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Flotillin 2 Is Distinct from Epidermal Surface Antigen (ESA) and Is Associated With Filopodia Formation

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Abstract ECS-1, a monoclonal antibody (MoAb) raised to cultured human keratinocytes, stains the intercellular glycocalyx with a pemphigus-like pattern and recognizes a 35-kDa epidermal surface antigen (ESA) on Western blotting of keratinocyte extracts. When ECS-1 MoAb was used to screen a keratinocyte expression library, a unique cDNA was identified that predicted a 42-kDa globular protein of unknown function. This putative ESA was conserved between mice and humans and was encoded by a gene on chromosome 17q11–12 in linkage with neurofibromin. Homology between the cDNA sequence has been reported with flotillin 1, a caveolae associated protein, as well as Reggie 1 and 2, neuronal proteins expressed during axonal regeneration present in activated GPI-anchored cell adhesion molecules in non-caveolar-associated micropatches. In order to determine whether the cDNA predicted protein and ECS-1 antigen were identical, we compared ECS-1 with the immunoreactivity of a new antibody raised to the cDNA fusion protein in epidermis and cultured cells. The cDNA fusion protein was expressed in bacteria and in cos cells with his, FLAG, and EGFP reporter tags and by stable transfection as an EGFP fusion protein. The fusion protein and native protein of 42 kDa were detected by the new antibody, but not by the original ECS-1. Thus, the ECS-1 antigen, ESA (35 kDa), is clearly distinct from the protein predicted by the cDNA (renamed flotillin 2). Stable transfection of ESA/flotillin 2 fusion protein in cos cells induced filopodia formation and changed epithelial cells to a neuronal appearance. Thus, the function of flotillin 2 may resemble that of the goldfish optic nerve neuronal regeneration proteins, Reggie 1 and 2. J. Cell. Biochem. 75:147–159, 1999.

Key words: caveolae; flotillins; epidermal surface antigen; epidermal adhesion; cell signaling; melanocytes; neuron axonal regeneration; Reggie

ECS-1 antibody raised to cultured human keratinocytes recognizes a 35-kDa epidermal protein, epidermal surface antigen (ESA) [Negi et al., 1986]. When ECS-1 was used to isolate one novel cDNA from a human keratinocyte library, it was assumed to encode the ESA antigen [Schroeder et al., 1994]. However, the keratinocyte cDNA predicted a larger protein of 41.7 kDa, with little homology to known proteins [Schroeder et al., 1994]. As detected by ECS-1, ESA is a cell surface protein with a pemphigus-like staining pattern in epidermis appearing in skin at the time of desmosome assembly (day 16 in mouse and 9 weeks in human fetus). ESA is hypothesized to function in keratinocyte adherence [Negi et al., 1986]. The isolated cDNA was used to map its gene on chromosome 17q11–12 telomeric to the neurofibromatosis 1 gene [Schroeder et al., 1991; Kayes et al., 1992]. The mouse cDNA was also cloned and the coding and regulatory sequences were highly conserved with the human, suggesting an important, but unknown, function [Cho et al., 1995].

Recently, other investigators have shown homology of the putative ESA cDNA to two other novel proteins, Flotillin 1 and Reggie 1, and suggested related functions [Bickel et al., 1997; Schulte et al., 1997]. A protein identical in sequence to the predicted ESA cDNA was also isolated with the 45-kDa protein, called Flotil-

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lin 1, from murine lung tissue, brain, and endothelium [Bickel et al., 1997]. Flotillin 1 is a caveolae-associated, integral membrane protein, 47% homologous to the putative ESA cDNA, and hence the new name flotillin 2 was suggested to replace ESA as defined by the cDNA sequence [Bickel et al., 1997]. Flotillins are characterized by insolubility in Triton-X-100 and buoyant density in sucrose gradients [Bickel et al., 1997], which also contain a number of other membrane associated signal transduction molecules [Lang et al., 1998].

Caveolae, small blebs on the inner cell membrane, sequester cellular receptors and signaling proteins such as epidermal growth factor receptor (EGF-R), insulin receptor, G-proteins, endothelial nitric oxide synthase (eNOS), and H-ras [Li et al., 1995; Parton, 1996]. Caveolin 1 is a 21- to 24-kDa integral membrane protein that forms large aggregates and that tethers other molecules to inner membranes of caveolae [Sargiocomo et al., 1995]. Caveolin 1 may play an important role in cell signal transduction by regulating epidermal growth factor and insulin receptors [Yamamoto et al., 1998]. Caveolins have been implicated in the pathogenesis of diabetes, cancer, and Alzheimer's disease [Engelman et al., 1998].

The function of flotillins, newly described caveolae-associated proteins, is unknown [Bickel et al., 1997]. Flotillin 1 shares only 47% identity with Flotillin 2 (the putative ESA), including six repeats of A/G,E,A/G,E in the C-region, and several phosphorylation sites that may be important for membrane signaling. The brain is not known to contain caveolae [Parton, 1996], but invertebrate flotillins are expressed in developing *Drosophila* embryo central nervous system (CNS)[Galbiati et al., 1998]. Human flotillin 2 (putative ESA) was isolated from fetal brain RNA as an expressed tag [Adams et al., 1993].

Reggie 1, a 48-kDa neuronal surface protein [Schulte et al., 1997], is 80% homologous to the putative ESA/flotillin 2 cDNA sequence [Schroeder et al., 1994]. Reggie 1 was first isolated from severed and regenerating fish optic neurons. Reggie 1 and 2 colocalize with endothelial cells and with newly regenerating ganglion axons in the fish retina. Reggie proteins, like flotillin 2, lack transmembrane and membrane anchor domains. Reggies localize to newly differentiated neuronal cell surface in association with filopodia formation [Schulte et al., 1997]. They have recently been shown to cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons [Lang et al., 1998].

In order to determine the relationship between the original ECS-1 antibody and its 35-kDa keratinocyte antigen and the ECS-1-detected cDNA predicted protein of 41.7 kDa, we expressed the ESA cDNA with identical sequence to flotillin 2 as a fusion protein (ESA/flotillin 2). A new antibody raised to the ESA/flotillin 2 fusion protein was compared with the original ECS-1 in native cell lines and in transfected cells. These studies show that the ECS-1 defined 35-kDa ESA antigen is clearly distinct from the predicted cDNA protein, now called flotillin 2. Stable expression of flotillin 2 confers filopodia formation to epithelial cells giving them a neuronal appearance, thus the function of flotillin 2 may be in neuronal signaling.

MATERIALS AND METHODS

Cells

Neonatal human foreskins treated with 0.1% dispase (Boehringer-Mannheim, Indianapolis, IN) were used to isolate keratinocytes, melanocytes, and fibroblasts as described previously [Kitano and Okada, 1983]. Keratinocytes and melanocytes were cultured in serum-free KGM and MGM media (Clonetics, San Diego, CA). Cos-1 monkey kidney simple epithelial cells were purchased from American Type Cell Culture (ATCC). Fibroblasts and cos-1 cells were grown in Dulbecco's modified Eagle's emdium (DMEM) (Irvine Scientific, Irvine, CA) with 10% bovine calf serum. Squamous cell carcinoma cells [Duvic et al., 1994], obtained from MD Anderson Cancer Center, were cultured in DMEM/F12 media (Gibco-BRL, Gaithersburg, MD) with 10% bovine calf serum.

