Distribution of Fodrin in the Keratinocyte In Vivo and In Vitro

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Distribution of fodrin in the keratinocyte, both in vivo and in vitro, was examined by immunofluorescence microscopy. In the rat epidermis in vivo, fodrin was localized in the cell periphery of the spinous layer of all the skins studied. In only the basal layer of the thick skin, however, fodrin was seen intensely in the cytoplasm.

As in vitro keratinocytes, a mouse cell line (Pam 212) cultured in low (0.06 mM) as well as standard (1.87 mM) Ca^{2+} was examined. In low Ca^{2+}, fodrin was observed throughout the cytoplasm without marked accumulation irrespective of the cell density. The cytoplasmic labeling in low Ca^{2+} looked filamentous and became aggregated when cells were treated with cytochalasin B; at least some of the aggregates coexisted with those of F-actin. In contrast, fodrin distribution was not affected with colchicine. On the other hand, in standard Ca^{2+}, the protein became concentrated along the cell periphery and less conspicuous in the cytoplasm as the cells reached confluency. When cells were transferred from low to standard Ca^{2+}, the distribution of fodrin changed accordingly within 180 min.

The present results indicate that fodrin in the keratinocyte is likely to be associated with actin filaments and that it takes two different ways of distribution both in vivo and in vitro. The peripheral and the cytoplasmic labeling of in vivo and in vitro cells are likely to correspond. It may be that fodrin changes its localization according to the cell's proliferative activity. *J Invest Dermatol 94:724–729, 1990*

In the cultured epidermal cells, many changes are induced by increasing extracellular Ca^{2+} concentration. In medium with low Ca^{2+} (<0.1 mM), the cells proliferate with the characteristics of basal cells and do not form cell-cell contacts; when transferred to medium with a standard Ca^{2+} concentration ($>$ 0.1 mM) ("calcium switch"), they begin to stratify and form desmosomes as well as adherence junctions [1–5]. As assumed from the formation of intercellular junction, changes occur in the cytoskeleton. Distribution of keratin filaments [3] and actin filaments [4,5] has been reported to change, due to the calcium switch.

Fodrin is one of the spectrin-like proteins found in a wide variety of cells and is assumed to comprise the skeletal framework of the plasma membrane [6]. Although several functions have been postulated about fodrin, the most interesting characteristic is that the protein in conjunction with other peripheral membrane proteins can bind to both integral membrane proteins and cytoskeletal proteins, and thus may be able to transmit alterations occurring on the cell surface to the cell interior (for reviews, see [7,8]). Although a protein immunoreactive with erythrocyte spectrin was reported to exist in the epidermal cell of both in vivo and in vitro [9], it has not been examined whether there is any change in the proteins' localization in correlation with the cell's proliferation and differentiation.

We have conducted immunolocalization studies of fodrin in keratinocytes, in vivo and in vitro. We will present the results of immunofluorescence microscopy of fodrin in the rat epidermis in vivo and Pam 212 cells [10] kept in low as well as standard Ca^{2+}. The cell line retains characteristics of epidermal cells and has the ability to differentiate [10]. The results show that distribution of fodrin is different between basal and spinous cells of the in vivo thick epidermis and that the two different distributions are reproduced in keratinocytes in vitro by changing extracellular Ca^{2+} concentration. This is the first report to show that fodrin translocates between the cell periphery and the cytoplasm by a change of cellular ionic environment.

MATERIALS AND METHODS

Preparation of Antibodies and Immunoblotting Fodrin was isolated and purified from rat brains as described [6]. Spectrin was prepared from rat erythrocytes [13]. Antibodies to fodrin and spectrin were produced in rabbits and purified by affinity chromatography using the respective antigen immobilized on glutaraldehyde-activated AcA 22 Ultrogel (LKB, Sweden) [14]. Monospecificity of the antibodies was confirmed by immunoblotting. Extracts of the rat epidermis and Pam 212 cells were prepared in a solubilizing buffer (15 mM Tris/HCl, pH 8.0, 2.5% sodium dodecyl sulfate [SDS], 20% sucrose, 2% β-mercaptoethanol, and 0.02% bromophenol blue), separated on 7.5% polyacrylamide slab gels containing 0.1% SDS according to Laemmeli [15], and transferred electrophoretically to nitrocellulose paper as described [16]. The nitrocellulose paper was incubated with the primary antibodies for 180 min, and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, USA) for 30 min at room temperature. Positive bands were visualized by diaminobenzidine reaction.

