Localization of Adducin in Epidermis

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On the cytoplasmic side of the plasma membrane of erythrocytes there is a dense protein filament matrix that maintains the shape of the cells. The main constituents of this system, actin and spectrin, which have also been detected in keratinocytes and fibroblasts, are known to be linked in erythrocytes in a network structure by additional proteins such as band 4.1 and adducin. The interaction between actin and spectrin, mediated by adducin, is regulated by calmodulin and protein kinase C. Because we have previously found adducin in cultured keratinocytes, we investigated epidermis by immunochemical techniques. We found adducin to be localized at cell-cell contact sites in epidermis using affinity-purified antibodies against human erythrocyte adducin. Immunofluorescence of epidermis revealed an intense fluores-

cence in the basal layer, whereas stratum spinosum and stratum granulosum showed moderate staining. There was intense staining at sites of cell-cell contact in cultured human keratinocytes. Immunoblot analysis indicated the presence of adducin polypeptides of 103 kd and 97 kd in epidermis, but in cultured keratinocytes only the higher molecular weight form could be detected. This study indicates adducin, a regulatory protein in erythrocytes, is also present in epidermis. Its localization suggests that it may be involved in the formation of the microfilament matrix of the membrane skeleton at cell-cell contact sites. Key words: intercellular junctions/spectrin/membrane skeleton/keratinocyte. *J Invest Dermatol* 101:783-788, 1993

Adducin is a structural protein associated with the spectrin-based membrane skeleton of cells [1-3]. The spectrin skeleton has been intensively investigated in erythrocytes (for review, see [4-6]), but in recent years spectrin isoforms have also been detected in cultured rodent keratinocytes and skin [7], and in cultured human keratinocytes [8-10] and human epidermis [8,10]. The protein has been localized in human epidermis at the cytoplasmic surface of the cell membrane in all epidermal layers except the stratum corneum [8]. The spectrin-based membrane skeleton can be viewed as a system of interacting proteins coordinated in an integrated structure. Electron microscopy of preparations of membrane skeleton proteins of human erythrocytes made from erythrocyte ghosts revealed a regular, lattice-like organization with rod-shaped spectrin molecules linked to short actin filaments to form a sheet of five- and six-sided polygons [11,12]. In biochemical assays the major constituents of the microfilament system, actin and spectrin, reveal only low affinity for each other [1] and require the presence of other proteins to form complex structures, visualized by electron microscopy [13].

Therefore, we focused on additional proteins associated with actin. One of the first proteins identified to promote the binding of actin to spectrin was protein band 4.1 [14,15], named after its mobility on sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels of human erythrocyte ghosts [16]. Biochemical assays [14,15,17] suggested that band 4.1 was present at the actin-spectrin junction, where it formed a ternary complex. Proteins of the band 4.1 family have recently been reported to be present in cultured keratinocytes and epidermis [10,18]. An immunohisto-

chemical study found that band 4.1 was localized preferentially in the basal layer of skin [18]. Mutha *et al* were able to isolate cDNA clones from a human keratinocyte library [10], confirming the active transcription of spectrin (fodrin) and band 4.1 genes in keratinocytes.

In this study we report on the localization of adducin in epidermis. Adducin is a member of the proteins found associated with spectrin-actin complexes and has been shown to recruit additional molecules of spectrin to the ternary complex containing spectrin, actin, and adducin [1,2]. Adducin has been characterized in erythrocytes as a heterodimer with subunits of 97 kd (α) and 103 kd (β) [19]. Adducin is localized in various tissues and cells including brain [2], kidney [2], lens [2,9], Madin-Darby canine kidney (MDCK) cells [9,20], fibroblasts [21], and normal human keratinocytes [9]. In human erythrocytes [22] and fibroblasts [21], adducin is localized diffusely on the cytoplasmic surface of the cell membrane. In human keratinocytes, adducin has also been localized diffusely in the cytoplasm in a low-calcium medium [9], in which cells do not form intercellular junctions [23]. Upon induction of intercellular contact by an increase in calcium ion concentration, adducin became concentrated at sites of cell-cell contact at earlier stages of junctional formation than did adherens junctions and desmosomes [9]. The function of this protein is not fully understood, but biochemical data indicate a possible role in cell signalling processes, because adducin is a substrate for both protein kinase C and protein kinase A [24,25]. In addition, adducin is a calmodulin-binding protein [3,19], and the calcium-calmodulin complex can regulate the affinity of adducin for the spectrin-actin complex in a calcium-dependent manner [1,3]. This study supports the idea of a subplasmalemmal network in keratinocytes, the structure of which may be modified by extracellular signals.