Reagents

The putative ESA cDNA from 122 to 379, first cloned by our laboratory [Schroeder et al., 1994], was expressed as a fusion protein and a monoclonal IgG1 antibody was raised to it. The ESA fusion protein IgG antibody and caveolin 1 antibody were purchased from Transduction Research. ECS-1, a mouse IgM MoAb, was kindly provided as mouse ascites fluid (Roc-1) by Dr. Ann Haas and Al Lane (University of Rochester, NY). Secondary antibodies were purchased from Jackson Immunodiagnostics (Bar Harbor,

ME). Bovine desmosome snout proteins on nitrocellulose membranes were kindly provided by Kathleen J. Green, (Northwestern University, Chicago, IL). Chemicals were purchased from Sigma Chemical CO. (St. Louis, MO) and membranes from Schleicher and Schuell (Keene, NH).

Immunofluorescence Detection of Proteins

Human foreskin keratinocytes and cos-1 cells were dissociated with 0.25% trypsin and plated at 80,000 cells per well on Nunc Permanox 4-well chamber slides (Nunc, Napirville, IL). Immunofluorescence detection was performed as previously described [Konohana et al., 1988]. Cells were incubated with ECS-1 (1:200), flotillin 2 (1:20), or no antibody in 0.01 M phosphate-buffered saline (PBS) with 4% bovine serum albumin (BSA), pH 7.3, for 30 min at 37°C. Fluorescein labeled goat anti-mouse IgG or IgM at 1:40 dilution was incubated with slides for 30 min [Negi et al., 1986]. Sections were examined using a Zeiss Axioplot epifluorescence microscope.

Fresh human skin frozen sections or transfected cos-1 cells were first permeabilized in −20°C acetone for 10 min, washed in PBS, and blocked with 10% goat serum for 30 min at 25°C. Sections were incubated with ESA fusion protein antibody (1:20) or with ECS-1 (1:100) for 2 h at 25°C, and washed 3× in PBS for 10 min. Sections were incubated with rhodamine-conjugated goat anti-mouse IgG (1:50 dilution) or anti-mouse IgM for 1 h at 25°C, washed with PBS, and rinsed with distilled water. Slides were mounted in elvanol (Dupont, Wilmington, DE) and examined using red laser excitation at 543 nm for rhodamine.

For visualization, a Zeiss Confocal Laser Scanning microscope was used with LSM v 2.0 software equipped with an internal Helium-Neon laser (543-nm emission, power 5 mW) and an external Argon laser (488 and 514 emission lines, power 10 mW). Signals were collected by photomultipliers with 590-nm longpass filter and 530–560 bandpass filte, respectively (570–640 bandpass). Digitized images were transmitted to a Mac based image analysis system through a GPIB interface using BDS-LSM software (Biological Detection Systems, Pittsburgh, PA). Image processing used IPLab (Signal Analytics, Vienna, VA) and NIH Image v 1.53 software. Images were assembled using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Construction and Expression of His-Tag ESA Fusion Protein in Bacteria

For expression of the full-length mouse putative ESA/flotillin 2 cDNA, 1.182 kb of coding sequences initiating at position 232 was amplified using Taq (Perkin-Elmer, Emoryville, CA) polymerase chain reaction (PCR). The fragment was cloned into a TA PCR cloning vector PCRII (Invitrogen, Carlsbad, CA), excised with *Spe*I and *Xba*I, and recloned into *Spe*I and *Xba*I sites of p-Pro-ex-1 expression vector (Promega, Madison, WI) [Polayes et al., 1994]. The correct reading frame and orientation were confirmed by automated DNA sequencing.

Synthesis of a his-tag fusion protein was induced in bacteria DH5α strain (Gibco-BRL), using 1 mM IPTG for 3 h at 37°C. The fusion protein was purified by Ni^{2+} affinity resin chromatography over His Bind resin (Novagen, Madison, WI) under denaturing conditions, according to the manufacturer's instructions. The fusion protein was eluted from the column with 200 mM imidazole.

Construction of Mammalian Expression Vectors for Mouse ESA

The full-length mouse putative ESA/flotillin 2 cDNA starting at nucleotide 232 was also excised from p-PCRII-MESA with *Xba*1 and *Kpn*1 and ligated into the same sites of the vector p-FLAG-CMV (Eastman Kodak, New Haven, CT). FLAG-fusion proteins expressed in mammalian cells were detected with p-FLAG antibody. An *Eco*R1 fragment excised from p-FLAG was next ligated into the p-EGFP-C2 cloning vector (Clonetech, Palo Alto, CA) to express a putative ESA/flotillin 2-GFP (green fluorescence protein) fusion protein in mammalian cells.

Transfection of Putative ESA/Flotillin-2 cDNA in Cos-1 Cells

Cos-1 monkey kidney epithelial cells plated overnight at 2×10^5 cells per 35-mm dish were transfected with 1.5 µg DNA (vector or vector plus insert) in 10 µl of lipofectamine and 1 ml of OPTIMEM (Gibco-BRL). After a 5-h incubation at 37°C in 5% CO_2, media was replaced with DMEM containing 10% bovine calf serum. Cells were analyzed at 48 h for transient transfection using Western blotting and immunofluorescence staining.

Additional transfection reactions were grown for 28 days in genticin selection media and plated by limited dilution to select stable colonies. Vector and nontransfected cells served as negative controls. Cells selected in genticin were monitored by plating on coverslips and observing fluorescence at 488 nM for GFP.

Extraction of Protein and Immunoblotting

Proteins from transfected or cultured control cells were extracted with 10 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (NP-40) and complete protease inhibitor cocktail (Boehringer-Mannheim) as described previously [Negi et al., 1986]. Protein concentrations were determined with BCA reagent (Pierce, Rockford, IL). Proteins at 5 µg per lane were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a Mini Protein II apparatus (BioRad, Hercules, CA) at 170 V for 1 h.

Western blots were performed as previously described [Duvic et al., 1994]. Blocking was in 5% non-fat dry milk in phosphate-buffered saline, pH 7.4, with 0.1%Tween 20 (PBST). Incubation with primary antibody was for 2 h at room temperature, followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (for flotillin 2) or IgM (for ECS-1) for 1 h at 1:5,000 dilution. Detection was with ECL chemiluminescence on Hyperfilm film (Amersham, Arlington Heights, IL). Prestained markers (BioRad) were myosin, 205 kDa; phosphorylase B, 106 kDa; BSA, 80 kDa; ovalbumin, 49.5 kDa; carbonic anhydride, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; and lysozyme, 18.5 kDa.