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Abbreviations:
LC: low calcium
SC: standard calcium

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Preparation of Tissues In Vivo The rat skin was excised from the nipple and immediately fixed with 3% formaldehyde (freshly depolymerized from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.4, for 60 min at room temperature. The tissue was stored in 0.5% formaldehyde at 4°C until cryosectioning.

Cells Pam 212 cells, a generous gift from Dr. Stuart H. Yuspa [10], were maintained in Dulbecco's modified Eagle's minimal essential medium no. 1 (DMEM) with penicillin (100 units/ml) and streptomycin (100 μg/ml), and supplemented with 15% fetal bovine serum (GIBCO, USA). For subculturing, 0.2% trypsin and 0.25 mM EDTA were used to detach cells. 2×10^5 cells were plated in 35-mm plastic dishes with 2 ml DMEM and incubated at 37°C in a humidified incubator with 95% air and 5% CO_2. Low Ca^{2+} medium was prepared using calcium-free DMEM supplemented with 15% chelex-100 (Bio-Rad, USA) treated fetal bovine serum and the Ca^{2+} concentration was adjusted to 0.06 mM by adding an appropriate amount of $CaC1_2$.

To produce an "instant" confluent monolayer of cells, 2×10^6 cells trypsinized and resuspended in low Ca^{2+} medium were replated in a 35-mm dish. The medium change, from low Ca^{2+} (LC) to standard Ca^{2+} (SC), was instituted at 24 h after initiating the cultures.

Some of the cells were treated with 30 μM cytochalasin B (dissolved in DMSO at the final concentration of 0.5%) [11] or with 20 μM colchicine [12] and incubated for 1 h at 37°C in the CO_2 incubator. Control cultures were incubated in the medium with 0.5% DMSO.

Indirect Immunofluorescence Microscopy of Cells on Coverslips PAM 212 cells cultured on coverslips were indirectly immunolabeled for fodrin. The cells in the LC and SC media were fixed with 3% formaldehyde (freshly depolymerized from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min at room temperature; rinsed with PBS containing 10 mM glycine; and treated sequentially with 1% Triton X-100 and 2% gelatin for 10 min each. They were then incubated with the rabbit anti-fodrin

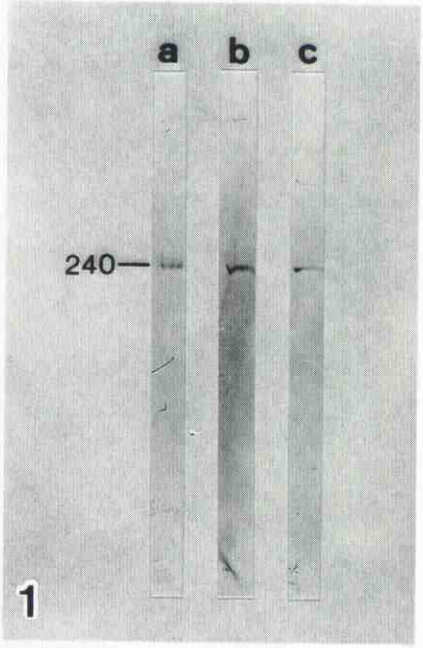


Figure 1. Immunoblots of rat epidermis and Pam 212 cells with anti-fodrin antibody. Erythrocyte ghosts *(lane a)*, extracts of rat epidermis *(lane b)*, and Pam 212 cells *(lane c)* were subjected to SDS-PAGE, and transferred to nitrocellulose paper and incubated with rabbit anti-spectrin *(lane a)* or rabbit anti-fodrin *(lanes b* and *c)*. The anti-fodrin antibody reacted solely with a 240-kd band in extracts of rat epidermis and Pam 212 cells. The anti-fodrin antibody did not react with spectrin.