MATERIALS AND METHODS

Preparation of Affinity-Purified Antibodies Against Adducin Adducin was purified from human erythrocytes according to Bennett and Gardner [19] with slight modifications. In brief, after ghosts were made

Manuscript received December 10, 1992; accepted for publication June 8, 1993.

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Abbreviations: MDCK, Madin-Darby canine kidney.

from human erythrocytes by lysis in hypotonic buffer, erythrocyte ghosts were extracted in 10 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), containing 1% v/v Triton X-100 and were then washed in high-salt buffer (1 M NaCl, 7.5 mM sodium phosphate, pH 7.4, 1 mM EDTA, and 1 mM DTT). After dialysis against 10 mM sodium phosphate, pH 7.4, containing 50 mM NaCl, 0.25 mM EDTA, and 1 mM DTT, the protein was applied to a 30-ml column of diethylaminoethyl(DEAE)-Sephacel (Pharmacia, Freiburg, Germany) equilibrated with the same buffer and eluted with a linear salt gradient (0.05–0.5 M NaCl). Finally, fractions containing adducin as determined by SDS polyacrylamide gel electrophoresis were loaded on a hydroxylapatite column equilibrated in 0.01 M potassium phosphate, pH 7.4, containing 0.5 M sodium bromide and 0.02% Tween and eluted with a linear gradient of potassium phosphate (0.01–0.3 M) in the same buffer.

Antibodies were raised in a New Zealand rabbit by injecting 100 μ g of protein with complete Freud's adjuvant, the animal was boosted with four subsequent injections of incomplete Freud's adjuvant at 3-week intervals and was bled 2 weeks after the final injection. Antiadducin serum from rabbits was affinity purified on adducin run on SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Biorad, Munich, Germany). Bound antibody was dissociated by incubating the membranes with phosphate-buffered saline (PBS) at 56°C for 30 min [26].

Keratinocyte Culture Normal human keratinocytes were obtained from newborn foreskins and initiated into culture by a modification [27] of the method of Rheinwald and Green [28]. Cells were subcultured in a serum-free MCDB 153 medium [29] containing 0.1 mM calcium supplemented with hydrocortisone (0.4 μ g/ml), insulin (5 μ g/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), epidermal growth factor (5 ng/ml), bovine pituitary extract (150 μ g/ml), histidine (2×10^{-4} M), isoleucine (7.5×10^{-4} M), methionine (9×10^{-5} M), phenylalanine (9×10^{-5} M), tryptophan (4.5×10^{-5} M), and tyrosine (7.5×10^{-5} M). Cells were subcultured at 5000 cells/cm². For the immunoblots and immunofluorescence studies, cells were grown for 3 d on either 100-mm Petri dishes or coverslips.

Immunoblotting Studies The presence of adducin was investigated in human erythrocytes, rat brain, human cultured keratinocytes, and human epidermis. For the detection of adducin in erythrocytes, erythrocyte ghosts were prepared according to Bennett [16] and diluted 1:5 times with PBS, pH 7.5. Rat brain was removed after perfusion of animals with 5 mM sodium phosphate, 0.15 M NaCl, 2.5 mM NaEDTA, 5 mM diisopropylfluorophosphate (DFP), pH 7.5 via the left ventricle after incision of the right atrium.

