

Expression of Functional Domains of Beta_G-Spectrin Disrupts Epithelial Morphology in Cultured Cells

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Abstract. Spectrin is a major structural protein associated with the cytoplasmic surface of plasma membranes of many types of cells. To study the functions of spectrin, we transfected Caco-2 intestinal epithelial cells with a plasmid conferring neomycin resistance and encoding either actin-binding or ankyrin-binding domains of beta_G-spectrin fused with beta-galactosidase. These polypeptides, in principle, could interfere with the interaction of spectrin with actin or ankyrin, as well as block normal assembly of alpha- and beta-spectrin subunits. Cells expressing the fusion proteins represented only a small fraction of neomycin-resistant cells, but they could be detected based on expression of beta-galactosidase. Cells expressing spectrin domains exhibited a progressive decrease in amounts of endogenous beta_G-spectrin, although alpha-spectrin was still present. Beta_G-spectrin-deficient cells lost

epithelial cell morphology, became multinucleated, and eventually disappeared after 10–14 d in culture. Spectrin-associated membrane proteins, ankyrin and adducin, as well as the Na⁺,K⁺-ATPase, which binds to ankyrin, exhibited altered distributions in cells transfected with beta_G-spectrin domains. E-cadherin and F-actin, in contrast to ankyrin, adducin, and the Na⁺,K⁺-ATPase, were expressed, and they exhibited unaltered distribution in beta_G-spectrin-deficient cells. Cells transfected with the same plasmid encoding beta-galactosidase alone survived in culture as the major population of neomycin-resistant cells, and they exhibited no change in morphology or in the distribution of spectrin-associated membrane proteins. These results establish that beta_G-spectrin is essential for the normal morphology of epithelial cells, as well as for their maintenance in monolayer culture.

SPECTRIN is an elongated actin-binding protein that is the principal component of a system of structural proteins associated with the cytoplasmic surface of plasma membranes of most metazoan cells (reviewed by Bennett and Gilligan, 1993). Spectrin is comprised of two subunits, termed alpha and beta, that are aligned side-to-side to form heterodimers, and the dimers are linked head-to-head to form tetramers. Beta subunits contain most of the recognition sites of spectrin for other proteins including ankyrin, protein 4.1 actin, as well as the site for ankyrin-independent association of spectrin with membranes. Beta_G-spectrin is the most common type of beta subunit, and it is expressed in most vertebrate tissues (Hu et al., 1992). The structure and function of spectrin has been best characterized in mammalian erythrocytes from both in vivo and in vitro studies (Palek and Lambert, 1990; Delaunay and Dhermy, 1993; Gallagher and Forget, 1993; Bennett and Gilligan, 1993). Erythrocyte spectrin forms a membrane-associated polygonal network by the associations of spectrin with actin filaments and with integral membrane proteins through linkages

with ankyrin and protein 4.1. Defects and deficiencies in components of the spectrin-actin network result in abnormally fragile erythrocytes in humans and mice, suggesting that one function of spectrin is to physically stabilize the phospholipid bilayer.

Structures similar to the spectrin-based membrane skeleton of erythrocytes are likely to exist in other tissues. Genes encoding proteins closely related to erythrocyte spectrin and spectrin-associated proteins are expressed in brain, as well as most other tissues (reviewed by Bennett and Gilligan, 1993). In vitro studies also show that tissue spectrins can interact with isoforms of spectrin-associated proteins including ankyrin (Davis and Bennett, 1984) and adducin (Bennett et al., 1988). However, the organization of spectrin-based membrane structures and their cellular roles are likely to be more complex in nonerythroid tissues. Major differences between the spectrin-based membrane skeleton of erythrocytes and that of more complex cells include the presence of multiple isoforms of spectrin and associated proteins caused by alternate exon usage and distinct genes, additional proteins associating with spectrin that do not occur in erythrocytes, and localization of spectrin in areas in addition to the plasma membrane.

A candidate function of spectrin and associated proteins,

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inferred from their localization in tissues and association with proteins in *in vitro* assays, is in assembly and/or maintenance of specialized domains on the cell surface. Sites of cell-cell contact in epithelial tissues are the best-characterized example of a role of membrane skeletal proteins in a membrane domain (reviewed in Nelson, 1992; Nelson et al., 1991). Direct evaluation of functions of spectrin in non-erythroid cells has been approached by several methods. Microinjection of fibroblasts with antibodies against spectrin induced the formation of spectrin aggregates as well as the condensation of intermediate filaments, but had little effect on cell shape, actin filaments, or microtubules (Mangeat and Burridge, 1984). In contrast, properties of alpha-spectrin mutants in *Drosophila* demonstrate that alpha-spectrin is essential for larval survival and development, and furthermore, that spectrin plays essential roles in maintenance of cell shape and cell-cell interactions (Lee et al., 1993).

To further understand the functions of spectrin, we have expressed functional domains of beta_G-spectrin in cultured epithelial cells with the expectation that these domains would interfere with the activity of endogenous beta-spectrin. Results of these experiments demonstrate that spectrin plays an essential role in maintenance of epithelial cell morphology in culture.

Materials and Methods

Reagents

Affinity-purified antibodies against brain adducin, Na⁺,K⁺-ATPase, erythrocyte ankyrin, and beta_G spectrin (repeats 4-9) were prepared from rabbit antisera as described (Davis and Bennett, 1984). Antibody against alpha-spectrin was kindly provided by Dr. Velia Fowler (Scripps Research Institute, La Jolla, CA). Monoclonal antibody against beta-galactosidase was from Promega Corp. (Madison, WI). Polyclonal antibody against beta-galactosidase was from 5 Prime-3 Prime, Inc. (Boulder, CO). Monoclonal antibody against E-cadherin was from Sigma Immunochemicals (St. Louis, MO). Fluorescein- and rhodamine-conjugated secondary antibodies were from Pierce Chemical Co. (Rockford, IL). Fluorescein-labeled phalloidin was obtained from Molecular Probes (Eugene, OR). Restriction enzymes were from New England Biolabs Ltd. (Mississauga, Ontario). Reagents for lipofectin transfection were from Gibco BRL (Gaithersburg, MD), and those for immunoblot staining were from Bio Rad Laboratories (Hercules, CA).

Plasmid Construction

DNA encoding actin- and ankyrin-binding domains of beta_G-spectrin was inserted into a mammalian expression vector containing a beta-actin promoter, and conferring neomycin resistance (pHbetaApr-1 neo-CB-17). The pHbetaApr-1 neo-CB-17 vector was kindly provided by Dr. Elwood Linney (Duke University) (Espeseth et al., 1989). DNA fragments encoding the actin-binding domain (amino acids 1-396) and ankyrin-binding domain (amino acids 1661-1990) of beta_G-spectrin were made from beta_G spectrin cDNA (Hu et al., 1992) by a PCR reaction using primers to introduce a HindIII restriction site and an ATG initiation site at the 5' end of the DNA sequence, and BamHI site at the 3' end. HindIII- and BamHI-restricted DNA fragments were inserted into the pHbetaApr-1 neo-CB 17 mammalian expression vector. The BamHI site was made in-frame with the beta-galactosidase gene to produce a COOH-terminal fusion protein. For a control plasmid, an ATG initiation site was inserted in the pHbetaApr-1 neo-CB-17 vector such that beta-galactosidase was expressed by itself in cells. Beta-galactosidase fusion proteins were detected either by immunofluorescence of fixed and permeabilized cells or by enzymatic activity of living cells. The DNA sequence of various constructs was confirmed by direct sequence analysis.