RESULTS

ECS-1 Antibody and the Putative ESA/Flotillin 2 Fusion Protein Antibody Have Distinct Staining Patterns in Epidermis and Cells

Negi et al. [1986] previously showed that ECS-1 MoAb raised to human cultured keratinocytes recognizes a 35-kDa epidermal surface antigen (ESA) found in epidermal glycocalyx with an intercellular, pemphigus vulgaris-like pattern. To compare ECS-1 determined ESA antigen with the protein encoded by a putative ESA cDNA clone identified with ECS-1, an antibody raised to the fusion protein, ESA/flotillin 2, was used for immunofluorescence staining of fresh frozen skin sections and cultured human foreskin keratinocytes. As shown in Figure 1, both antibodies stained the epidermis in whole skin mounts. No staining was seen with secondary antibody. Permeabilization was required to visualize staining of the cDNA fusion protein ESA /Flotillin 2 antibody, suggesting an intracellular localization (Fig. 1A). This antibody stained the epidermis uniformly without staining the nucleii (Fig. 1A). By contrast, ECS-1 stained the stratum Malpighii layer more strongly than other layers (Fig. 1C). The fusion protein antibody did not stain cultured keratinocytes or Cos-1 cells even with permeabilization and when viewed under confocal microscopy (not shown). However, ECS-1 stained the intercellular spaces between individual cultured keratinocytes examined under laser confocal microscopy (Fig. 1E). When the ESA/flotillin 2 fusion protein was expressed transiently in cos-1 cells, fluorescence was visualized as EGFP direct fluorescence (Fig. 2A) and with ESA/flotillin 2 antibody in the cell cytoplasm (Fig. 2B). Confocal scanning laser microscopy cross sections of cos cells also showed cytoplasmic localization of the transiently expressed fusion protein (Fig. 2C).

ECS-1 and ESA/Flotillin 2 Fusion Protein Antibodies Differ in Their Reactivity to Bovine Snout Proteins, Intrinsic Cos-1 Cell Proteins, and cDNA-Fusion Protein Expressed in Bacteria

To determine whether ECS-1 antigen (ESA) and the putative ESA/flotillin 2 protein are expressed in bovine snout desmosomes or in cos-1 cells (derived from simple kidney epithelia), we performed Western blotting. The antibody raised to ESA/flotillin 2 fusion protein recognizes a 41.7-kDa band present in

Fig. 1. Immunofluorescence staining of human skin and cultured keratinocytes. **A:** Flotillin 2 antibody (1:20) raised to the epidermal surface antigen (ESA) cDNA fusion protein amino acids 122–357 (Transduction Research) was incubated with whole frozen skin sections and detected with rhodamine conjugated goat-anti-mouse IgG (1:50) and examined under Zeiss axioplot epifluorescence microscopy at 543 nm. ×400. **B:** Rhodamine-conjugated goat anti-mouse IgG (1:50) as negative control. ×400. **C**: ECS-1 ascites fluid (1:100) was detected with rhodamine-conjugated goat-anti-mouse IgM (1:50). ×400. **D:** Rhodamine- conjugated goat-anti-mouse IgM (1:50) as negative control. ×400. **E:** Cultured human foreskin keratinocytes on slides were stained with ECS-1 (1:200) and detected with fluorescein-conjugated goat-anti-mouse IgM (1:40) using laser scanning confocal microscopy. ×1,000.

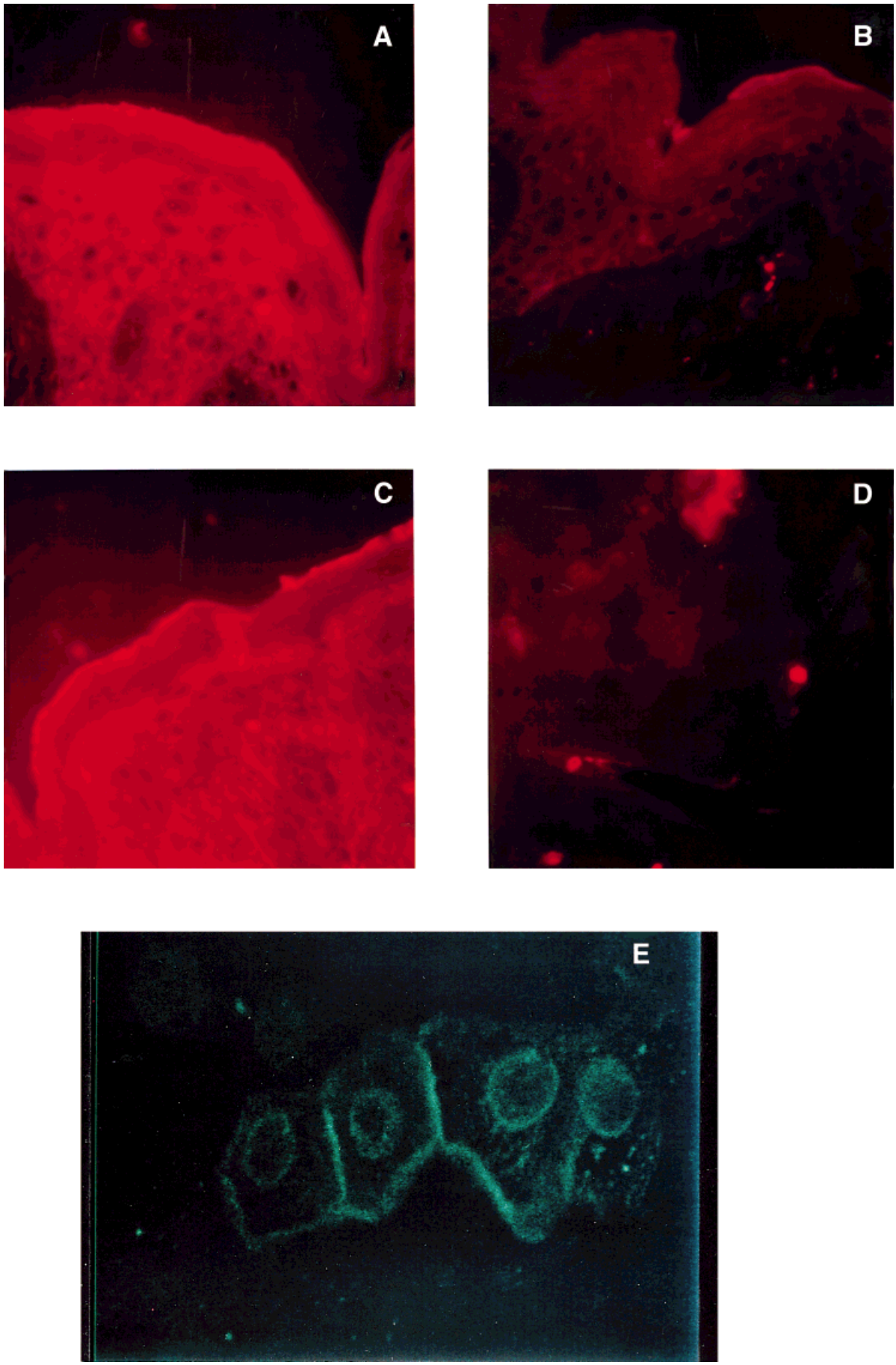

Figure 1.