For immunoblot studies, cultured human keratinocytes were incubated with PBS, pH 7.5, containing 5 mM EDTA and 5 mM DFP for 15 min at 4°C before being scraped off with a rubber policeman. Normal human epidermis was obtained by placing biopsies in hot PBS (95°C) with 2 mM phenylmethyl-sulfonyl-fluoride for 1 min. Following this procedure, epidermis could be collected with forceps, minced, and homogenized in Fairbanks' sample buffer [30]. Cells and tissues were dissolved in Fairbanks' sample buffer for 10 min at 68°C. Proteins were separated on 1.5-mm slab gels with a 3.5%–17% exponential gradient in 0.2% w/v SDS and transferred electrophoretically to PVDF membranes (Biorad) according to the method of Towbin *et al* [31]. After blocking of nonspecific binding with 2% protease-free bovine serum albumin (ICN, Meckenheim, Germany) for 4 h at room temperature, membranes were incubated overnight at 4°C with affinity-purified antibodies against adducin (1:1,000) in incubation buffer consisting of PBS containing 2% bovine serum albumin (BSA) and 0.1% (v/v) Tween 20. Blots were washed with 0.2% Tween 20 in PBS and for 30 sec with 0.1 M glycine buffer, pH 8.2, containing 2 M urea, 1% (v/v) Triton X-100, followed by one high-salt wash in 1 M NaCl in PBS, pH 7.4, to reduce nonspecific background. Final washes were performed with incubation buffer containing no BSA.

Goat-anti-rabbit antibodies covalently coupled to horseradish peroxidase (Biorad), were applied for 90 min at 4°C in 1:3000 dilution in PBS with 2% BSA. After washing as described above, bound antibodies were visualized by chemiluminescence-ECL (Amersham, Braunschweig, Germany) by exposing the membranes briefly to Kodak XAR film.

Immunofluorescence Studies Samples of skin, spinal cord, and spinal root were immediately frozen in isopentane precooled with liquid nitrogen. Four-micrometer cryostat sections were cut and mounted on ovalbumin-coated glass slides. Monkey esophagus was obtained from Bios GmbH, Munich, Germany. Human erythrocytes were collected from healthy donors, smeared on slides, and air-dried. Normal human keratinocytes were grown on eight-well tissue culture slides (ICN, Meckenheim, Germany) for 3 d after being plated at 5000 cells/cm². Cells were shifted to a final calcium

Figure 1. Epidermis contains the α - and β -subunits of adducin. Proteins were separated by electrophoresis on a 3.5–17% gradient SDS polyacrylamide gel and transferred to PVDF membranes, which were then incubated with anti-adducin antibody as described. Molecular weight markers are shown in kd at left. Lane 1, human erythrocyte ghosts; lane 2, rat brain; lane 3, human keratinocytes cultured in MCDB 153 at 0.1 mM Ca⁺⁺ with supplements for 3 d and shifted to 1.1 mM Ca⁺⁺ for 3 h before extraction; lane 4, human epidermis.

concentration of 1.1 mM in MCDB 153 for 3 h. Erythrocyte smears and keratinocytes were fixed with 2% paraformaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS, pH 7.4. Sections and cells were incubated for 30 min with 2% protease-free BSA in PBS, pH 7.4, at room temperature and then overnight with affinity-purified anti-adducin antibodies (1:20) at 4°C. Slides were washed in PBS, pH 7.4, containing 0.01% Tween for 15 min and then incubated with rhodamine-labeled goat-anti-rabbit IgG (Dianova, Hamburg, Germany) in a 1:200 dilution for 30 min at room temperature. Slides were washed again and mounted in 40% glycerol in PBS. Cells and sections were viewed with a Zeiss Axiophot equipped with epifluorescence, photographed with Kodak TRI-X film, and developed with Ultrafin SF (Tetenal, Norderstedt, Germany) at 400 ASA.