Mammalian Cell Culture

Human Caco-2 intestinal epithelial cells were grown in MEM medium, supplemented with 20% fetal bovine serum, MEM nonessential amino acids, and penicillin/streptomycin. Medium was changed every 3 d, and the cells were replated when they grew to confluency. G418 (0.5 mg/ml) was added to select transfected cells 48 h after transfection, and fresh G418 medium was added every 3-4 d.

Immunofluorescence Staining and Detection of Beta-Galactosidase Activity

Caco-2 cells were grown on 1-ml glass slide chamber coated with 1 mg/ml of poly-D-lysine. The cells were transfected by lipofectin as described in the Gibco protocol, washed three times with PBS, and fixed with 3% paraformaldehyde/PBS at 4°C for 30 min. In some experiments, cells were stained for beta-galactosidase activity before fixation, and transfected cells were detected as described (Espeseth et al., 1989).

After 3% paraformaldehyde/PBS fixation, cells were extracted with 0.25% Triton X-100 in PBS for 5 min at room temperature. The cells were then incubated in 1% BSA, 10% normal goat serum, and 0.1% Tween-20 in PBS at room temperature for 15 min to block nonspecific binding. 1 μg/ml of monoclonal anti-beta-galactosidase antibody was added to the blocking buffer and further incubated overnight at 4°C. After washing thoroughly with PBS, fluorescein- or rhodamine-labeled goat anti-mouse secondary antibodies in blocking buffer were added to label the primary antibody. Cells were then washed five times with PBS and mounted with 5 mg/ml *n*-propyl gallate in 30% glycerol. Double immunofluorescence was performed to determine the localization in cells producing beta-galactosidase fusion proteins, endogenous spectrin, and spectrin-associated proteins, such as ankyrin, adducin, and Na⁺,K⁺-ATPase. The beta_G spectrin antibody was directed against repeats 4-9, and they did not recognize either the actin or ankyrin-binding domains expressed by transfected cells. The same protocol was used as described above, but antibody incubations were performed by mixing antibodies from rabbit with antibodies from mouse. A mixture of rhodamine-labeled goat anti-rabbit antibody and fluorescein-labeled goat anti-mouse antibody was used to visualize the antigens. Double immunofluorescence for colocalizing beta-galactosidase expression and E-cadherin was performed stepwise to decrease the cross-reaction of the goat anti-mouse secondary antibody with rat monoclonal anti-E-cadherin antibody. Cells were first incubated with mouse monoclonal anti-beta-galactosidase antibody, followed by rhodamine-labeled goat anti-mouse antibody. After washing thoroughly with PBS buffer, the cells were incubated with rat monoclonal anti-E-cadherin antibody, followed by the incubation with fluorescein-labeled goat anti-rat antibody. The stained slides were then examined by epifluorescence using a Bio Rad confocal microscope.

4',6'-diaminophenylindole (DAPI)¹ was used to visualize cell nuclei by fluorescence microscopy. Cells were stained with rhodamine-conjugated anti-beta-galactosidase antibody and goat anti-mouse antibody, as described above. DAPI in PBS buffer was then added into the glass chamber and incubated for 5 min at room temperature.

Immunoblotting Studies

Cell extracts of transfected Caco-2 cells were obtained by solubilizing cells in SDS-electrophoresis buffer. The cell extracts were passed through a 23-gauge needle several times to shear DNA, heated for 15 min at 65°C, electrophoresed on SDS-polyacrylamide gels, and transferred by electrophoresis onto nitrocellulose paper (Davis and Bennett, 1983). After blotting, the paper was incubated in TTBS blocking buffer: 20 mM Tris HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20, and 3% nonfat dry milk for 20 min at room temperature. Monoclonal anti-beta-galactosidase antibody was added to the buffer and incubated with the paper for overnight at 4°C. After washing with TTBS buffer, goat anti-mouse antibody conjugated with alkaline phosphatase was used to label the anti-beta-galactosidase antibody on the nitrocellulose paper. The color on the paper was developed as described in the Bio Rad protocol.

1. Abbreviation used in this paper: DAPI, 4',6'-diaminophenylindole.

Results

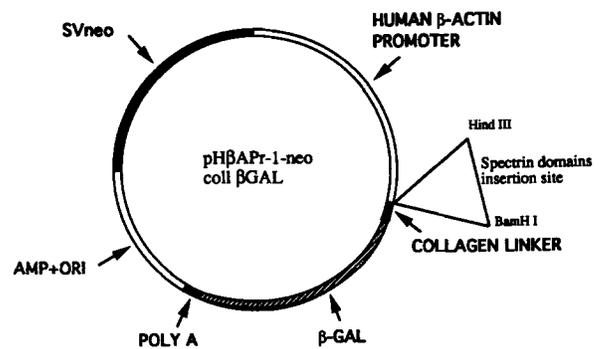
Strategies for Evaluation of Beta_G-Spectrin Function in Cultured Cells

Experiments were designed to disrupt endogenous spectrin function by either using antisense DNA or expression of spectrin domains. A mammalian expression vector containing antisense sequence of beta_G-spectrin under control of a cytomegalovirus promoter and conferring neomycin resistance was transfected into epithelial cell lines. After G418 selection, permanent clones of transfected cells were obtained. The cells lost their normal epithelial morphology, and they eventually disappeared from culture (data not shown). Unfortunately, because of loss of cells, it was difficult to study the specific function of spectrin by the method of antisense expression. Therefore, we chose the approach of interfering with endogenous spectrin by expression of truncated forms of beta_G-spectrin as fusion proteins with beta-galactosidase. It was possible to follow the transfected cells using beta-galactosidase as a marker at early stages and to characterize individual cells by immunofluorescence and morphology.

Constructs encompassing two important protein-binding domains of beta_G-spectrin, the actin- and ankyrin-binding domains, were expressed in Caco-2 epithelial cells. The binding site of beta_G-spectrin for ankyrin is localized to repeat 15 (Kennedy et al., 1991), and the site for actin is located in the NH₂-terminal domain from Ala47 to Lys186 (Karinich et al., 190). Constructs used in this study extend from residues 1-396 and 1661-1990, and they are referred to as the actin- and ankyrin-binding domains of beta_G-spectrin, respectively. The expression vector conferred neomycin resistance, and it contained a beta-actin promoter (Fig. 1 A). Beta_G-spectrin domains were linked to the 5' end of truncated beta-galactosidase in the vector (see Materials and Methods). For control experiments, an ATG initiation site was introduced at the 5' end of the beta-galactosidase gene (Materials and Methods) (Fig. 1 B, a).

