Multiple Mechanisms of Dissociated Epidermal Cell Spreading

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ABSTRACT To test the possibility that epidermal cells use a common basement membrane protein whenever they spread, in vitro experiments were conducted using trypsin-dissociated guinea pig epidermal cells and the following proteins: human serum, bovine serum albumin, serum fibronectin, Type IV collagen, laminin, and epibolin (a recently described serum glycoprotein which supports epidermal cell spreading; Stenn, K. S., 1981, *Proc. Natl. Acad. Sci. U. S. A.* 78:6907.). When the cells were added to media containing the specific proteins, all the tested proteins, except for serum albumin, supported cell spreading. Added to protein-coated substrates in defined media, the cells spread on fibronectin, epibolin, and laminin-Type IV collagen, but not on albumin or whole serum. In none of these experiments were the results qualitatively affected by the presence of cycloheximide. Antibodies to a specific protein blocked cell spreading on that protein but not on the other active proteins, e.g. whereas antibodies to epibolin blocked cell spreading on epibolin, they did not affect spreading on fibronectin, collagen, or laminin. In a second assay in which the cells were allowed to adhere to tissue culture plastic before the protein-containing medium was added, the cells spread only if the medium contained epibolin. Moreover, under these conditions the spreading activity of whole serum and plasma was neutralized by antiepibolin antibodies. These results support the conclusion that dissociated epidermal cells possess multiple spreading modes which depend, in part, on the proteins of the substrate, proteins of the medium, and the sequence of cell adhesion and protein exposure.

Considerable experimental effort has been directed toward defining the biochemical substrate upon which cells adhere and spread (1, 2). For dissociated epithelial cells a group of basement membrane-associated molecules including fibronectin, laminin, and Type IV collagen (3–6) have been implicated in in vitro adhesion and spreading. Immunofluorescence studies have also implicated these molecules in the movement of epithelial sheets in wound closure (7, 8). Recently, we isolated from human plasma a glycoprotein called epibolin (9), which also supports dissociated epithelial cell and epithelial sheet spreading in vitro.

It was the purpose of this study to consider the role of each of these molecules—fibronectin, laminin, Type IV collagen, and epibolin—in the spreading of dissociated epidermal cells and to test the possibility that one is more important or is the common spreading molecule under all conditions. The results indicate that epidermal cells will spread maximally on various protein substrates if the cells are added to the protein-containing medium or to the protein-coated dish. However, if the cells are allowed to adhere to a noncoated dish, maximal spreading will not occur unless the medium contains epibolin. In all cases, spreading occurred independent of protein synthesis. These results suggest that epithelial cells employ several mechanisms of spreading and that none of the molecules studied is crucial to every spreading mode.

MATERIALS AND METHODS

Protein Chemistry: Protein concentration was measured by absorbance at 280 nm and by the Lowry procedure (10). Chemicals were of reagent grade purchased from local suppliers. Water was singly or doubly distilled. Dialysis bags were prepared as described previously (11). Bovine serum albumin was purchased (Sigma Chemical Co., St. Louis, MO). Human plasma from normal donors was obtained from the Yale-New Haven Hospital Blood Bank 1–2 wk after bleeding and cleared by centrifugation (5,000 *g* for 20 min at 4°C in a Beckman J6-B centrifuge, Beckman Instruments, Inc., Fullerton, CA). Human serum was prepared from the cleared plasma by dialysis against 10 mM Tris HCl, pH 8.0 plus 100 mM NaCl plus 20 mM $CaCl_2$ at 4°C for 24 h.

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Epibolin was purified from human plasma as previously described (9). In all experiments, step-5 purified material was used. Human plasma fibronectin was purified and characterized as previously described (12) using a modified procedure of Engvall and Ruoslahti (13). Laminin, Type IV collagen, and Type V collagen were prepared and characterized as previously described (14–17). Protein-coated culture dishes were prepared from bacteriological Petri dishes (5).

Dissociated guinea pig epidermal cells were prepared as previously described (9); however, the defined medium was Earle's medium 199 (199E) (prepared by the media laboratory, Yale University School of Public Health). The media were sterilized by Millipore filtration (0.45 μm pore diameter). All incubations were conducted at 37°C in an H_2O-saturated 95% air/5% CO_2 environment for varying periods of time. Spreading was quantitated after specified intervals by counting the number of cells in a radial or polar spread pattern (18) by light microscopy after fixing and staining in Giemsa stain (Fisher Scientific Co., Pittsburgh, PA) (19). For those assays under conditions of reduced protein synthesis, the cells were continuously exposed to media containing 50 μg/ml cycloheximide (Sigma Chemical Co.) beginning 4 h before the experimental start. The effect of cycloheximide on protein synthesis was measured by pulsing a spread cell preparation for 2 h with [^{3}H]proline (3 μCi/ml, 35 Ci/mM) (Amersham Corp., Arlington Heights IL) followed by autoradiography as previously performed (20). In these experiments, after 4 h in the presence of 50 μg/ml of cycloheximide, amino acid incorporation was reduced by >90%.