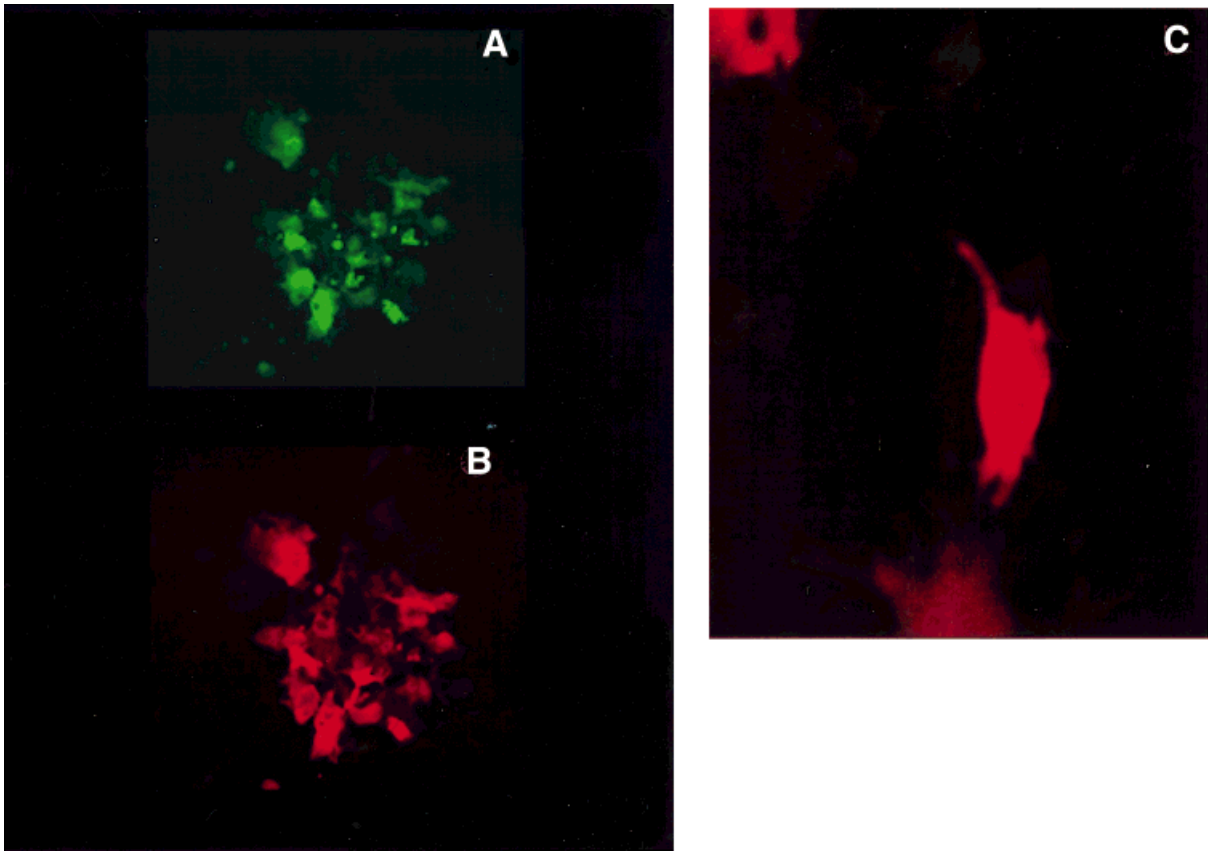

Fig. 2. Immunofluorescence detection of expressed epidermal surface antigen (ESA)/flotillin 2 protein in Cos-1 cells. **A:** Cos-1 cells transfected with p-EGFP-ESA/flotillin 2 and selected in G418 media for 28 days were studied for expression using a Zeiss axioplot epiluminescence microscope. The GFP tag was detected at 488-nm excitation. ×200. **B:** Cells were observed using the ESA/flotillin 2 fusion protein antibody (1:20) detected with rhodamine-conjugated goat anti-mouse IgG (1:50) at 543-nm excitation. ×200. **C:** Cos-1 cell as in B were observed under laser zoom scanning confocal microscope in 10-µm sections. ×400.

untransfected cos-1 cells (Fig. 3A, lane 1) but does not react to desmosomal bovine snout proteins (Fig. 3A, lane 2). By contrast, ECS-1 antibody recognizes a 35-kDa band in nontransfected Cos-1 cells (Fig. 3A, lane 3) and also a 70-kDa band in bovine desmosomes (Fig. 3A, lane 4).

Putative ESA/Flotillin 2 cDNA was expressed in bacteria as a p-Pro-Ex fusion protein with a his-tag, allowing for nickel agarose chromatography purification. The antibody to the ESA/Flotillin 2 fusion protein recognized a 41.7-kDa band in cos-1 cells (Fig. 3B, lane 1), while a band of 35-kDa was detected using ECS-1 antibody (Fig. 3B, lane 4). ESA/Flotillin 2 antibody (Fig. 3B, lanes 2, 3), but not ECS-1 antibody (Fig. 3B, lanes 5, 6), recognized the expected size fusion proteins expressed in *Escherichia coli*. Although ECS-1 has cross-reactivity with other proteins found in cos-1 cells (Fig. 3B, lane 4), a 41-kDa band where flotillin 2 runs was not present.

Transient Expression of ESA-Flotillin 2 Fusion Proteins in Cos-1 Cells

We prepared two constructs containing different tags for the purposes of expressing the putative ESA/flotillin 2 in cos-1 cells. The mouse cDNA was inserted into a pFLAG-CMV expression vector with the epitope tag (DYKDDDDK) at the N-terminus [Shelness et al., 1988] and was transiently expressed in cos-1 cells [Gluzman, 1981]. For stable transfection, pEGFP-C2 containing the SV-40 promoter, selectable markers kanamycin and neomycin resistance genes, and the green fluorescence protein EGFP tag of 27 kDa was selected. Western blotting of cos-1 cells transiently transfected with these constructs detected the expression