Growth of cells expressing beta-galactosidase was determined during G418 selection, using X-gal as a chromogenic substrate (Fig. 2 A). For the control plasmid, >95% of the surviving cells were neomycin resistant and beta-galactosidase positive (Fig. 2 A). However, <5% of cells transfected with beta_G-spectrin actin-binding domain, and 40% of cells transfected with ankyrin-binding domain expressed beta-galactosidase after 15 d of G418 selection (Fig. 2 A). These data suggested that expression of actin- and ankyrin-binding domains of beta_G spectrin resulted in selective advantage for those neomycin-resistant cells that did not express the fusion proteins. Cells expressing beta_G-spectrin domains may die during culture, have a reduced rate of cell division, and/or detach from the epithelial monolayer as a result of loss of cell-cell contacts (see below). Cells transfected with ankyrin-binding domain had a higher percentage of blue-stained cells than cells transfected with actin-binding domain, suggesting that expression of actin-binding domain was more disruptive than expression of the ankyrin-binding domain. The effects of beta_G-spectrin actin-binding domain were not caused by a generalized competition for other actin-binding proteins since the ankyrin-binding domain yielded

A



B Expressed fusion proteins

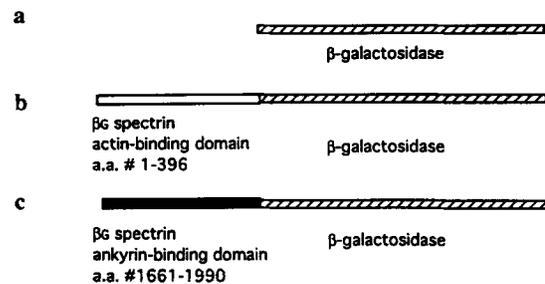


Figure 1. Schematic model of mammalian expression plasmid used for transfection experiments. (A) Mammalian expression vector, pHbetaApr-1-neo coll bGAL (pHbetaAPr-1-neo-CB-17), contains unique restriction sites for HindIII and BamHI, which are used as insertion sites for beta_G-spectrin-domains. (B) Expressed fusion proteins in mammalian cells after insertion of initiation site or beta_G-spectrin-domains. a, ATG initiation codon; b, actin-binding domain of beta_G-spectrin; c, ankyrin-binding domain of beta_G-spectrin.

qualitatively similar results, and since F-actin morphology was unperturbed (see the next section). Moreover, perturbation of the cells was not caused by the transfection procedure or to beta-galactosidase itself because the cells transfected with control plasmid were still normal and >95% of the cells still expressed beta-galactosidase.

To determine if the constructs expressed the correct proteins, immunoblot analysis of transfected cells was performed using antibody against beta-galactosidase to detect the fusion proteins. Proteins of 100, 143, and 136 kD corresponding to predicted sizes of beta-galactosidase alone, beta-galactosidase plus beta_G-spectrin actin-binding domain (amino acids 1-396), and beta-galactosidase plus beta_G-spectrin ankyrin-binding domain (amino acids 1661-1990) were detected in the extracts of cells transfected with plasmids encoding control, actin-binding domain, and ankyrin-binding domain, respectively (Fig. 2 B, lanes 1-3). These results indicate that the fusion proteins were expressed, at least in a subpopulation of cells. The relative amount of expressed fusion proteins in transfected cells correlated well with the percentage of blue-stained cells (Fig. 2 B). Express-

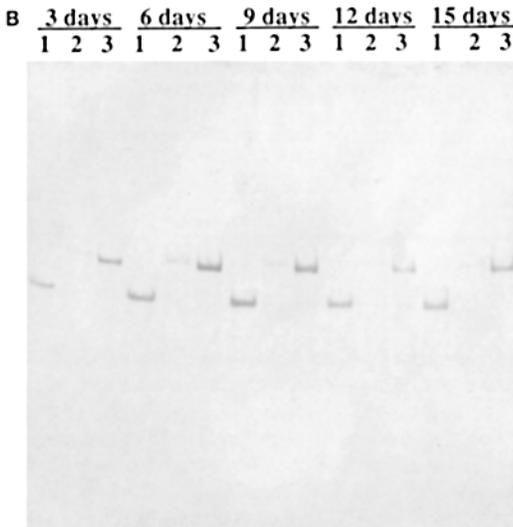
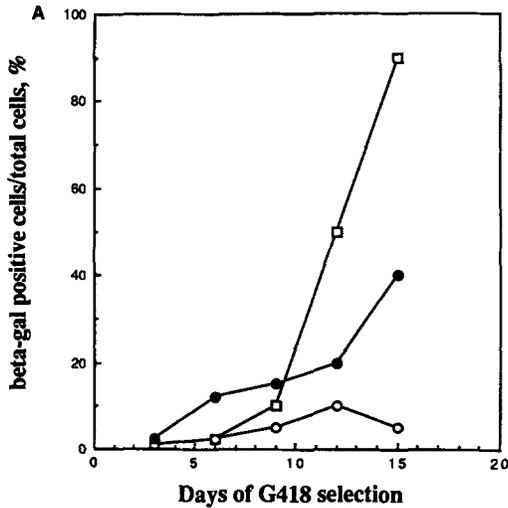


Figure 2. Caco-2 cells expressing the actin- or ankyrin-binding domain of β _G-spectrin disappear from monolayer culture during G418 selection. Caco-2 cells were transfected with vectors encoding beta-galactosidase, beta-galactosidase fused with actin-binding domain, or ankyrin-binding domain of β _G-spectrin. (A) Transfected Caco-2 cells were fixed and evaluated for beta-galactosidase enzyme activity using X-gal as a chromogenic substrate. The percentage of blue-stained cells is plotted as a function of time of G418 selection. Caco-2 cells transfected with beta-galactosidase alone (□), actin-binding domain of β _G-spectrin-beta-galactosidase fusion protein (○), and ankyrin-binding domain of β _G-spectrin-beta-galactosidase fusion protein (●) were compared. (B) Immunoblot analysis of transfected cell extracts after 3, 6, 9, 12, and 15 d of G418 selection. A monoclonal antibody against beta galactosidase was used to stain the expressed fusion proteins. Lane 1, Caco-2 cells expressing beta-galactosidase alone; lane 2, cells expressing actin-binding domain of β _G-spectrin-beta-galactosi-

dase fusion protein; lane 3, cells expressing ankyrin-binding domain of β _G-spectrin-beta-galactosidase fusion protein. The fact that the ankyrin-binding domain fusion proteins were expressed at higher levels than the other constructs at the beginning of the experiment may be caused by a higher transfection efficiency for this construct. 100–200 cells were counted for each construct and each time point.