Antibody Preparations: Antibody to epibolin was prepared by injecting 100 μg of step-VI purified epibolin (9) emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) into flank skin of rabbits for three 2-wk intervals. Serum was collected two weeks after the last injection. The immunoglobulin fraction was isolated by making the antiserum 40% saturated in $(NH_4)_2SO_4$ (at 4°C) and collecting by centrifugation (5,000 *g* at 4°C). After being washed three times with 40% $(NH_4)_2SO_4$), the precipitate was dissolved in an equal volume of 10 mM Tris-HCl, pH 8.0 plus 100 mM NaCl and dialyzed overnight against the same buffer (4°C). This antibody preparation had a titer of >2,500 by the ELISA assay (21) and showed one precipitin arc by Ouchterlony immunodiffusion against human serum (as previously done [12]). No precipitin was observed when this antibody was diffused against serum albumin, fibronectin, laminin, Type IV, or Type V collagens.

Antibodies to Type IV collagen, laminin, and fibronectin were prepared and characterized as previously described (15, 16, 17).

RESULTS

Epidermal Cell Spreading on Tissue Culture Dishes Not Previously Coated With Protein

As shown in Table I (*a* and *b*), no cell spreading was observed over the course of 24 h when the cells were incubated in defined medium alone or in medium containing bovine serum albumin (*BSA*). In contrast, spreading occurred if the medium contained fibronectin (*FN*), epibolin, human serum, Type IV collagen, or laminin. Blocking protein synthesis with cycloheximide did not affect spreading. It is notable that at 24 h there was less spreading in the presence of FN and laminin than in the presence of collagen, serum, and epibolin.

Epidermal Cell Spreading on Coated Dishes in Defined Medium

If the substrate was coated with FN, epibolin, or laminin-Type IV collagen, maximal cell spreading was observed (Table II: *b*, *c*, and *d*). Once again, BSA alone was unable to support spreading. It was surprising that human serum-coated dishes did not support spreading even though serum contains FN as well as epibolin. Two possible explanations for this observation were considered. First, that the FN and epibolin in serum were both denatured during the drying process. This seemed unlikely because both purified epibolin and purified FN were active after drying as demonstrated (Table II: *b* and *c*). The second possibility was that an inactive serum protein, present in high concentration (e.g. albumin is 30 mg/ml serum), covers the binding sites of the plastic surface and thus limits the adhesion of such active molecules as FN (~0.3 mg/ml serum) and epibolin. Indirect evidence for this interpretation is given in Table III (*a* and *b*). When the dish was coated with the inactive molecule BSA even though the medium contained FN and epibolin, no spreading occurred. This result was observed previously by others (22). Grinnell (2) reported that BHK cells were unable to spread on BSA-coated dishes even if the medium contained 5% fetal calf serum. A corollary to this conclusion is that the proteins supporting spreading have a higher affinity for plastic than BSA when complete serum is added to the medium (Table 1: *e*). Indeed, Grinnell (2) asserts that the adhesion-promoting protein(s) has a 50- to 1,000-fold higher affinity for the substratum than BSA. Once again, in