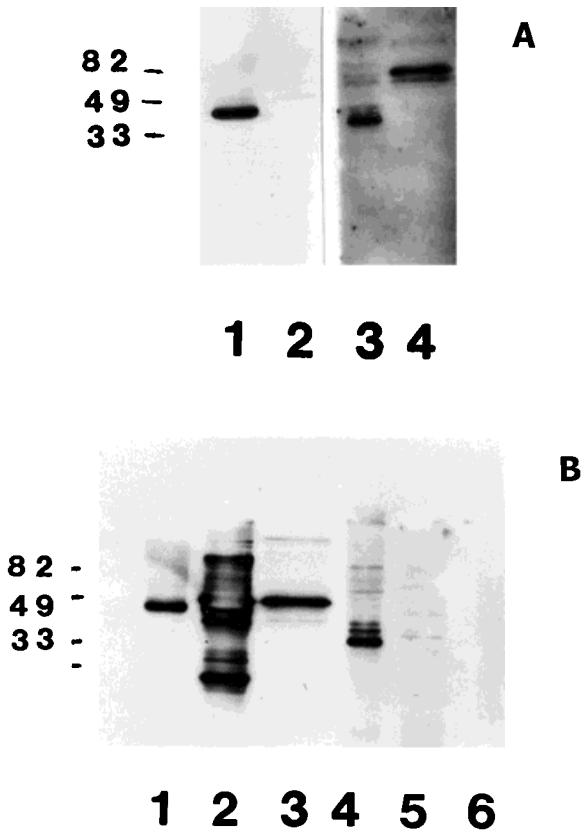

Fig. 3. Expression of epidermal surface antigen (ESA) and flotillin in desmosomes and cos-1 cells. **A:** Western blot of bovine desmosome versus cos-1 proteins. Protein extracted from Cos-1 cells **(lanes 1, 3)** or bovine snout desmosome preparations (kindly provided by Kathy Greene) were loaded at 5 µg protein per lane and electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Incubation was with Flotillin 2 antibody (1:5,000) **(lanes 1, 2)** and with ECS-1 MoAb (1:2,000) **(lanes 3, 4)**. Detection was using horseradish peroxidase-conjugated goat anti-mouse IgG or IgM secondary antibodies at 1:5,000. Detection of signal was with Amersham ECL-S chemiluminescence with Hyperfilm. Markers were prestained BioRad low-molecular-weight markers. **B:** Differences in staining of Cos-1 and bacterial fusion proteins by flotillin 2 and ECS-1. A total of 5 µg of cos-1 cell proteins **(lanes 1, 4)**, crude bacterial extracts of flotillin 2 fusion proteins **(lanes 2, 5)**, and Ni^{2+} agarose purified p-Pro-Ex fusion proteins expressing flotillin 2 **(lanes 3, 6)** were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and used for Western blotting as in A. ESA/Flotillin 2 antibody is shown in **lanes 1–3** and ECS-1 in **lanes 4–6**.

of ESA/flotillin 2 FLAG and GFP fusion proteins (Fig. 4). ESA/Flotillin 2 p-FLAG-fusion protein ran at about 42 kDa (Fig. 4, lane 2) and the GFP-fusion protein ran at 69 kDa as predicted (Fig. 4, lane 4). Vector only transfections (Fig. 4, lanes 1, 3) did not show reactivity.

Cultured Skin Cells Have Differential Expression of ESA Antigen, ESA/flotillin 2, and Caveolin 1 In Vitro

Since the original ESA was initially identified with an antibody raised to cultured keratinocytes [Negi et al., 1986], and the predicted cDNA ESA/ flotillin 2 was purified by another laboratory from brain and lung [Bickel et al., 1997], the expression of proteins recognized by the two antibodies was studied in cultured lines from different origins. Duplicate Western blots containing proteins extracted from cultured cells were probed with ESA/Flotillin 2 fusion protein antibody (Fig. 5A) or ECS-1 (Fig. 5B). ESA/ Flotillin 2 antibody recognized a strong ≃42-kDa band of equal intensity among fibroblasts, melanocytes, and squamous carcinoma cell lines. There was also a weak higher band in all cells, except melanocytes (Fig. 5B, lane 2). ECS-1 antigen was weak in fibroblasts (Fig. 5B, lane 1), almost absent in melanocytes (Fig. 5B, lane 2) and was strong in all the keratinocyte derived cells (Fig. 5B, lanes 3–9). ECS-1 also showed cross-reactivity with high-molecular-weight bands. Three strong high-molecular-weight bands of unknown significance were present in fibroblasts (Fig. 5B, lane 1).

We also compared ESA/flotillin 2 protein expression Fig. 6A with that of caveolin, the 21-kDa major component of caveolae Fig. 6B, in cultured cell lines. Untransfected cos-1 cells (Fig. 6A, lane 2) and transiently tranfected cos-1 cells (Fig. 6A, lanes 3, 4) expressing pFLAG or GFP ESA/flotillin 2 fusion proteins as controls were examined by Western blotting. ESA/Flotillin 2 fusion protein antibody (Fig. 6A) detected the expressed fusion proteins as well as the native 41-kDa protein bands. Caveolin 1 antibody (Fig. 6B) reacted strongly with a 21-kDa band in keratinocytes (Fig. 6B, lanes 6, 7), weakly with untransfected cos-1 cells (Fig. 6B, lane 3), and did not react with ESA/flotillin 2 fusion proteins (Fig. 6B, lanes 4, 5). Caveolin 1 was not seen among proteins extracted from foreskin melanocytes (Fig. 6B, lanes 8, 9).

ESA/Flotillin 2 Expression in Cos-1 Cells Is Cytoplasmic

Transfected Cos-1 cells were selected for 28 days in genticin, cloned by limited dilution, and checked for EGFP by fluorescence at 488 nM (Fig. 2A) and with the antibody raised to the fusion protein (Fig. 2B). There was cytoplasmic