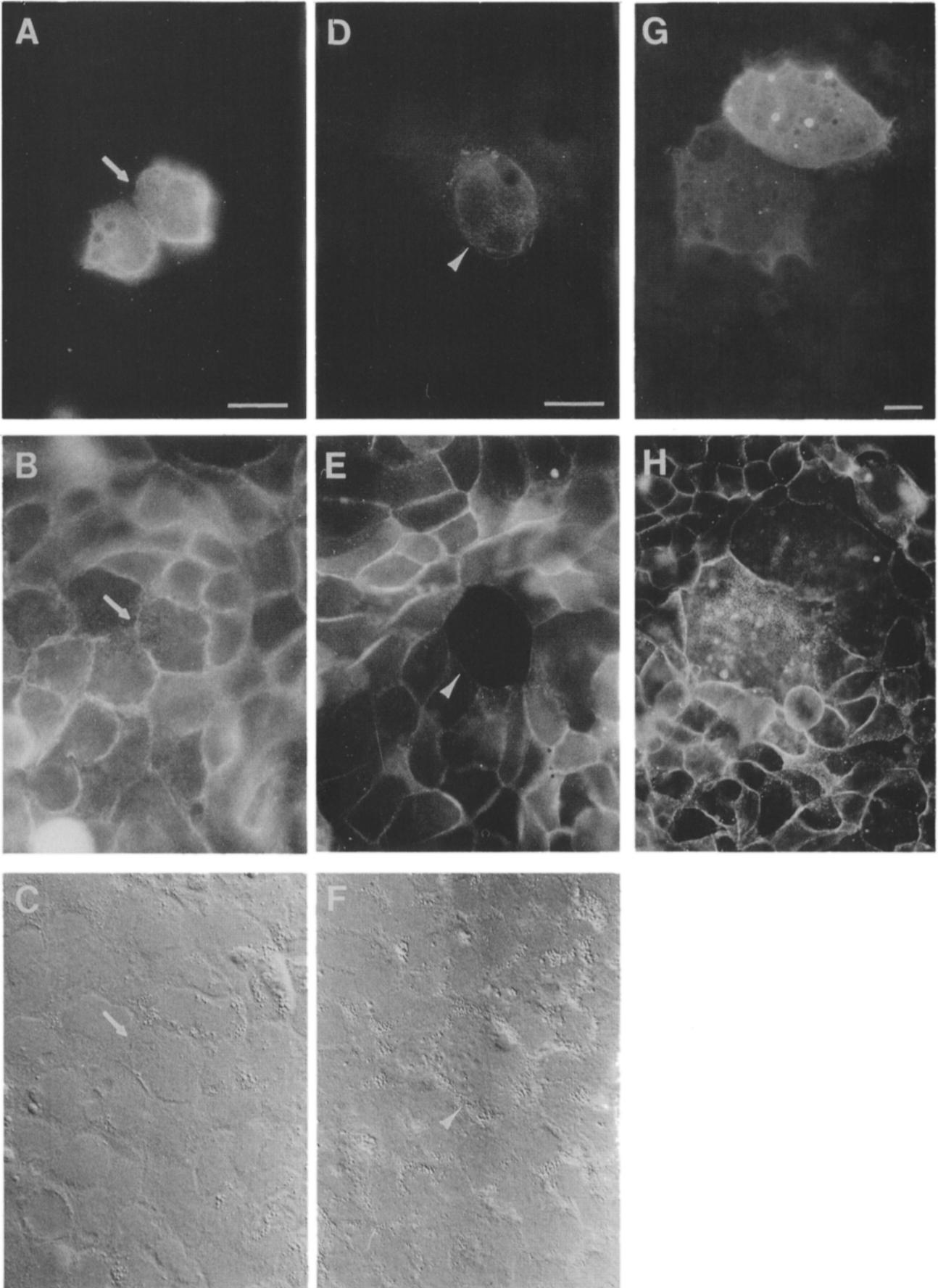
Expression of Domains of β _G-Spectrin in Caco-2 Cells Results in Decreased Levels of Endogenous β _G Spectrin

Immunofluorescence double-staining with antibodies against beta-galactosidase and spectrin was used to examine effects of actin-binding domain or ankyrin-binding domain of β _G-spectrin on levels and pattern of distribution of endogenous spectrin. Endogenous β _G-spectrin was visualized using an antibody that specifically recognizes repeats 4–9 of β _G-spectrin, but not the actin-binding domain or ankyrin-binding domain. After 3 d of G418 selection, transfected cells were still viable and surrounded by untransfected normal epithelial cells. Cells transfected with the control plasmid still retain a normal epithelial morphology and anti- β _G-spectrin antibodies stained both around cell-cell junctions and on the cell surface (Fig. 3 A–C, arrows show the same cells). Cells transfected with actin-binding domain lost β _G-spectrin staining on the cell surface, and the staining at cell junctions was very weak (Fig. 3 D–F, arrowheads show the same cell). Therefore, expression of the actin-binding domain of β _G-spectrin in cells reduced levels of endogenous β _G-spectrin. Similar results were obtained for the cells transfected with the ankyrin-binding domain of β _G-spectrin. Endogenous β _G-spectrin was either displaced or greatly reduced, depending on the amount of fusion protein expressed in the cells. As shown in Fig. 3, G and H, the cell containing more fusion protein had less endogenous spectrin. A punctate staining of spectrin was occasionally observed, suggesting the possibility that the endogenous spectrin that is expressed is present in cytoplasmic structures instead of in the plasma membrane.

Alpha-spectrin, in contrast to the β _G subunit, was

dase fusion protein; lane 3, cells expressing ankyrin-binding domain of β _G-spectrin-beta-galactosidase fusion protein. The fact that the ankyrin-binding domain fusion proteins were expressed at higher levels than the other constructs at the beginning of the experiment may be caused by a higher transfection efficiency for this construct. 100–200 cells were counted for each construct and each time point.

Figure 3. Expression of β _G-spectrin domains decreases the amount of endogenous β _G-spectrin in transfected Caco-2 cells. Transfected cells double stained with mouse monoclonal antibody against beta-galactosidase (A, D, and G) and rabbit polyclonal Ig against β _G-spectrin, repeats 4–9 (B, E, and H). Fluorescein-labeled goat anti-mouse and rhodamine-labeled goat anti-rabbit secondary antibodies were used to detect the primary antibodies. The spectrin antibody can recognize endogenous β _G-spectrin, but not the actin- and ankyrin-binding domains of β _G-spectrin. A–C are cells expressing beta-galactosidase alone; D–F are cells expressing actin-binding domain of β _G-spectrin-beta-galactosidase fusion protein; G and H are cells expressing ankyrin-binding domain of β _G-spectrin-beta-galactosidase fusion protein. C and F are DIC micrographs. The apparent reduction in beta-spectrin in some of the nontransfected cells is the result of the focal plane. Bar, 20 μ m.



present at the same levels of intensity in untransfected cells, and in cells transfected with beta-galactosidase alone or with the ankyrin-binding domain-beta-galactosidase fusion protein, as visualized by immunofluorescence (Fig. 4). Alpha-spectrin was evident in the cytoplasm as well as in association with the plasma membrane of transfected cells (Fig. 4).

Beta_G-Spectrin Is Essential for Normal Epithelial Cell Morphology

Caco-2 epithelial cell morphology was disrupted after expression of actin-binding or ankyrin-binding domains of beta_G-spectrin (Fig. 5, C-F). Caco-2 cells still retained the normal morphology as epithelial cells after the cells were transfected with the control plasmid (Fig. 5, A and B). Cells expressing either beta_G-spectrin actin-binding domain or ankyrin-binding domain became enlarged 5-10 times the size of normal epithelial cells, and they contained numerous vacuoles. Cells observed by confocal microscopy frequently overlaid other epithelial cells, and they apparently grew without contact inhibition (data not shown).

Another effect of expression of spectrin domains was that cells became multinucleated. On different days of G418 selection, transfected cells were double stained with anti-beta-galactosidase antibody and a nuclear staining dye, DAPI. The percentage of multinucleated cells in the total cells that expressed fusion protein was counted (Fig. 6). More than 50% of the cells transfected with actin-binding domain or ankyrin-binding domain were multinucleated after 10 d of G418 selection, while <25% of cells transfected with control plasmid contained more than one nucleus. However, there appeared to be no significant difference in the number of pyknotic nuclei in these cells, indicating that the cells were still viable at the time of observation. Therefore, loss of beta_G-spectrin may cause either malfunction of some step in cell division such as cytokinesis, or it may result in cell-cell fusion. Further experiments are required to distinguish these two possibilities.