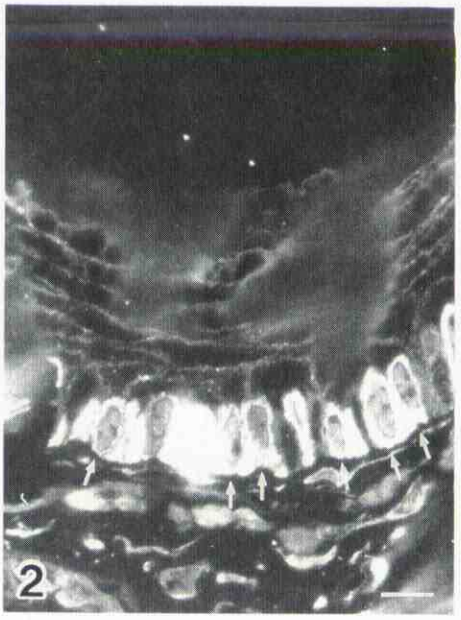
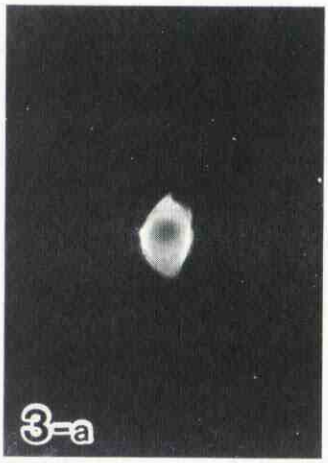


Figure 2. Immunofluorescence microscopy of fodrin. Semi-thin frozen sections of the rat thick skin in the nipple. Fodrin is labeled diffusely in the cytoplasm of the basal cells *(arrows)*, but is confined to the cell periphery in upper layers. *Bar*, 10 μm.



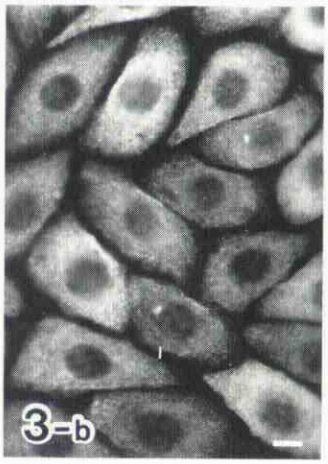


Figure 3. Immunofluorescence microscopy of fodrin in Pam 212 cells in low Ca^{2+}. *(a)* Single and *(b)* confluent cells. In both cases, labeling for fodrin is seen in the cytoplasm. In confluent cells, filamentous pattern is particularly apparent with little accumulation along the cell surface. *Bar*, 10 μm.

antibody (10 μg/ml) for 30 min, rinsed, incubated with fluorescein-labeled goat anti-rabbit IgG (Cappel, USA) (20 μg/ml) for 30 min, rinsed, and mounted on glass slides with 90% glycerol. Some cells were also labeled with rhodamine-phalloidin (Molecular Probes, USA). Control was taken by omitting the primary antibody, resulting in minimal background fluorescence. Cells were observed with a VANOX photomicroscope (Olympus, Japan) equipped with epifluorescent illumination and a phase-contrast device. The specimens were photographed on Kodak Tri-X film.

Indirect Immunofluorescence Microscopy of Semi-Thin Frozen Sections The rat skin in vivo was prepared as above. Pam 212 cells grown on plastic dishes in the LC and SC media were detached with repeated pipetting, rinsed with PBS, and fixed with a mixture of 3% formaldehyde and 0.05% gluataraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min at 4°C. The rat skin and the cell pellet embedded in 1% agar were infused with 2.3 M sucrose and rapidly frozen in liquid nitrogen. Semi-thin frozen sections (0.5–1.0 μm in thickness) were cut in a Reichert-Jung ULTRACUT E ultramicrotome with a cryoattachment (Austria) [17]. Sections treated with 1 mg/ml sodium borohydride for 10 min to quench autofluorescence were immunolabeled and observed as described above for cells on coverslips.