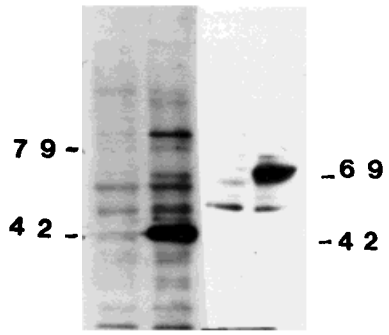

fig.4

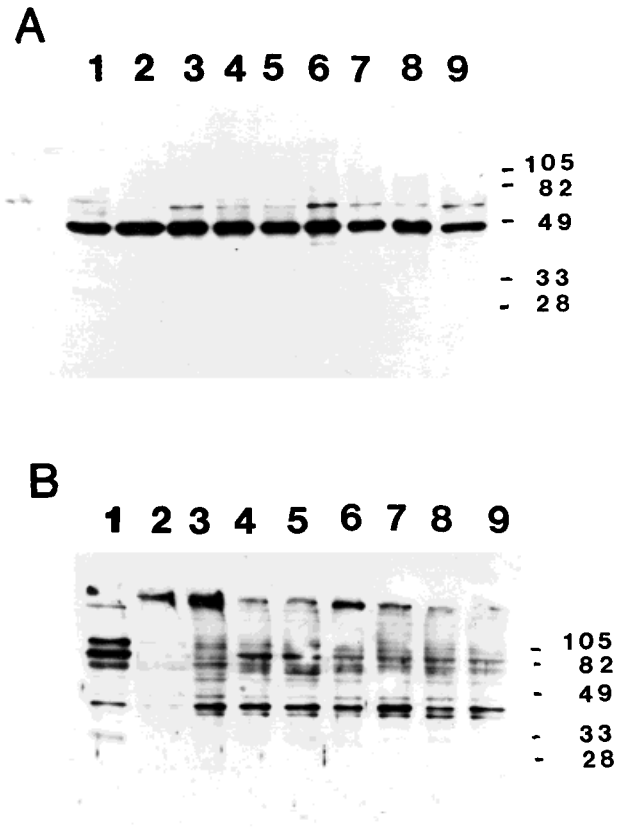

fig.5

Fig. 4. Transient expression of epidermal surface antigen (ESA)/Flotillin 2 fusion proteins in Cos-1 cells. 2×10^5 cos-1 cells plated in 35-mm dishes were transfected with 1.5 µg of vector with or without insert in serum-free OPTIMEM media containing 10 µl of lipofectamine (Gibco) for 5 h. Dulbecco's modified Eagle's medium (DMEM) with 10% calf sera was added for 48 h. Proteins extracted in sodium dodecyl sulfate (SDS) lysate buffer with protease inhibitors were electrophoresed at 5 µg/lane on 12% polyacrylamide gel electrophoresis (PAGE). Nitrocellulose membrane-bound proteins were detected with pFLAG **(lanes 1, 2)** or GFP monoclonal antibody (MoAb) (1:500) **(lanes 3, 4)** and horseradish peroxidase-conjugated goat anti-mouse secondary (1:5,000). ECL-S detection (Amersham) was for 1 min. **Lane 1**, pFLAG-CMV vector; **lane** 2, P-FLAG-flotillin 2; **lane 3**, pEGFP-C2 vector control; **lane 4**, p-EGFP-C2-flotillin 2. Secondary antibody alone did not react (not shown).

Fig. 5. Expression of epidermal surface antigen (ESA), ESA/flotillin 2, and caveolin 1 proteins in cultured cell lines. A total of 5 µg of proteins from cell lines were electrophoresed on 12% polyacrylamide gel electrophoresis (PAGE) gels, and transferred to nitrocellulose filters. BRL molecular-weight standards were used. **A:** ESA/flotillin 2 monoclonal IgG1 antibody at 1:5,000 was detected with secondary goat anti-mouse IgG (1:5,000). Lanes loaded: 1-normal skin fibroblasts, 2-melanocytes, 3-psoriasis keratinocytes, squamous carcinoma lines 4–9. **B:** Same filter as A. ECS-1 Ig M antibody at 1:2,000 detected with secondary biotinylated goat anti-mouse IgM (1:5,000), using ECL-S Amersham chemiluminescence kit and exposed to Hyperfilm for 4 min.

staining of the transfected cells. Selected cells were also analyzed by Western blots to confirm expression of ESA/flotillin 2 fusion protein (Fig. 7). ESA/Flotillin 2 antibody stains untransfected cos-1 cells (Fig. 7, lane 1) and cells expressing the control EGFP vector (Fig. 7, lane 2), as well as the cells expressing EGFP-ESA/flotillin 2 fusion protein (Fig. 7, lane 3). By contrast, ECS-1 MoAb showed reactivity only to the native 35-kDa ESA band in untransfected cos-1 cells (Fig. 7, lane 4) and the intensity of band was decreased in stably transfected cos-1 cells (Fig. 7, lanes 5, 6).

Stable Expression of ESA/flotillin 2 Is Associated With Morphologic Changes and Filopodia Formation

Cos-1 cells stably transfected with ESA flotillin 2- EGFP fusion protein vector DNA were examined by epiluminescence microscopy to detect EGFP fluorescence. The morphology of the unselected cos-1 cells (Fig. 8A) and the EGFP