Localization of Actin, Ankyrin, Adducin, Na⁺,K⁺-ATPase, and E-Cadherin in Cells Expressing the Ankyrin-binding Domain of Beta_G-Spectrin

Spectrin associates with actin in *in vitro* assays through contact with the actin-binding domain of the beta subunit, and spectrin-actin complexes are the basic structural unit of the spectrin-skeleton in erythrocytes (Bennett and Gilligan, 1993). It was, therefore, of interest to determine the effect of beta_G-spectrin deficiency on F-actin localization. The localization of F-actin was evaluated in cells expressing the ankyrin- and actin-binding domains of beta_G-spectrin using fluorescein-labeled phalloidin to identify actin filaments and polyclonal antibody against beta-galactosidase to identify the cells expressing foreign proteins (Fig. 7). F-actin was present in stress fibers (Fig. 7, C and F) and in close association with the plasma membrane (Fig. 7, B and E) in cells expressing beta-galactosidase alone or either of the beta_G-spectrin domain fusion proteins. The apparently normal distribution of F-actin in transfected cells is in contrast to their bizarre morphology and lack of beta_G-spectrin. These results suggest that proteins other than beta_G-spectrin are responsible for targeting of F-actin to the plasma membrane, and that the altered cell morphology in beta_G-spectrin-

deficient cells is not a consequence of perturbation of the population of actin molecules detected using phalloidin as a probe.

Cells were double stained with anti-beta-galactosidase antibody (Fig. 8, a2-e2) and antibodies against E-cadherin (Fig. 8, a1-d1). E-cadherin colocalizes with spectrin, and it coisolates with spectrin and ankyrin in extracts of epithelial cells (Nelson and Hammerton, 1989). Moreover, expression of E-cadherin induces a polarized distribution of spectrin and ankyrin to sites of cell-cell contact (McNeill et al., 1990). E-cadherin was localized at cell-cell junctions of cells expressing the ankyrin-binding domain of beta_G-spectrin, even though these cells had lost other aspects of epithelial morphology (Fig. 8 d1). Similar results were obtained for the cells expressing actin-binding domain fusion protein (data not shown). The normal targeting of E-cadherin to cell-cell junctions suggests that E-cadherin sorts to the plasma membrane by a pathway independent from beta_G-spectrin, and it is consistent with observations that E-cadherin is concentrated at cell-cell contact before appearance of spectrin (McNeill et al., 1993).

Ankyrin and adducin associate with spectrin and are colocalized with spectrin at sites of cell-cell contact in epithelial cells (Nelson and Veshnock, 1987a; Nelson and Hammerton, 1989; Kaiser et al., 1989). Na⁺,K⁺-ATPase interacts with ankyrin in *in vitro* assays, and it is colocalized with spectrin and ankyrin in epithelial cells (Nelson and Veshnock, 1987b; Koob et al., 1988; Morrow et al., 1989). All three antibodies stain normal epithelial cells at sites of cell-cell contact. The cells transfected with control plasmid showed normal morphology of epithelial cells, and a normal distribution of adducin at cell-cell junctions (Fig. 8 e), as well as a normal distribution of ankyrin, Na⁺,K⁺-ATPase, or E-cadherin (data not shown). Cells transfected with ankyrin-binding domain of beta_G-spectrin were stained with antibodies against ankyrin (Fig. 8 a1), Na⁺,K⁺-ATPase (Fig. 8 b1), or adducin (Fig. 8 c1). The distribution of ankyrin, Na⁺,K⁺-ATPase, and adducin was diffuse in the cytoplasm, and was not restricted to cell-cell junctions. Na⁺,K⁺-ATPase and adducin also exhibited punctate staining in the cytoplasm of some of these cells. The altered distribution of ankyrin, adducin, and the Na⁺,K⁺-ATPase is in contrast to relatively normal patterns for alpha-spectrin, F-actin, and E-cadherin.

Discussion

Expression of actin- and ankyrin-binding domains of beta_G-spectrin in cultured epithelial cells was used in this study as an initial step in resolving functions of spectrin *in vivo*. Cells expressing beta_G-spectrin domains exhibited a progressive reduction in levels of endogenous beta_G-spectrin, based on results of immunofluorescence, and they eventually disappeared after 10-14 d in culture. Beta_G-spectrin-deficient cells exhibited dramatic changes in cell morphology, including increased numbers of multinucleated cells, 5-10-fold enlargement of cell size, formation of many vacuoles, and growth on top of the monolayer of normal epithelial cells. The cells continued to express cadherin, F-actin, and alpha-spectrin normally, but they failed to target adducin, ankyrin, and the Na⁺,K⁺-ATPase to their correct subcellular loca-