RESULTS

Adducin is Present in Epidermis To analyze the specificity of the affinity-purified polyclonal antibody against adducin, we performed immunoblots on lysates of erythrocytes, brain, cultured normal human keratinocytes, and epidermis (Fig. 1, lanes 1–4). We identified two polypeptide bands of 103 kd and 97 kd in the erythrocytes (Fig 1, lane 1). With the antibody raised against the erythrocyte isoform of adducin used in our study, we also detected a protein at 117 kd in brain lysates (Fig 1, lane 2). (The presence of higher M_r isoforms of adducin in brain has been reported previously [2].) Lysates of human keratinocytes cultured in 0.1 mM calcium [9] and also of fibroblasts [20] have been shown to react with only one band comigrating with the α -subunit of adducin on immunoblots. Anti-adducin antibodies used in this study confirm this result for human keratinocytes cultured in serum-free medium and shifted to 1.1 mM calcium for 3 h before preparation of the lysates (Fig 1, lane 3). To investigate the presence of adducin-like proteins in normal human epidermis, we separated epidermis from dermis by heating and performed immunoblotting, which showed two polypeptide bands comigrating with the α - and β -subunits of adducin (Fig 1, lane 4), in contrast to the findings in cultured human keratinocytes. To exclude contamination of the epidermal lysate with erythrocytes,

Figure 2. β -adducin in epidermis is not caused by contamination of the sample of epidermis with erythrocytes. Human epidermis was separated from dermis as described in *Materials and Methods*. The same lanes (lane 1, human erythrocytes; lane 4, human epidermis) used in Fig 1 were heated and washed to separate bound anti-adducin antibodies and then reprobbed with antibodies to erythrocyte band 3.

which might account for the finding of the β -adducin band in the epidermal extract, the same membranes were reprobbed with antibodies against the cytoplasmic domain of band 3 (kindly provided by D. Drenckhahn), a protein that is present only in erythrocytes. Immunoblotting with anti-band 3 IgG revealed a band at 95 kD in lysates of erythrocytes (Fig 2, lane 1), but lysates of normal human epidermis did not react with the antibody (Fig 2, lane 2), indicating that both α - and β -subunits of adducin are intrinsic components in epidermis.

Immunofluorescence Studies

Adducin in Other Tissues: To test the specificity of the antibody and in addition to obtain more information about the possible function of adducin, other tissues were examined with the antibody against adducin. In erythrocytes intense staining was detected in the cell membrane after permeabilization with non-ionic detergent (Fig 3A). Because adducin is known to be present in central and peripheral nervous tissue [2,9], we investigated spinal cord and spinal root. In spinal cord (data not shown) and spinal root (Fig 3C), antibodies against adducin stained regions near the cell membrane of the axoplasm. Peripheral nerves in sections of esophagus (Fig 3B) were also stained by antibodies to adducin. The epithelium of esophagus (Fig 3D) showed staining in the periphery of the cell. In contrast to the staining in skin, however, all layers of the epithelium showed a similar intensity of fluorescence. Control sera did not react (data not shown).

Epidermal Localization of Adducin, Actin, and Spectrin: Because Western blots indicated that adducin was present in epidermis, we incubated cryostat sections of normal human epidermis with affinity-purified polyclonal antibodies against adducin. In epidermis a strong fluorescence was detected in the lateral aspects of the basal keratinocytes (Fig 4A). The cell membranes of the spinous layer were only moderately stained, but staining was of higher intensity in the granular layer (Fig 4A, large arrowheads). A punctate pattern suggesting staining of cell-cell junctions could be visualized

in some areas of the basal keratinocytes at higher magnification (Fig 4B).

Biochemical investigations have shown that adducin binds to actin and to actin-spectrin complexes. To investigate the localization of actin and spectrin and to determine their localization with respect to adducin, epidermis was incubated with rhodamine-

Figure 3. Localization of adducin in other tissues. Anti-adducin antibody was used to examine human erythrocytes (A) or cryostat sections of peripheral nerve in esophageal mucosa (B), spinal root (C), and esophagus epithelium (D) by indirect immunofluorescence. Cells (A) or sections (B,C,D) were incubated overnight with antibodies against adducin, and bound antibody was visualized by rhodamine-labeled goat-anti-rabbit IgG as described. Bar, 40 μ m for A, 20 μ m for B, C, and D.