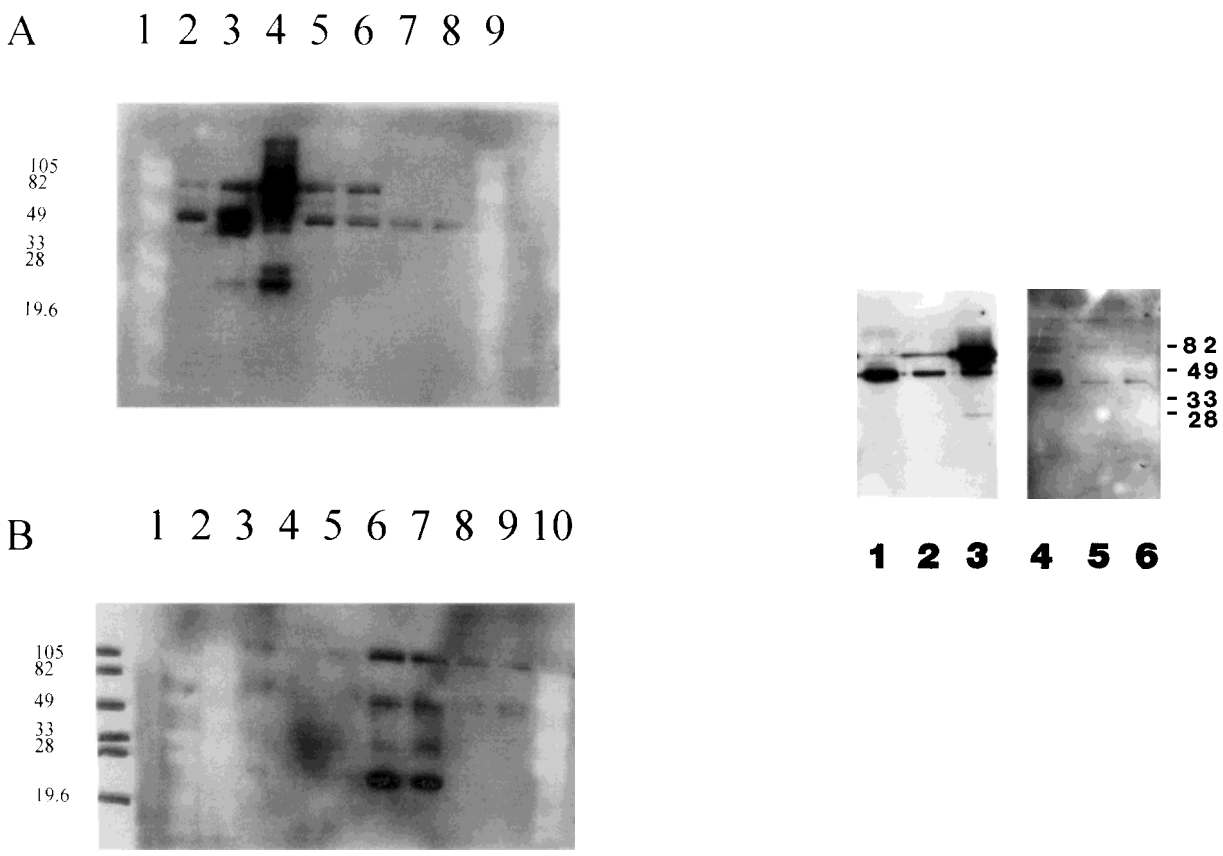

Fig. 6. Differential expression of Flotillin 2 and caveolin 1 in skin cell lines. **A:** Proteins from transiently transfected cos-1 cells were compared with proteins from normal cells for expression of Flotillin 2 detected with epidermal surface antigen (ESA)/flotillin 2 antibody (1:1,000). Biorad prestained low molecular weight markers **(lane 1)**, untransfected cos-1 cells **(lane 2)**, p-Flag-ESA/flotillin 2 transfected cos-1 cells **(lane 3)**, EGFP-ESA/flotillin 2 transfected cos-1 cells **(lane 4)**, normal foreskin keratinocytes **(lanes 5, 6)**, normal human foreskin melanocytes **(lanes 7, 8)**. **B:** Proteins reacted with antibody to caveolin 1 (1:1,000), detected by Western and ECL-S method. Kaleidoscope markers **(lanes 2, 10)**; BioRad low-molecular-weight markers **(lane 1)**; untransfected cos-1 cells **(lane 3)**, p-Flag flotillin transfected cos-1 cells **(lane 4)**, EGFP-transfected cos-1 cells **(lane 5)**, normal foreskin keratinocytes **(lanes 6, 7)**, normal human foreskin melanocytes **(lanes 8, 9)**.

Fig. 7. Stable transfection of p-EGFP-C2-ESA/Flotillin 2 in Cos-1 cells by Western blotting. After transfection, Cos-1 cells were grown for 28 days in GM418 media and plated by limited dilution to isolate single colonies. For comparison, untransfected Cos-1 cell proteins are in **lanes 1, 4**. p-EGFP-C2 vector control cells are shown in **lanes 2, 5**. p-EGFP-C2-ESA/flotillin 2 transfected cells are shown in lanes 3 and 6. Epidermal surface antigen (ESA)/Flotillin 2 fusion protein antibody was used in **lanes 1–3** and ECS-1, in **lanes 4–6**.

vector only control transfected cos-1 cells (Fig. 8B) were the same. Cos-1 cells stably transfected with the ESA/flotillin-2 EGFP vector at confluency had a different appearance from that of the controls (Fig. 8C,D). Control cells were small and polygonal in shape and formed confluent sheets of cells that covered their dishes. By contrast, selected clones of cos-1 cells expressing ESA/flotillin 2-EGFP had numerous dendritic processes that made contact with other cells (Fig. 8D). This resulted in spaces between individual cells in confluent cultures (Fig. 8C). Each cell formed several long filopodia and had a neuronal appearance (Fig. 8D).

We then examined cells under 488 nm fluorescence to see expression of EGFP. The vector transfected cells (Fig. 8E) were flat, and both nuclei and abundant cytoplasm were fluorescent (Fig. 8E). Cells with ESA/flotillin 2-GFP fusion protein expression were smaller and had narrow dendritic processes that were fluorescent (Fig. 8F,G, white arrows). Thus, the fusion protein appeared to be expressed within the filopodia of cells.

DISCUSSION

These studies show for the first time that the 35-kDa ECS-1 antigen (ESA or epidermal sur-

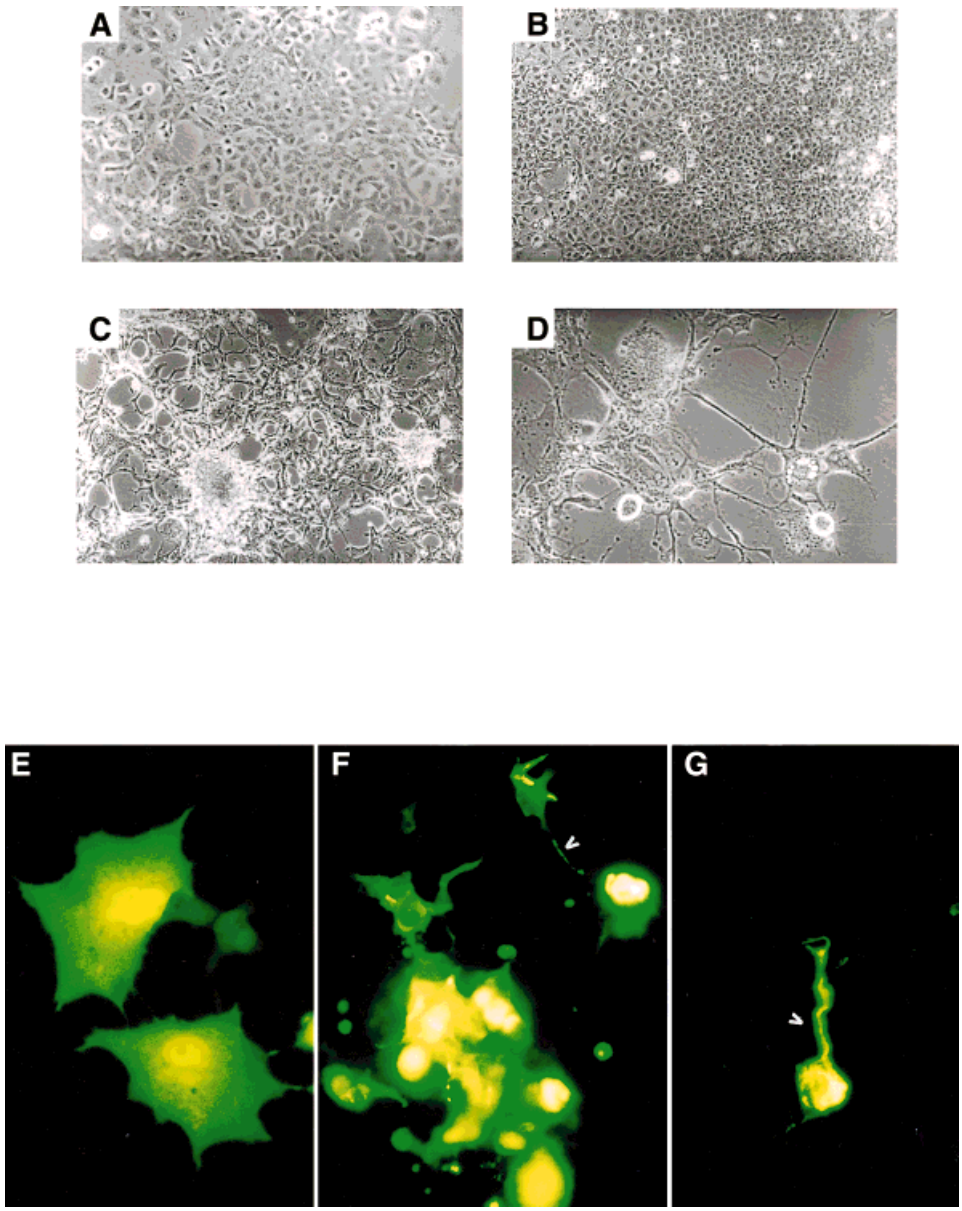

Fig. 8. Stable transfection of Cos-1 cells with flotillin 2 gives a dendritic phenotype. **A–D:** Phase-contrast microscopy of untransfected and unselected cos-1 cells (A), cos-1 cells transfected with EGFP vector control and selected with genticin (B), cos-1 cells transfected with epidermal surface antigen (ESA)/flotillin 2-EGFP vector and selected with genticin (C), and clone of flotillin 2 tranfectants after further selection (D). Cells were grown for >28 days in genticin selection media. A–C: ×100; (D) ×200. **E,G:** Fluorescence of the EGFP epitope tag was observed using epifluorescence microscopy at 488 nm. Vector control cells (E); Selected clones expressing flotillin 2-GFP fusion protein (**F,**G). White arrows show filopodia in which flotillin 2 is expressed. ×400.