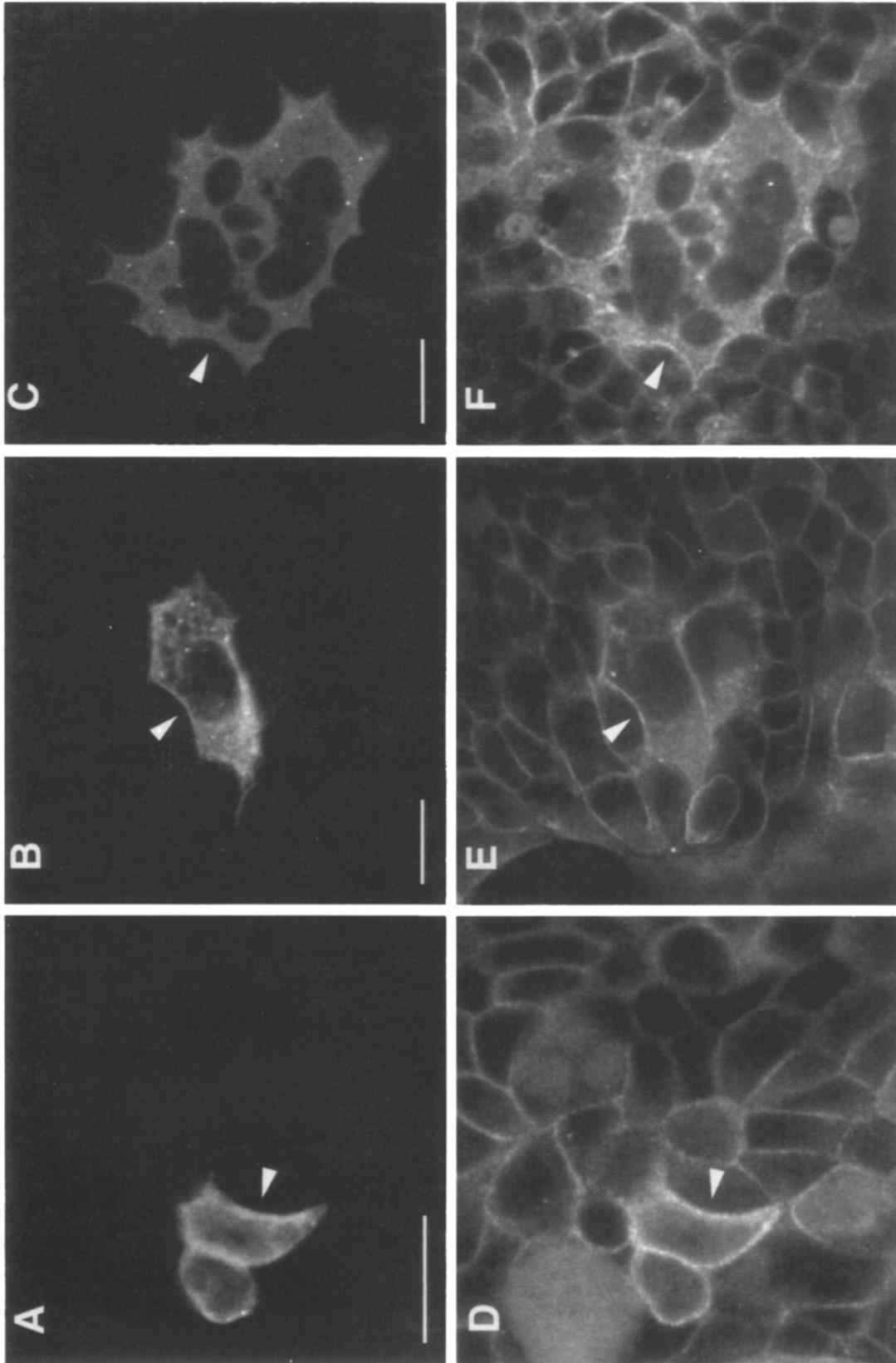


Figure 4. Alpha-spectrin expression persists in Caco-2 cells transfected with beta-spectrin ankyrin-binding domain. Cells were transfected with beta-galactosidase alone (*A* and *D*) or with the ankyrin-binding domain of beta-galactosidase (*B*, *C*, *E* and *F*). Transfected cells were detected using an anti-beta-galactosidase monoclonal antibody followed by fluorescein-conjugated secondary antibody. Alpha-spectrin was stained using a subunit-specific antibody followed by rhodamine-conjugated secondary antibody. Note that alpha-spectrin continues to stain the regions of cell-cell contact, even in cells demonstrating extensive changes in cell morphology (*C* and *F*). Raw confocal images were processed in COMOS, followed by Adobe Photoshop 2.5, and printed using a Tektronix Phaser II SDX Printer. Bars, 25 μ m.

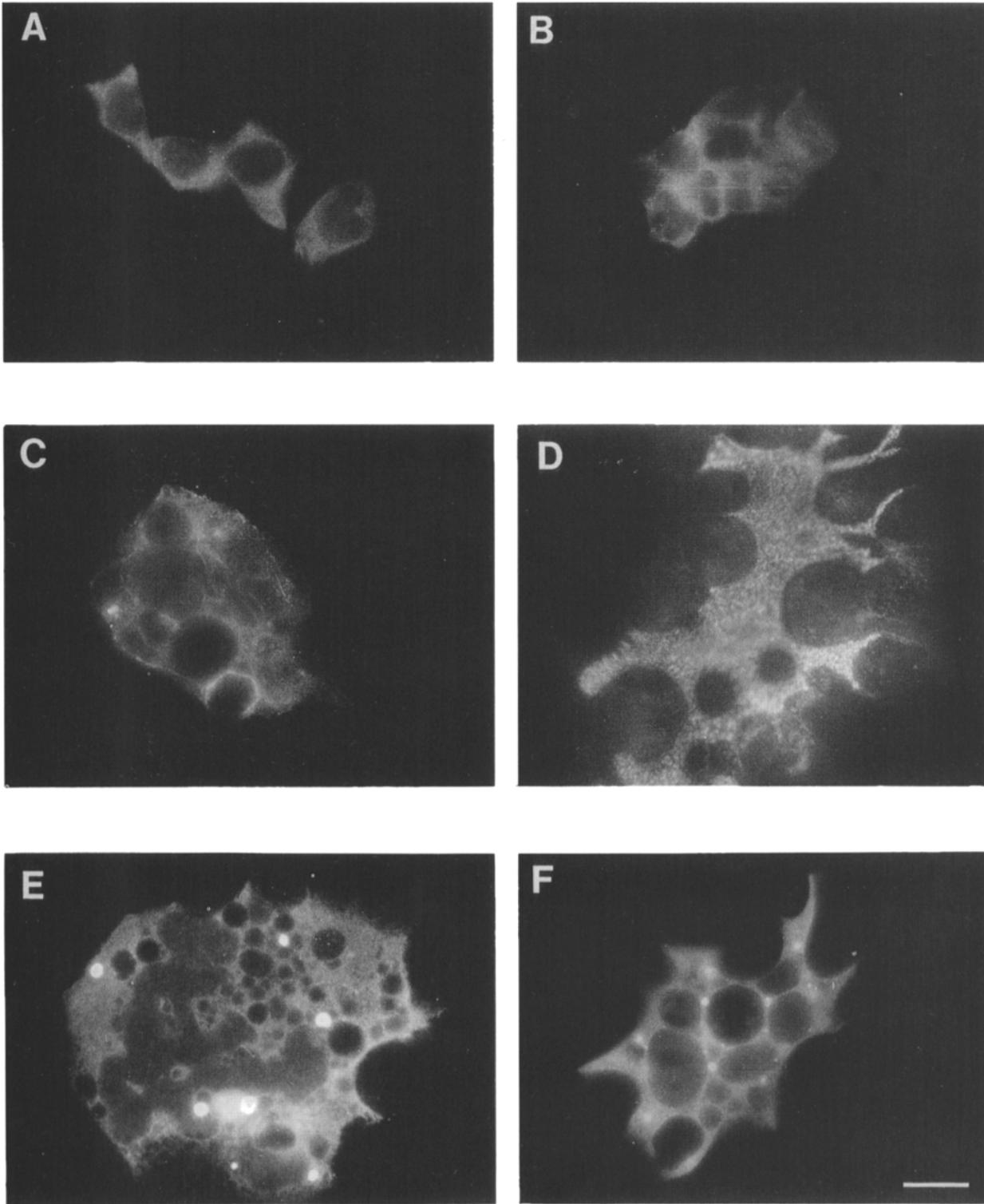


Figure 5. Expression of β -spectrin actin-binding domain and ankyrin-binding domain in Caco-2 cells results in loss of normal epithelial morphology. Transfected cells were stained with monoclonal antibody against beta-galactosidase. (A and B) Cells expressing beta-galactosidase alone. (C and D) Cells expressing actin-binding domain of β -spectrin-beta-galactosidase fusion protein. (E and F) Cells expressing ankyrin-binding domain of β -spectrin-beta-galactosidase fusion protein. Bar, 20 μ m.

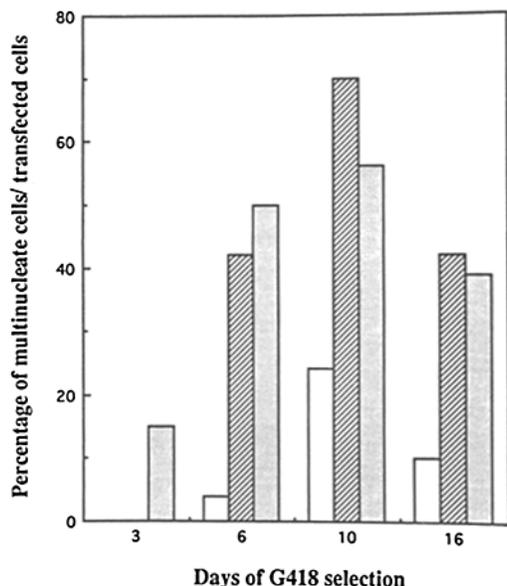


Figure 6. Expression of beta_G-spectrin actin- and ankyrin-binding domains results in an increased percentage of multinucleated Caco-2 cells. Caco-2 cells were cultured in the presence of G418 after transfection, and cells expressing beta-galactosidase and beta-galactosidase fusion proteins were stained with anti-beta-galactosidase monoclonal antibody and detected by rhodamine-labeled goat anti-mouse secondary antibody. Nuclei of cells were visualized by DAPI staining. The percentages of multinucleated cells versus total transfected cells were calculated after 3, 6, 10, and 16 d of G418 selection. *Clear bars*, cells expressing beta-galactosidase alone; *cross-hatched bars*, cells expressing actin-binding domain of beta_G-spectrin-beta-galactosidase fusion protein; *stippled bars*, cells expressing ankyrin-binding domain of beta_G-spectrin-beta-galactosidase fusion protein. 100–180 cells were counted for each time point.