RESULTS

Immunoblotting Immunoblots were made with the polyclonal anti-fodrin and anti-spectrin antibodies (Fig 1). Erythrocyte ghosts were reacted with the anti-spectrin antibody to show the level of 240 kd. In extracts of the rat epidermis and Pam 212 cells, only a band at the same level as α-spectrin reacted positively with the anti-fodrin antibody.

The Rat Epidermis In Vivo The thick skin taken from the nipple was examined for localization of fodrin. The protein showed different distribution in the basal cells and in the other keratinocytes: in the former, it was observed diffusely in the cytoplasm, while in the latter it was seen only along the cell periphery (Fig 2). The difference between basal and spinous layers both in staining pattern and intensity was consistent. No specific fluorescence was observed in the stratum corneum.

The Keratinocyte In Vitro Pam 212 cells grown on coverslips were immunolabeled for fodrin and observed en face by immunofluorescence microscopy. In single cell in the LC medium, fodrin was observed in the cytoplasm (Fig 3*a*). Confluent cells in the LC medium showed filamentous labeling of fodrin throughout the cytoplasm and accumulation of the immunolabeling was not seen in the cell periphery (Fig 3*b*). In contrast, cells in the SC medium showed different distribution of fodrin dependent upon the cell density; in cells isolated singly (Fig 4*a*) and of small colony (Fig 4*b*), the protein was seen to be in the cytoplasm, while in cells at confluency (Fig 4*c*) it appeared markedly concentrated along cell-cell contacts and the cytoplasmic labeling was not seen.

When confluent cells were transferred from the LC medium to the SC medium, cell-cell contacts became apparent within 60 min by phase-contrast observation. But at this point, fodrin labeling occurred in the cytoplasm as well as in the cell peripery (Fig 5*a*). The cytoplasmic labeling decreased gradually and finally fixed at 180 min after the transfer, fodrin was seen to be almost confined to cell-cell contacts (Fig 5*b*).

To confirm the cytoplasmic labeling in the LC medium as well as the surface labeling in the SC medium, semi-thin frozen sections of confluent Pam 212 cells detached from culture dishes were prepared and immunolabeled for fodrin. The labeling was seen intensely in the cytoplasm of the confluent cells grown in the LC medium (Fig 6*a*), whereas it was observed only along the cell surface, and not in the cytoplasm, of the confluent cells in the SC medium (Fig 6*b*).

In order to examine if the filamentous localization of fodrin in cells in low Ca^{2+} is related to actin filaments and/or microtubules, cells were treated with either cytochalasin B or colchicine, fixed, and labeled for fodrin. By the addition of cytochalasin B, labeling