face antigen) first identified by ECS-1 MoAb [Negi et al., 1986] is distinct from a protein predicted from a cDNA isolated from a keratinocyte expression library with ECS-1 [Schroeder et al., 1994]. The 41.7-kDa predicted protein encoded by this cDNA has been renamed flotillin 2, based on its limited homology to flotillin 1, a novel caveolae-associated protein [Bickel et al., 1997]. However, flotillin 2 may be more closely related to Reggie 1, a goldfish optic nerve regeneration protein identified in GPI-micropatches in neurons [Schulte et al., 1997; Lang et al., 1998].

Epidermal surface antigen or ESA should be reserved for the original ECS-1 antigen the identity of which remains undetermined. An

antibody raised to the putative ESA/flotillin 2 cDNA fusion protein and transfection experiments has allowed comparison with ECS-1 expression. Both ESA and flotillin 2 are expressed by simple epithelial cos-1 cells and are present in human epidermis. ECS-1 antibody does not recognize the flotillin 2 cDNA fusion protein when expressed in bacteria or in cos-1 cells that show reactivity. Thus, it is unclear why ECS-1 did react with lambda gt11 fusion protein expressing cDNA clones encoding flotillin 2.

ECS-1, but not flotillin 2 antibody, reacts with a 70-kDa bovine snout protein extracted from desmosomes, and this could represent a dimer of 35-kDa ESA. ESA is hypothesized to be a desmosome associated protein with a role in keratinocyte adherence, first suggested by Negi. ESA antigen first appears at the time of desmosome formation and ECS-1 antibody causes keratinocyte detachment in vitro [Negi et al., 1986]. ESA can be detected on the surface of keratinocytes with accentuation between epidermal keratinocytes, whereas the ESA/flotillin 2 fusion protein antibody requires permeabilization for visualization of flotillin 2, and in this regard is similar to the cytoplasmic Reggie proteins [Lang et al., 1998].

ESA has more limited expression than flotillin 2 since the latter appears to be constitutively expressed in keratinocytes, fibroblasts, and melanocytes. Although the function of flotillin 2 is still unknown, the protein has been isolated from murine lung tissue, brain, and endothelium in association with another new 48-kDa, caveolae associated protein, flotillin 1 [Bickel et al., 1997]. Flotillins 1 and 2 have 47% identity of amino acids including six repeats of A/G,E,A/G,E in the carboxyl-region. Flotillin 2 also has 46% homology with bullous pemphigoid antigen 1 and less homology with the alpha helical proteins: myosin, collagens, keratins, a rat neurofilament protein, and plectin [Schroeder et al., 1994; Cho et al., 1995; Guo et al., 1995].

Flotillin 2 mRNA was also isolated as a sequence tag from human fetal brain and is expressed in mouse brain mRNA [Adams et al., 1993; Cho et al., 1995]. Flotillin 1 and 2 proteins were initially isolated from brain [Bickel et al., 1997]. Although brain does not have caveolae, it may use other kinds of membrane regions for cell signaling [Parton, 1996; Olive et al., 1995; Bouillot et al., 1996]. Invertebrate flotillins are conserved and expressed in *Drosophila* CNS during embryonic development [Galbiati et al., 1998]. Whether flotillins have functions similar to caveolin is unknown. Flotillin-2 expression was found to differ from that of caveolin 1. Flotillin 2 protein was found in melanocytes, a neuronal derived cell line that migrates to skin but caveolin 1, the major caveolae protein, was not detected in cultured human melanocytes under the culture conditions used. Caveolin 1 was found in keratinocytes and cos-1 cells, as was flotillin 2.

The human flotillin 2 gene (formerly ESA) on chromosome 17q11.12 (Schroeder et al., 1991] is in linkage dysequilibrium with the neurofibromatosis 1 gene [Kayes et al., 1992]. NF-1 gene also contains an oligo-dendrocyte neuronal protein [Chen et al., 1994]. Both flotillin 2 and NF1 genes are highly conserved between mouse and humans [Bernards et al., 1993; Cho et al., 1995], suggesting they have important, conserved functions. Neurofibromin is known to be important for ras membrane signaling, profoundly influencing neuronal development [Cawthon et al., 1990; Lowy et al., 1993; Malhotra and Ratner, 1994]. Mouse flotillin 2 maps to the syntenic region of mouse chromosome 11 that contains a neuronal protein, the Evi-1 retrovirus insertion site [O'Connell et al., 1990], and the nude mouse gene, Wnt-1 [Nehls et al., 1994; Cho et al., 1995]. Wnt-1 is a proto-oncogene that is critical for brain development and is associated with cadherin/catenin adhesion proteins [Sanson et al., 1996; Peifer, 1997]. Thus, the flotillin 2 gene lies within a region that appears to contain several genes relating to neuronal development.

The colocalization of flotillin 2 with caveolin suggests that its function may relate to cell signaling. Flotillins 1 and 2 have conserved protein kinase C phosphorylation sites at residue 150 and at tyrosine 152 [Schroeder et al., 1994; Bickel et al., 1997]. Because caveolae are thought to sequester G-proteins and receptors for signal transduction [Parton and Simons, 1995; Parton, 1996], conserved PKC and tyrosine sites may be critical and their phosphorylation state may be important for cell membrane signaling. Whether flotillins can function like caveolins to tether molecules to the inner membrane, is not known. However, the reggie proteins are not caveolae associated, but rather are found in GPI-anchored micropatches in neuron filopodia [Lang et al., 1998].

In order to learn more about flotillin 2 function, we expressed it in cos-1 cells and selected stable transfectants. The phenotypic appearance of selected cells expressing flotillin 2, but not control vector, was dendritic. New filopodia formation between neighboring cells was seen and they extended several cell widths out, making connections with other cells. EGFP fusion protein was detected under fluorescence and appeared to be present within the filopodia. These findings support the hypothesis that flotillin 2 may help determine characteristics of the cell membrane which are important in outgrowth of neurons. Thus, human flotillins may be closely related in function to their homologous Reggie 1 and 2 proteins expressed in newly regenerating axons in non-caveolae GPI-anchored cell adhesion micropatches [Schulte et al., 1997; Lang et al., 1998].

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