tion. These experiments provide strong evidence that spectrin plays essential roles in maintaining cell morphology in cultured epithelial cells. These conclusions are in contrast to those of an earlier study of spectrin function based on microinjection of antibody against spectrin into fibroblasts (Mangeat and Burrige, 1984), and they could result from the following differences in experimental systems: (a) the present study involved epithelial cells that have a well-defined differentiated phenotype in culture; (b) the time of the perturbation in transfection studies was prolonged over a period of days compared to shorter term observations performed after microinjection; (c) the present study focused on the beta subunit, which may be the more important of spectrin subunits in terms of protein interactions.

The decrease in endogenous beta_G-spectrin in cells expressing domains of beta_G-spectrin was striking, although the molecular basis for the decrease is not known. Conjectures regarding mechanisms for beta-spectrin deficiency include the following possibilities. High levels of beta_G-spectrin domains could activate a normal cellular pathway for downregulating levels of spectrin expression. Alternatively, beta_G-spectrin domains could associate with alpha-spectrin subunits and compete with endogenous beta_G-spectrin for assembly of normal spectrin dimers and tetramers. These possibilities could be resolved by determining the levels of

beta_G-spectrin mRNA and the half-life of beta_G-spectrin polypeptides in transfected cells.

F-actin and E-cadherin were normally distributed in beta_G-spectrin-deficient cells (Figs. 7 and 8), suggesting that these proteins do not require beta_G-spectrin for their targeting. Alpha-spectrin is still expressed, and it exhibits localization at the plasma membrane (Fig. 4). It is not likely that alpha-spectrin would interact with F-actin based on results of *in vitro* assays (Davis and Bennett, 1983), although an interaction with E-cadherin is conceivable. The independent behavior of E-cadherin and beta-spectrin is consistent with observations that E-cadherin becomes localized at sites of cell-cell contact before spectrin (McNeil et al., 1993). Moreover, in the choroid plexus, cadherins are localized at distinct membrane domains from the Na⁺,K⁺-ATPase, as well as a subpopulation of spectrin and ankyrin (Marrs et al., 1993).

Ankyrin, adducin, and the Na⁺,K⁺-ATPase were localized primarily in the cytoplasm in spectrin-deficient cells instead of the plasma membrane at sites of cell-cell contact, as in normal epithelial cells (Fig. 8). A straightforward interpretation of this observation is that lack of spectrin results directly in the absence of these proteins at their normal location on the plasma membrane. In this case, the observation of lack of targeting provides evidence for *in vivo* interactions of spectrin with ankyrin and adducin, and for a requirement of a spectrin skeleton for maintenance of the Na⁺,K⁺-ATPase in a polarized distribution in epithelial cells. These conclusions are consistent with earlier observations of association of ankyrin and adducin with spectrin, and of ankyrin with the Na⁺,K⁺-ATPase in *in vitro* assays, and colocalization of these proteins at sites of cell-cell contacts in epithelial cells (reviewed by Bennett and Gilligan, 1993; Nelson, 1992). However, an important caveat is that beta_G-spectrin-deficient cells were so altered in overall morphology that missorting conceivably also could have resulted from indirect consequences of a major perturbation of the cells. It will be important in the future to design experiments to selectively interfere with individual spectrin-protein interactions without total disruption of the spectrin skeleton.

Increased numbers of multinucleated cells noted in cells expressing beta_G-spectrin domains could result from a defect at some stage of cell division or from cell fusion. A possible role of spectrin in dividing cells has been suggested previously by the observation that spectrin is phosphorylated and that it redistributes to the cytosol during mitosis (Fowler and Adam, 1992). It will be of interest in future experiments to follow the progression of spectrin-deficient cells through mitosis and to evaluate whether these cells are capable of forming cleavage furrows and undergoing cytokinesis. Spectrin could also be required for correct delivery of ion channels to precise locations on the plasma membrane to prevent altered levels of intracellular ions.

The transient transfection system used in this study permits conclusions that spectrin is essential for maintaining cell shape and possibly establishing polarized domains in the plasma membrane. A major limitation imposed by this system was that it was not possible to obtain a homogeneous population of cells lacking spectrin. In the future, it will be important to develop methods to disrupt spectrin expression synchronously in a population of cells and to selectively interfere with individual spectrin-protein interactions. Issues that can then be addressed include the role of spectrin in con-

