to inhibited cell movement. This conclusion is well supported by the following findings. Inhibition of cell proliferation with specific inhibitors, aphidicolin and hydroxyurea, exerted no
appreciable effects on S1 fibers in ARL cells. Also, the neutrophils (the terminally differentiated non-dividing cells), and the ARL cells that were brought to G0 state by serum starvation still exhibited prominent S1 fibers. In ARL cells, S1 fibers persisted under reduced cellular metabolic activities brought about by the serum starvation. Also, S1 fibers were commonly absent from most of the tissue cells, irrespective of their different levels of metabolic activity. These results suggested that S1 fibers are also unrelated to the metabolic activity of the cell. In confluent monolayers where cell migration is largely reduced, S1 fibers disappeared. However, S1 fibers appeared again when the cells were allowed to migrate into vacant space by scratching of confluent cell monolayers. Similarly, in the tilted culture experiment, the cells moving ahead the edge of the high cell density monolayer region had prominent S1 fibers, but the cells inside the dense region did not. S1 fibers were not detected in any of the non-migratory cells of various tissues examined. In contrast, neutrophils and monocytes, the migratory cells in the blood, exhibited large amounts of S1 fibers. Similarly, the fibroblasts in wound/healing, another representative migratory cells, contained significant amounts of S1 proteins in the cytoplasm, and contrasted to those in sham-operated tissues, which contained no detectable cytoplasmic S1 proteins. From these results, we concluded that S1 proteins become associated with VFs in migrating cells. Although no direct measurement of cell migration was made in the present study, the epithelial ARL cells that detached from high cell density regions in scratch-wound experiments, the cells moving ahead of dense areas in the tilted culture experiment, the neutrophils treated with chemotacticant IMLP and fibroblasts in tissue-remodeling (Alberts et al., 2002) as well as ARL cells at low cell-densities are all thought to be in migratory motion.

How well cultured cells develop S1 fibers depends on cell density. It is known that many cultured epithelial cells do move around, relative to their neighbors, even in confluent cells. This may be the reason why it took ARL cells more than a week to reach an undetectable level of S1 fibers. Even after reaching a confluent state, the cells increase in number and become smaller and denser. Thus time is required to form more packed monolayer, producing greater restraints on cell movement and leading to the disappearance of S1 fibers.

It is known that VFs are prerequisite for cell motility (Gailit and Clark, 1994; Eckes et al., 1998; Gilles et al., 1999). The function or the role of S1 proteins on VFs may be understood in terms of formation/reorganization of VFs. First, inhibition of RNA synthesis brought about relatively rapid reduction in S1 fibers. Second, appearance of S1 proteins in the cytoplasm seemed coupled with increased vimentin synthesis as shown with augmented vimentin mRNA and protein levels in tissue-healing fibroblasts in the gastric ulcer. Similarly, whenever S1 fibers were formed in ARL cells, VFs and their networks became more prominent. From these findings, we propose that S1 fibers may be involved in the synthesis of vimentin proteins. Furthermore, in ARL cells that were allowed to migrate by scratching of the confluent cell-monolayer, prominent S1 fibers appeared and VF networks were apparently reorganized in densely orienting, seemingly migrating directions (Fig. 6). Also, S1 fibers reappeared even in confluent cells when VFs underwent reorganization by treatment with okadaic acid (Inagaki et al., 1987). Hence S1 proteins may be involved in the formation/reorganization of VFs, which are needed for cell migration.

S1 proteins C2 and D2 act as hnRNA binding proteins in the nucleus (Inoue et al., 2001), and as presented in this study, appear to be associated with VFs in the cytoplasm. It is interesting to speculate whether the S1 proteins participate in the localization of a particular set of mRNAs on VFs, thereby facilitating the site-oriented production of proteins required for formation or reorganization of VFs. However, these possibilities await clarification by further study. In conclusion, we found that S1 proteins localize on VFs and propose that they may be involved in cell migration through participation in the formation/reorganization of VFs.

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