Figure 4. Spectrin and actin, like adducin, are located at the cell periphery in epidermis. Cryostat sections of epidermis were incubated with antibodies and then with secondary antibodies or with rhodamine-phalloidin (for actin) as in *Materials and Methods*. (A,B) anti-adducin antibodies; (C) antibodies against brain spectrin; (D) rhodamine-phalloidin. Arrowheads, staining of adducin in the basal cells (small arrowheads) and in the stratum granulosum (large arrowheads). Bar, 20 μ m (A,B); 100 μ m (C,D).

labeled phalloidin (Fig 4C), a specific probe for F-actin, and with anti-brain spectrin antibody (Fig 4D). The strong staining of epidermis indicated that actin and spectrin were present in similar or identical locations in the region of the cell membrane in all layers of the epidermis except the stratum corneum.

Localization of Adducin in Cultured Human Keratinocytes: In cultured keratinocytes, intense staining for adducin was noted at sites of cell-cell contact, but also modest cytoplasmic staining was present. No concentration of adducin could be detected at free cell borders at the edge of the colony, which were not in contact with other cells (Fig 5). This result is consistent with the localization of adducin in the lateral aspects of cells in the basal layer of epidermis and with the notion that keratinocytes in culture may have some similarity to basal cells *in vivo*.

DISCUSSION

In this study the presence of a protein reactive with affinity-purified anti-adducin antibody and comigrating with adducin bands on immunoblots was demonstrated in human epidermis and cultured keratinocytes. In cultured human keratinocytes, adducin was concentrated at cell-cell contact sites. The localization of adducin in the lateral region of basal cells in epidermis is consistent with the localization of adducin in cell culture. Adducin was colocalized with actin and spectrin in epidermis, but the intensity of staining for actin and spectrin was more continuous throughout the stratum basale, stratum spinosum, and stratum granulosum in comparison to adducin. Staining for adducin was pronounced on the surface of basal cells. Possible explanations could be the reduction of adducin expression, the expression of different adducin-isoforms, or the capping of antigenic sites on adducin during cell differentiation. In mammalian erythropoiesis adducin has been shown to be an early-expressed protein switching from a triton-soluble pool to binding sites on the membrane skeleton [22].

Immunoblot analysis of the epidermis showed a crossreactivity of the antibody against adducin with two polypeptide bands at 103 kd and 97 kd, the molecular weights of the two known human erythrocyte isoforms. In cultured keratinocytes, only the higher molecular weight subunit could be detected, suggesting that differentiation *in vivo* is associated with the expression of the β -subunit. A less likely explanation is that cells in epidermis distinct from keratinocytes, such as Langerhans cells and melanocytes, could contain mostly β -adducin. Adducin isoforms have been shown to be present in various cells and tissues. In fibroblasts, only α -adducin was detected [21], whereas in the MDCK cell, a kidney cell line derived from dog kidney, both the α - and β -subunits were detected by immunoblot analysis [9]. Additional support for the presence of different isoforms of adducin is provided by the observation that antibodies were generated against human-erythrocyte-adducin that stained epidermis, in contrast to previous results obtained with antibodies against

Figure 5. Adducin is localized at the cell periphery in cultured keratinocytes. Neonatal foreskin keratinocytes were maintained in MCDB 153 at 0.1 mM Ca^{++} for 3 d and shifted to 1.1 mM Ca^{++} 3 h prior to fixation. After lysis with isotonic buffer containing Triton X-100 and washing, cells were incubated overnight with affinity-purified anti-adducin antibodies and then with rhodamine-labeled goat-anti-rabbit IgG as described. Bar, 20 μ m.

bovine-brain adducin, which stained nerve fibers but failed to stain epidermis [9].