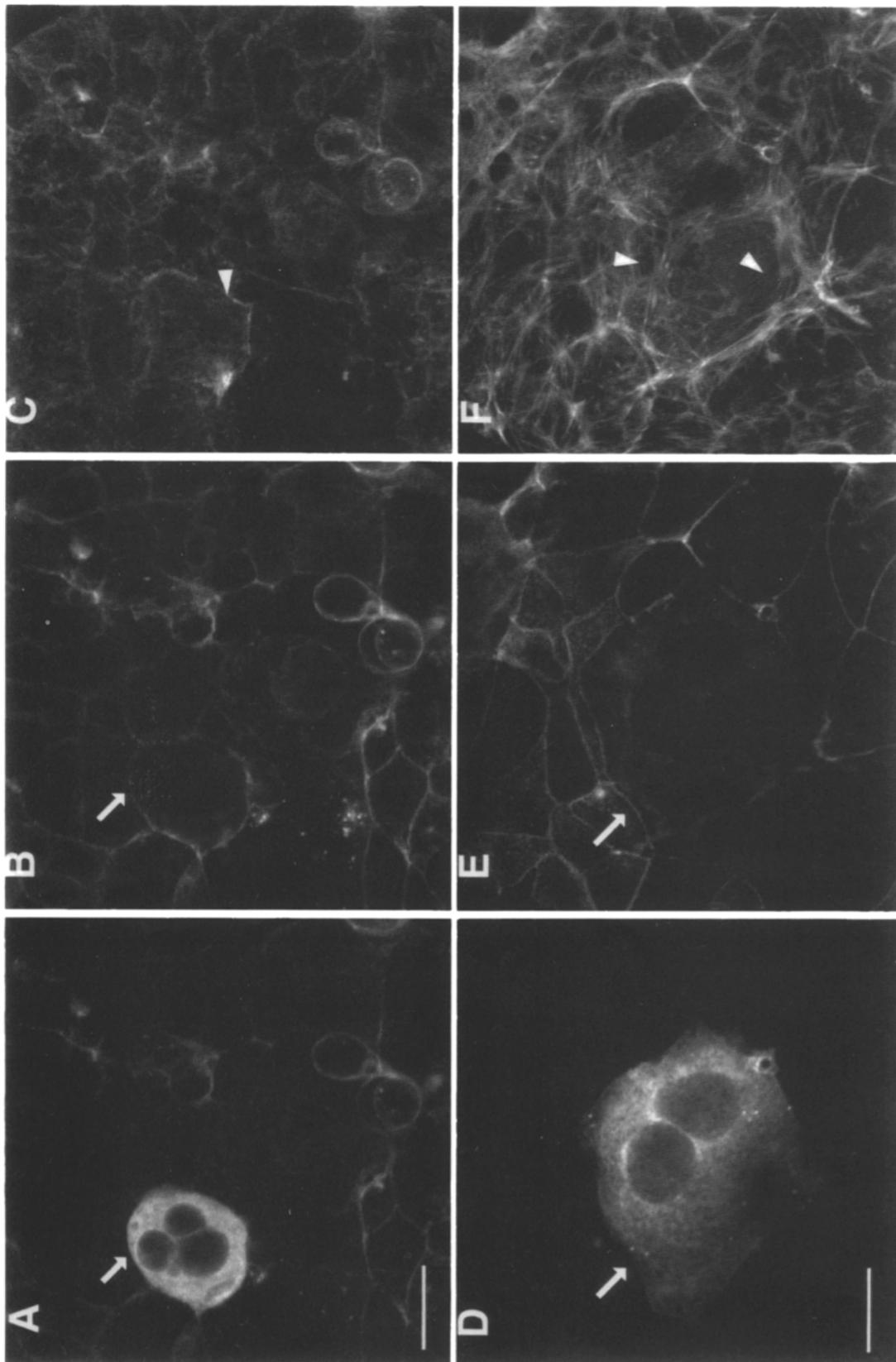


Figure 7. F-actin is distributed in stress fibers and cortical regions of Caco-2 cells expressing either the actin- or ankyrin-binding domains of beta-galactosidase. Cells were transfected with the ankyrin-binding (*A-C*) or actin-binding (*D-F*) domain of beta-galactosidase. Transfected cells were visualized using an anti-beta-galactosidase polyclonal antibody, followed by rhodamine-labeled secondary antibody (*A* and *D*). Actin was detected using fluorescein-labeled phalloidin (*B*, *C*, *E*, and *F*). Note that both cortical actin (*B* and *E*, *arrows*) and stress fiber actin (*C* and *F*, *arrowheads*) appear normally distributed. Raw confocal images were processed in COMOS, followed by Adobe Photoshop 2.5, and printed using a Tektronix Phaser II SDX Printer. Bars, 25 μ m.

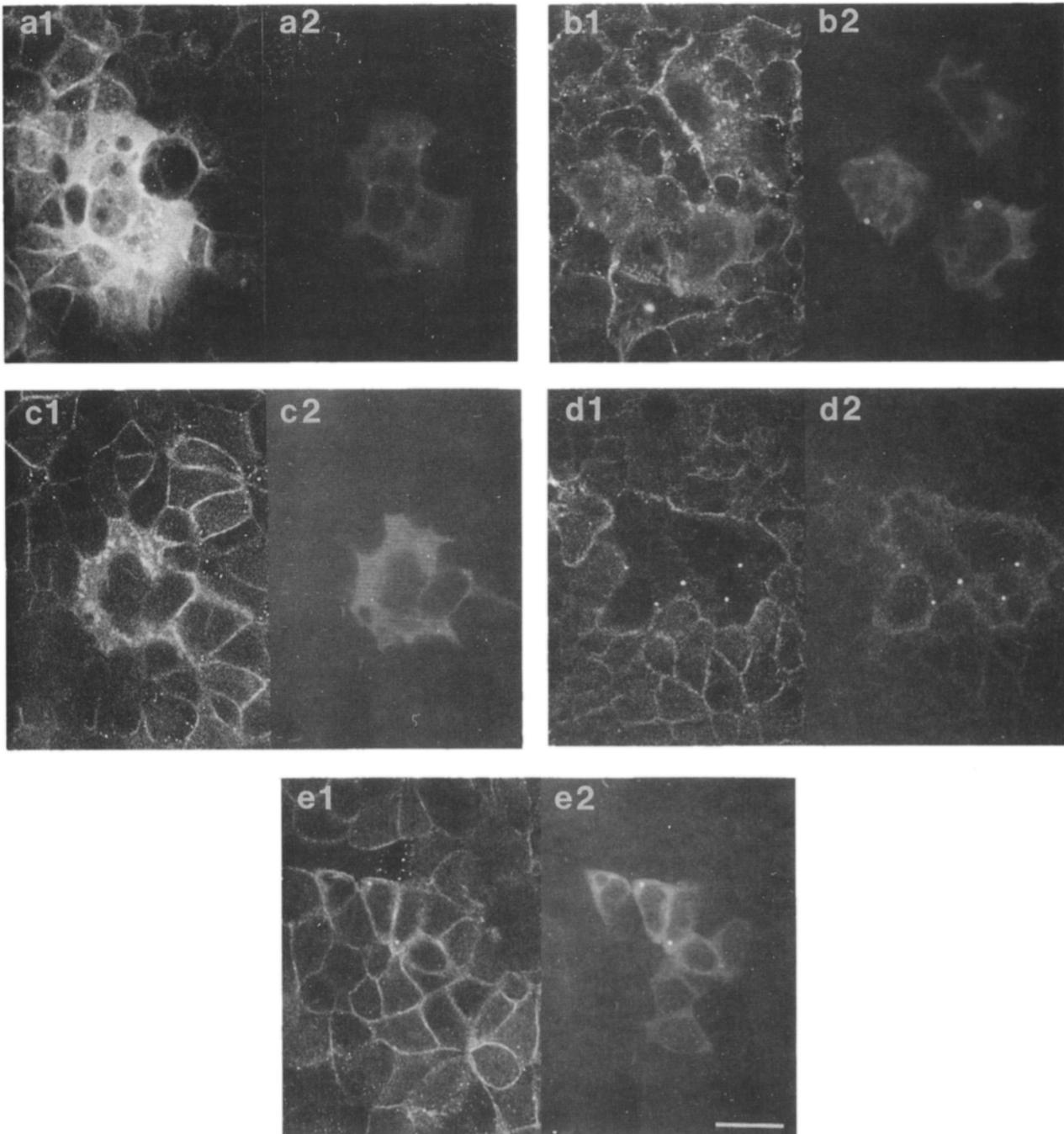


Figure 8. Localization of ankyrin, adducin, Na⁺,K⁺-ATPase, or E-cadherin in Caco-2 cells transfected with the ankyrin-binding domain of beta_G-spectrin. Caco-2 cells transfected with ankyrin-binding domain of beta_G-spectrin were double stained with anti-beta-galactosidase monoclonal antibody and with affinity-purified antibody against one of the following proteins: ankyrin, adducin, Na⁺,K⁺-ATPase, or E-cadherin. The stained cells were visualized by epifluorescence using a confocal microscope. (a1-d1) Cells transfected with plasmids encoding the ankyrin-binding domain of beta_G-spectrin-beta-galactosidase fusion protein and stained with antibody against ankyrin (a1), Na⁺,K⁺-ATPase (b1), adducin (c1), and E-cadherin (d1). (a2-d2) Cells stained with anti-beta-galactosidase monoclonal antibody. (e1 and e2) Cells transfected with plasmid encoding beta-galactosidase alone and stained with adducin antibody (e1) and anti-beta-galactosidase monoclonal antibody (e2). Bar, 20 μm.

trolling dynamic behavior of specific proteins in the plasma membrane, and design of experiments to distinguish between targeting and stabilization of proteins in specialized membrane domains.

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