TABLE I

Spreading of Guinea Pig Epidermal Cells on Plastic: Cells Added to Media

		Average % spread cells ± 1 SE (n)				
		Control				
Medium	Substrate	1 h	2 h	3 h	4 h	24 h
a. 199E	T.C. plastic	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (4)	0 ± 0 (2)
b. 199E + BSA	T.C. plastic	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (1)
c. 199E + FN	T.C. plastic	30 ± 6 (8)	50 ± 3 (7)	43 ± 3 (7)	51 ± 2 (8)	17 ± 5 (7)
d. 199E + Epi	T.C. plastic	13 ± 1 (3)	48 ± 5 (6)	67 ± 2 (3)	67 ± 3 (3)	61 ± 3 (9)
e. 199E + HS	T.C. plastic	62 ± 2 (4)	59 ± 2 (5)	59 ± 2 (4)	62 ± 5 (4)	51 ± 2 (4)
f. 199E + C_{IV}	T.C. plastic	49 ± 2 (4)	46 ± 5 (8)	50 ± 3 (8)	47 ± 2 (9)	63 ± 4 (4)
g. 199E + Lam	T.C. plastic	18 ± 4 (4)	52 ± 4 (6)	35 ± 1 (3)	42 ± 5 (4)	26 ± 1 (3)
		Cycloheximide				
h. 199E + FN	T.C. plastic	45 ± 3 (7)	46 ± 4 (7)	49 ± 4 (5)	38 ± 7 (6)	8 ± 3 (7)
i. 199E + Epi	T.C. plastic	0 ± 0 (6)	25 ± 12 (6)	51 ± 2 (7)	58 ± 3 (7)	60 ± 3 (6)
j. 199E + HS	T.C. plastic	58 ± 2 (3)	66 ± 2 (3)	65 ± 1 (3)	60 ± 1 (3)	70 ± 2 (3)
k. 199E + C_{IV}	T.C. plastic	—	—	—	58 ± 4 (8)	0 ± 0 (4)
l. 199E + Lam	T.C. plastic	—	—	—	48 ± 2 (3)	—

To tissue-culture (*T.C.*) plastic dishes (34 × 10, Costar, Data Packaging, Cambridge, MA) containing 2–3 ml of medium 199E plus 25–50 μg/ml of the specified protein (*BSA*, bovine serum albumin; *FN*, fibronectin; *Epi*, epibolin; *HS*, human serum; C_{IV}, type IV collagen; *Lam*, laminin) in the absence and presence of cycloheximide (50 μg/ml) was added 3 × 10^5 dissociated guinea pig epidermal cells. In general, 10% of added cells were adherent. Values are percent of spread cells per 10^2 attached cells. *n*, number of cells counted × 10^2 taken from more than one experiment.

TABLE II

Spreading of Guinea Pig Epidermal Cells on Various Substrates: Cells Added to Media

Medium	Substrate	Average % spread cells ± 1 SE (n)				
		Control				
		1 h	2 h	3 h	4 h	24 h
a. 199E	BSA	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (1)
b. 199E	FN	32 ± 3 (9)	31 ± 7 (9)	41 ± 6 (11)	24 ± 4 (10)	34 ± 5 (9)
c. 199E	Epi	18 ± 2 (3)	51 ± 2 (4)	62 ± 3 (4)	67 ± 1 (3)	75 ± 3 (3)
d. 199E	Lam plus C_{IV}	37 ± 4 (4)	48 ± 4 (4)	47 ± 1 (3)	47 ± 1 (3)	63 ± 4 (9)
e. 199E	HS	0 ± 0 (6)	0 ± 0 (6)	0 ± 0 (6)	2 ± 2 (6)	2 ± 1 (5)
		Cycloheximide				
f. 199E	FN	22 ± 4 (10)	31 ± 3 (13)	37 ± 5 (14)	28 ± 4 (12)	0 ± 0 (8)
g. 199E	Epi	23 ± 4 (4)	48 ± 1 (3)	70 ± 2 (3)	68 ± 3 (3)	30 ± 5 (4)
h. 199E	Lam plus C_{IV}	44 ± 2 (3)	71 ± 2 (3)	61 ± 5 (6)	61 ± 1 (3)	28 ± 3 (3)
i. 199E	HS	1 ± 1 (7)	6 ± 2 (10)	2 ± 1 (6)	1 ± 1 (3)	1 ± 1 (3)

Using bacteriological plastic Petri dishes (34 × 10, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) a volume of solution containing 50 μg/dish of protein was air-dried under ultraviolet radiation. The dried dishes were rinsed with 3 × 3 ml of sterile distilled H_2O. The experiments were performed and recorded as in Table I.

TABLE III

Spreading of Guinea Pig Epidermal Cells on Various Substrates in Various Media: Cells Added to Media

Protein in medium	Substrate	% Spread cells at 4 h
		Ave. ± 1 SE (n)
a. FN	BSA	0 ± 0 (6)
b. Epi	BSA	0 ± 0 (6)
c. Antiepi	Epi	0 ± 0 (7)
d. Anti-FN	Epi	56 ± 5 (4)
e. Antilam	Epi	62 ± 2 (3)
f. Anti-C_{IV}	Epi	62 ± 2 (3)
g. Anti-C_V	Epi	67 ± 1 (3)
h. no protein	FN	20 ± 3 (5)
i. Anti-FN	FN	0 ± 0 (3)
j. Antiepi	FN	16 ± 3 (4)
k. no protein	Lam plus C_{IV}	55 ± 4 (5)
l. Anti-C_{IV}	Lam plus C_{IV}	62 ± 4 (4)
m. Antilam	Lam plus C_{IV}	42 ± 7 (5)
n. Antilam plus Anti-C_{IV}	Lam plus C_{IV}	31 ± 6 (5)
o. Antiepi	Lam plus C_{IV}	45 ± 2 (4)
p. Anti-FN	Lam plus C_{IV}	59 ± 3 (5)