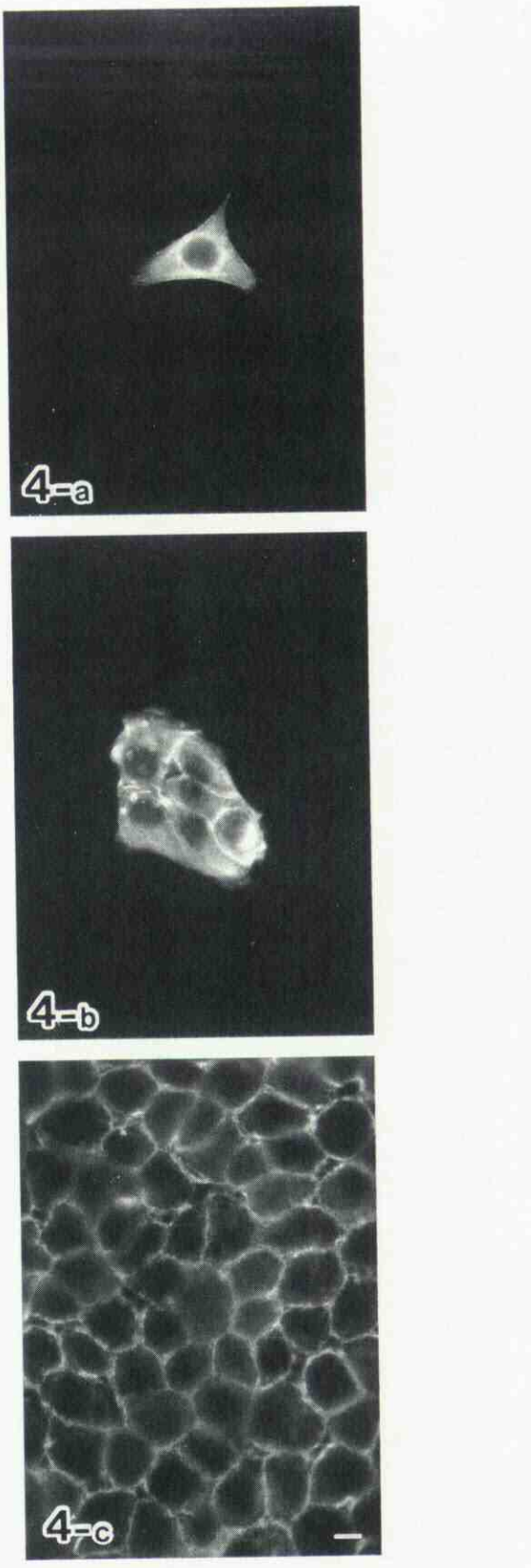


Figure 4. Immunofluorescence microscopy of fodrin in Pam 212 cells in standard Ca^{2+}. *(a)* Single cell, *(b)* small colony, and *(c)* confluent cells. In *(a)* and *(b)*, fodrin is labeled in the cytoplasm, while in *(c)* it is limited to cell-cell contacts and cytoplasmic labeling is scarce. *Bar,* 10 μm.

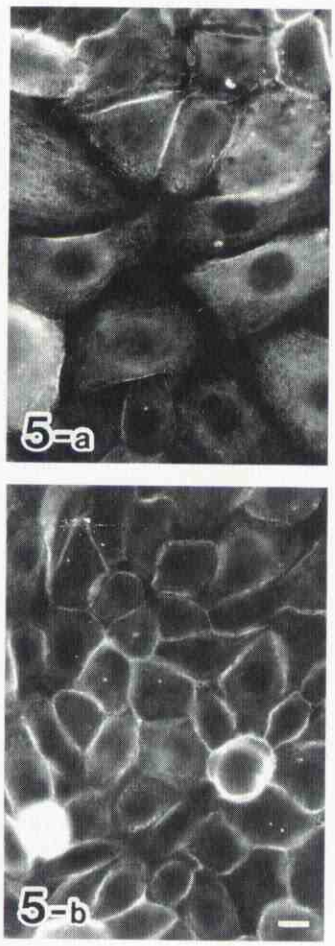


Figure 5. Immunofluorescence microscopy of fodrin in confluent Pam 212 cells transferred from low Ca^{2+} to standard Ca^{2+}. *(a)* 60 min and *(b)* 180 min after the transfer. At 60 min after the transfer *(a)*, cells are labeled in the cytoplasm as well as in the cell periphery, but at 180 min *(b)*, the labeling for fodrin is mostly in cell-cell contacts. *Bar,* 10 μm.

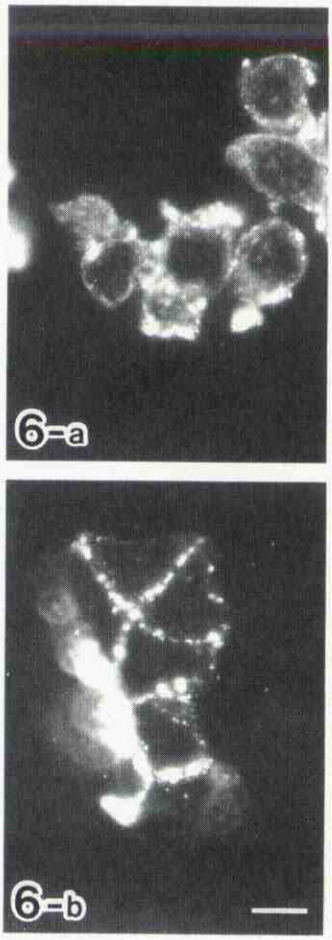


Figure 6. Immunofluorescence microscopy of fodrin in frozen semi-thin sections of Pam 212 cells. The cells grown confluent in culture dishes with low *(a)* and standard *(b)* Ca^{2+} were detached and embedded in agarose. Semi-thin frozen sections were immunolabeled with the affinity-purified antibody. Labeling for fodrin is seen diffusely in the cytoplasm except for the nucleus in the low Ca^{2+}. In contrast, the labeling is observed only along the cell-cell contact in standard Ca^{2+}. *Bar,* 10 μm.