Biochemical studies have revealed that adducin promotes the interaction of the major components of the subplasmalemmal cytoskeleton, actin and spectrin, which form a ternary complex [1,2,13] with adducin. In addition to adducin, the band 4.1 protein also induces actin-spectrin complexes [14,15]. Band 4.1 is present in erythrocytes and shares a high homology with band 4.1 protein in epidermis [10]; comparison of the amino acid sequences revealed that the keratinocyte protein is identical to band 4.1 in erythrocytes except for an 80-bp deletion between two *Hind* III restriction sites [10]. Protein 4.1 and adducin may interact at common sites on spectrin and actin complexes, as binding of adducin can be inhibited by 4.1 [13]. Though these proteins share identical functions, detailed biochemical investigations have indicated differences in their interactions. The band 4.1 protein does not directly interact with actin filaments [15] but associates at first with spectrin at the tails of spectrin tetramers with a 1:1 stoichiometry; this enhances the affinity of spectrin for actin [32–34]. Adducin, in contrast, does not bind to spectrin or actin alone [1,13], but associates with actin-spectrin complexes, which are then stabilized by adducin, and additional spectrin filaments then bind to this complex. This process has been shown to be regulated, as it can be inhibited by calcium in the presence of calmodulin [1,13].

Band 4.1 and adducin contain binding sites for additional proteins. Band 4.1 binds glycophorin C, or glyconectin [35], and band 3 [36,37], an anion exchanger of erythrocytes. Direct interaction of 4.1 with the lipid bilayer by association with phosphatidylserine has been reported [38]. No such interactions have been reported for adducin, but adducin has been identified as a major calmodulin-binding protein [19].

The presence of different proteins associated with actin but sharing similar functions could be important for a graded cellular response and could allow the differential assembly of specialized actin filaments in the vicinity of each other, because the affinities of the associated proteins for their substrates and for protein kinases are distinct. For example, Drenckhahn *et al* demonstrated that the spatial distribution of distinct actin-crosslinking proteins correlates with different supramolecular assemblies of actin filaments in hair cells of the auditory organ [39]. Thus, associated proteins may permit modulation of the interaction of actin and spectrin and of their interaction with the cell membrane.

The importance of filament-containing structures for the integrity of epidermis was demonstrated by Vassar *et al* in transgenic mice [40]. Bonifas *et al* were able to correlate defects in the sequence of keratin 14 with the development of epidermolysis bullosa simplex in two families [41], and mutations on single genes were subsequently found to correlate with the degree of severity of epidermolysis bullosa [42]. Until now, the elucidation of diseases associated with the (actin) microfilament system has been confined to erythrocytes and has provided a molecular basis for hemolytic diseases [4], e.g., the association of spherocytosis and elliptocytosis with the lack of protein 4.1 and spectrin. The identification of microfilament-associated proteins in brain has led to the discovery of molecular defects characterized by the degradation of Purkinje cells [43]. As suggested by Mutha *et al* [10], further investigations, including the microfilament system, might be important for clarifying our knowledge of dermatologic diseases characterized by the disintegration of normal epidermal structure.

This work was supported in part by the Deutsche Forschungsgemeinschaft Ka 852/1-1 to HWK and by AM 25871 from the National Institute of Arthritis and Metabolic and Skin Diseases to EO'K.

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For the second year, The American Skin Association will award prizes for meritorious research. The purpose of these prizes is to honor investigators for their Original Research in Cutaneous Biology. Three prizes will be awarded—each winner will receive a check for \$5000.

Discoveries in three fields will be considered: Dermatitis, Psoriasis, and Vitiligo.

Through these awards, the Directors of the American Skin Association hope to encourage new scientists to enter dermatological research. The intent is to recognize investigators who have made notable, recent observations and new discoveries in the fields being emphasized.

Recipients of the ASA Prizes in 1993 were Drs. Herbert Honigsmann and Klaus Wolff, Vitiligo Award, and Dr. John Hanifin, Dermatitis Award.

Nominations should be submitted in the form of a letter describing the contribution of the candidate. Include with the letter a copy of the curriculum vitae of the nominee. Candidates will be reviewed by the American Skin Association's Medical/Scientific/Policy Advisory Committee and the recipients of the three prizes will be selected in February, 1994.

Please mail application letters to:

The American Skin Association, Inc.
150 East 38th Street, 32nd Floor
New York, NY 10155-0002

Deadline for receipt of applications is January 15, 1994.