The substrates of the plastic dishes were prepared as in Table II (50 μg protein/dish) except for the laminin-Type IV collagen dishes, which contained 10 μg/dish. Cells were added to the dishes after the specified media had incubated in the coated dishes at room temperature for 15 min. *FN*, fibronectin, 50 μg/ml; *Epi*, epibolin, 50 μg/ml; *antiepi*, antiepibolin 900 μg/ml; *anti-FN*, antifibronectin 100 μg/ml; antilam, antilaminin 100 μg/ml; *anti-*C_{IV}, anti-type IV collagen 100 μg/ml; *anti-*C_{IV}, anti-type V collagen 100 μg/ml.

these experiments cell spreading was independent of protein synthesis.

Epidermal Cell Spreading on Protein-Coated Dishes in Media Containing Antibodies to the Active Proteins

In Table II(*c*) it is shown that epibolin-coated dishes supported epidermal cell spreading in defined medium; however, if antiepibolin antibody was added to the medium before the cells, no cell spreading was seen (Table III: *c*). In contrast, on epibolin-coated dishes antibody to FN, laminin, Type IV collagen, or Type V collagen did not prevent spreading (Table III: *d*, *e*, *f*, and *g*). Similarly, whereas cells spread on FN-coated dishes in defined medium (Table III: *h*), they did not spread in the presence of antiFN (Table III: *i*). Spreading on FN was unaffected by antiepibolin (Table III: *j*). Spreading on laminin-Type IV collagen dishes appeared more complex because neither antibody (Table III: *l* and *m*) acting alone or in concert (Table III: *n*) eliminated spreading. The extent of spreading in the presence of both antibodies, however, was significantly reduced (compare Table III: *k* and Table III: *n* where $P < 0.05$ by the Student's *t* test). This result may be due to the possibility that many more cell adhesion sites are present on the laminin-Type IV collagen surface than our antibodies recognize. Antiepibolin and anti-FN also did not interfere significantly with epidermal cell spreading on laminin-Type IV collagen-coated dishes (Table III: *o* and *p*).

Spreading of Epidermal Cells Adherent to Noncoated Plastic Dishes in Protein-containing Media

In contrast to the previous studies, in these experiments the epidermal cells were allowed to stick to the untreated tissue culture plastic dish for 2 h in defined medium before adding the spreading proteins. The requirements for spreading here were surprisingly different from those of the above experiments in which the cells were added to the media. Under these conditions, none of the proteins supported significant spreading except for plasma, serum (not shown), and epibolin (Table IV). Moreover, under these conditions, the antibody to epibolin blocked the spreading activity of plasma (Table V: *b* and *c*) and serum (not shown). Evidence that the antibody preparation was not nonspecifically toxic to the cells is shown in Table V: *e* where higher concentrations of plasma and epibolin (not shown) in the medium neutralized the inhibition. These results suggest that the important spreading protein in whole plasma (for epidermal cells under these conditions) is epibolin.

DISCUSSION

It was our intention in these studies to test the possibility that epidermal cells use a common basement membrane protein whenever they spread. Our results indicate not only that spreading during the first 24 h in culture is independent of protein synthesis, but that several different proteins will support

TABLE IV

Guinea Pig Epidermal Cell Spreading—Medium Added to Cells

Medium	Substratum	% Spread cells, average ± 1 SE (n)				
		Control				
		1 h	2 h	3 h	4 h	24 h
a. FN	T.C. plastic	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (9)
b. Epi	T.C. plastic	5 ± 3 (7)	52 ± 2 (7)	60 ± 2 (7)	59 ± 2 (8)	54 ± 4 (9)
c. Lam	T.C. plastic	0 ± 0 (6)	0 ± 0 (6)	3 ± 2 (8)	2 ± 2 (7)	0 ± 0 (3)
d. C_{IV} plus C_V	T.C. plastic	0 ± 0 (7)	0 ± 0 (3)	0 ± 0 (7)	2 ± 1 (8)	—
		Cycloheximide				
e. FN	T.C. plastic	0 ± 0 (4)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (6)
f. Epi	T.C. plastic	2 ± 1 (6)	30 ± 9 (7)	46 ± 5 (9)	54 ± 3 (8)	58 ± 3 (6)
g. Lam	T.C. plastic	0 ± 0 (3)	2 ± 2 (4)	1 ± 1 (4)	1 ± 0 (4)	0 ± 0 (3)
h. C_{IV} plus C_V	T.C. plastic	0 ± 0 (4)	0 ± 0 (4)	0 ± 0 (4)	0 ± 0 (7)	—