for fodrin became aggregated in the cytoplasm (Fig 7*a*). Labeling of the same specimen with rhodamine-phalloidin showed that F-actin also became aggregated in cytochalasin B-treated cells and that fodrin and F-actin coexist in some, if not all, aggregates (Fig 7*b*). On the contrary, colchicine did not affect the labeling for fodrin (photograph not shown). Cells in the SC medium did not show any change in the distribution of fodrin and F-actin even when treated with either cytochalasin B or colchicine (photograph not shown).

DISCUSSION

Cultured keratincoytes have been used frequently as a model system to study the mechanism of differentiation in vivo. The cells proliferate more rapidly in low Ca^{2+} than in standard Ca^{2+}, but only in the latter environment do they stratify and show some characteristics of keratinized cells. Many morphologic and biochemical changes caused by the change in Ca^{2+} concentration have been reported [1,2]. Pam 212 cells used in the present experiment also respond to the change of the Ca^{2+} concentration, and therefore compare well with the in vivo results. There have also been several studies on the cytoskeleton of the keratinocytes [3–5], but not much has been known about proteins of the cell membrane except for those in the desmosome.

Fodrin is a protein isolated from the brain [6], which shares many properties with erythrocyte spectrin [7,8]: ability to bind to ankyrin; taking a rod form of about 200 nm; ability to bind to actin, which affinity is increased by band 4.1; and ability to bind to calmodulin in a Ca^{2+} -dependent manner [18,19]. In collaboration with ankyrin and band 4.1-like proteins, fodrin can act as an intermediate between the cell membrane and the cytoskeleton.

A protein of the spectrin family, called p230 [20], was reported to exist along the periphery of in vivo human keratincoytes and in the ventral surface and cell-cell contact area of in vitro human keratinocytes [9]. But skins from the different body areas were not compared, nor was the influence of the extracellular Ca^{2+} concentration examined. Moreover, p230 crossreacts with erythrocyte α-spectrin, while fodrin does so only partially [7,8]. Although it needs further examination, it is possible that p230 and fodrin are not crossreactive and thus show different localization.

Changes in the extracellular calcium concentration have been reported to affect the solubility of fodrin in Madin-Darby canine kidney (MDCK) cells [21]. However, it was not known whether localization of fodrin changes under the experimental condition. In the present study, immunocytochemistry of frozen sections confirmed that fodrin, found only along the cell surface in SC medium, is localized in the cytoplasm of Pam 212 cells in LC medium. The mechanism which induces the translocation of fodrin is not known yet. Since de novo synthesis is not necessary to induce the change of the fodrin's solubility property in MDCK cells [21], it is likely that some post-synthetic modification of the protein is involved. Since fodrin is a major calmodulin-binding protein, calmodulin might