These experiments were performed by adding 3×10^5 dissociated guinea pig epidermal cells to tissue culture (*T.C.*) plastic dishes containing 2–3 ml of defined medium (*199E*). After 2-h incubation, the defined medium was discarded with loose cells, and the test medium was added. Cell spreading was followed over the indicated time period. The reults are recorded here as in Table I.

TABLE V

Antibody to Epibolin Blocks the Spreading Activity of Whole Plasma: Media Added to Cells

Medium	% Spread cells at 4 h
	(Ave. ± 1 SE) (n)
a. 199E + HP (1:500)	50 ± 2 (8)
b. 199E + HP (1:500) + antiepi (1:10)	0 ± 0 (8)
c. 199E + HP (1:500) + antiepi (1:100)	13 ± 2 (9)
d. 199E + HP (1:500) + antiepi (1:1000)	46 ± 2 (12)
e. 199E + HP (1:50) + antiepi (1:10)	60 ± 5 (4)

HP, human plasma (dilution); *antiepi*, antiepibolin (dilution). Experiments were performed and recorded as in Table IV.

maximal spreading and that blocking antibodies to no one of these proteins will affect spreading on the others. These results suggest that there are multiple modes of epidermal cell spreading, a conclusion made previously by other workers studying other cell types (23–27). Indeed, the FN-laminin studies of Johansson et al. (25) on hepatocytes directly corroborate our work with epidermal cells (their Fig. 6, our Table III). Although it is reasonable to conclude, as the latter authors did, that the different spreading proteins utilize distinct molecular regions and different cellular receptors, our studies are more phenomenological and do not elucidate this issue.

That dissociated epidermal cells do not require protein synthesis in the early stages of in vitro spreading may not be relevant to other cell types and to other stages and forms of cell spreading. Indeed, primary cell cultures—as used here—may have very different spreading properties than culture-adapted cells. In fact, these results contrast with our earlier studies (28), which suggest that epidermal cell sheet movement over several days requires protein synthesis and, more specifically, proline-rich protein synthesis. We believe that the phenomenon of dissociated cellular and cell sheet motility may be different. In neither case do we know what the actual adhesion and spreading mechanisms are, though these processes are clearly mediated by proteins of the media and substrate. Because the dissociated cells do not require protein synthesis in order to spread, those added proteins that support spreading would appear either to induce the liberation of nonprotein cellular products or to nonspecifically stimulate a metabolic process that establishes the spreading mode.

Whereas it is generally agreed that FN does not enhance the adhesion of epidermal (epithelial) cells to collagen (3, 5), there have been conflicting reports with regard to the role of FN in epidermal cell spreading on plastic. Although some workers observed that FN supports epithelial cell spreading (27, 29, 30), others have not (12). This discrepancy is elucidated by the studies reported here. If the cells were added to FN-coated, or FN-containing dishes, they spread; on the other hand, if the cells were first allowed to adhere to the dish, as they were in the early conflicting study (12), subsequent exposure to FN did not support cell spreading. In our experience, heretofore, only epibolin-containing media permitted significant epidermal cell spreading under the latter conditions. How epibolin acts differently from the other molecules studied is the focus of current investigation.

In the three assays we used here, there are crucial distinctions in how the cells were allowed to adhere. In one case, the cells adhered to a plastic substrate coated with the proteins; in the other case, the cells adhered to an uncoated plastic substrate. In the former case, the cells adhered to molecules upon which they were able to spread rapidly. In the latter case, the cells adhered to a surface that did not support spreading and would not spread unless exposed to a very specific serum protein. Observing two adhesion-to-spreading modalities in vitro begs the in vivo question. Because we do not know precisely upon what molecules within the basement membrane zone epidermal cells rest, we do not know which modality is relevant to the epithelial cells in situ. Do epidermal cells sit on the basement membrane in a spreading or nonspreading modality? Resolving such a question is crucial to understanding the initial mechanisms of epidermal cell movement in wound closure.

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