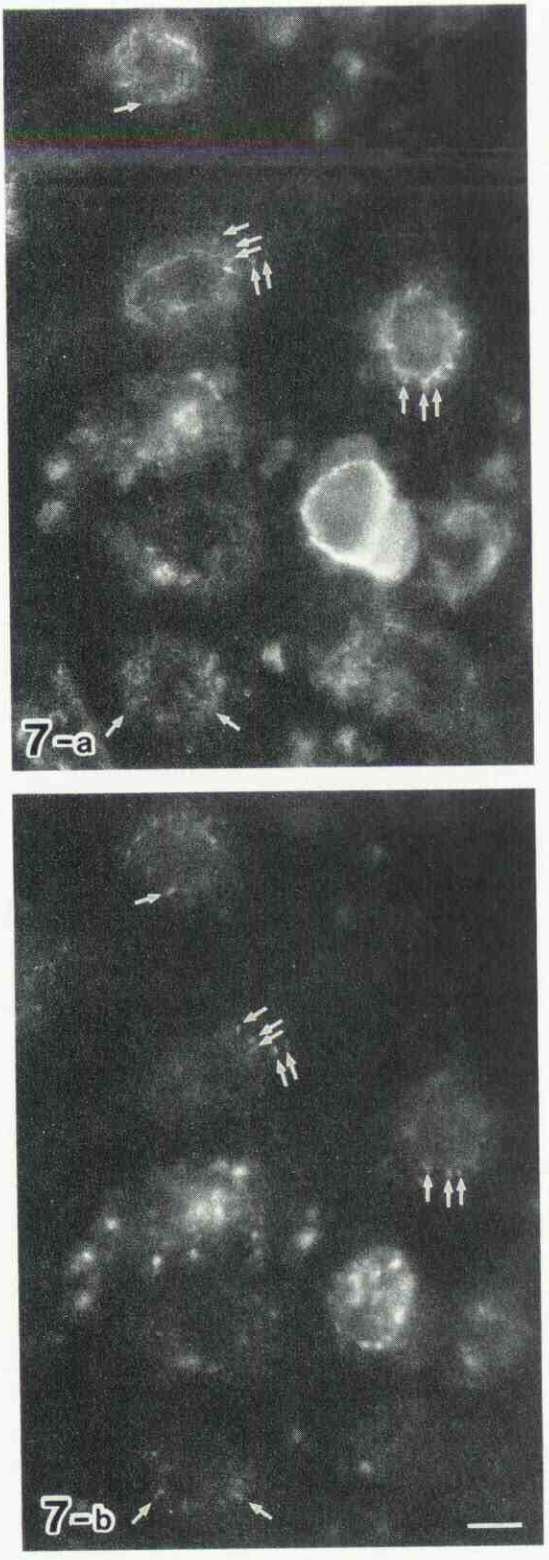


Figure 7. Double immunofluorescence microscopy of *(a)* fodrin and *(b)* F-actin in Pam 212 cells in low Ca^{2+}. Fodrin was labeled with affinity-purified antibody and FITC conjugated secondary antibody, while F-actin was labeled with rhodamine-phalloidin. Both fodrin and F-actin appear to form aggregates, some of which coincided with each other *(arrows)*. *Bar*, 10 μm.

affect fodrin's localization in some manner. The fact that proliferative keratinocytes in low Ca^{2+} contain more calmodulin than the basal monolayer cells in standard Ca^{2+} [22] does not contradict with the above assumption. Although only speculative, it is interesting to study whether binding of calmodulin to fodrin in a Ca^{2+}-dependent manner influences the distribution of fodrin.

By changing calcium concentration, desmosomal proteins in human keratinocytes are known to accumulate at the cell periphery in a similar time course as taken by fodrin [3]. Moreover, the accumulation of desmosomal proteins is accompanied by a rearrangement of keratin filaments. Although direct correlation between fodrin and desmosomal proteins and between actin filaments and keratin filaments may not exist, it is interesting that two different sets of peripheral membrane proteins and cytoskeletal filaments are changed in distribution almost simultaneously.

Although fodrin is confined to the cell membrane in most cells [6,23,24], several examples have been reported in which fodrin is found in the cytoplasm [14,25–32]: fodrin is seen either associated with actin filaments [14,25–27], microtubules [28–30], or membrane organelles [26,30,31], or distributed in the cytoplasm not associated with other known structures [14,32]. In the present experiment, it was shown that by treatment with cytochalasin B, a reagent which specifically depolymerizes actin filaments, distribution of fodrin was changed. Codistribution of fodrin and F-actin after cytochalasin B treatment indicates that fodrin is likely to be attached to actin filaments at least partially. But it is not clear whether the attachment is direct and to what extent it occurs. It cannot be denied that fodrin may also exist independent of actin filaments.

The keratinocytes which have abundant cytoplasmic occurrence of fodrin, basal cells in vivo, and keratinocytes cultured in low Ca^{2+} in vitro share another characteristic. That is, they are both rapidly proliferating cells. We do not know how the distribution of fodrin affects the cell growth, or vice versa. But presence of fodrin is reported to affect many properties of the plasma membrane including the fluidity of the lipid bilayer [33], distribution of integral membrane proteins [34]. The protein localization might be related to the very basic character of the cell.

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