Regulation of the SHP-2 Tyrosine Phosphatase by a Novel Cholesterol- and Cell Confluence-dependent Mechanism*§

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Endothelial cells approaching confluence exhibit marked decreases in tyrosine phosphorylation of receptor tyrosine kinases and adherens junctions proteins, required for cell cycle arrest and adherens junctions stability. Recently, we demonstrated a close correlation in endothelial cells between membrane cholesterol and tyrosine phosphorylation of adherens junctions proteins. Here, we probe the mechanistic basis for this correlation. We find that as endothelial cells reach confluence, the tyrosine phosphatase SHP-2 is recruited to a low-density membrane fraction in a cholesterol-dependent manner. Binding of SHP-2 to this fraction was not abolished by phenyl phosphate, strongly suggesting that this binding was mediated by other regions of SHP-2 beside its SH2 domains. Annexin II, previously implicated in cholesterol trafficking, was associated in a complex with SHP-2, and both proteins localized to adhesion bands in confluent endothelial monolayers. These studies reveal a novel, cholesterol-dependent mechanism for the recruitment of signaling proteins to specific plasma membrane domains via their interactions with annexin II.

Endothelial cells approaching confluence in culture undergo profound morphological and functional alterations, including the formation of intercellular junctions and the cessation of cell growth. These changes are associated with reductions in tyrosine phosphorylation levels of both receptor tyrosine kinases (1, 2) and cadherins and catenins (3), the major components of adherens junctions. The dephosphorylation of adherens junctions proteins appears to be necessary for the formation of stable, confluent endothelial monolayers, since increased tyrosine phosphorylation of adherens junctions proteins leads to the disruption of adherens junctions (4–6). The mechanisms by which cell density decreases tyrosine phosphorylation of endothelial membrane proteins are unknown.

One possible mechanism is that increased cell confluence modulates the local environment of signaling proteins in the plasma membrane, thereby altering their response to extracellular ligands. Recently (7), we identified membrane cholesterol as a potential determinant of tyrosine phosphorylation levels in growing endothelial monolayers. Membrane cholesterol increased markedly with cell density in cultures of growing endothelial cells, exhibiting levels 3–4-fold higher upon reaching confluence. Depletion of cholesterol from confluent monolayers of CPAE1 cells induced the tyrosine phosphorylation of multiple membrane proteins, including the adherens junctions proteins γ-catenin and pp120, and disrupted adherens junctions.

Cholesterol might regulate tyrosine phosphorylation levels through the localization of tyrosine kinases or phosphatases to specific plasma membrane loci. To begin to identify such kinases or phosphatases, we have used very low (0.01%) levels of the sterol-containing detergent digitonin to identify proteins bound to membranes in a cholesterol-dependent manner (8). At these concentrations, digitonin complexes specifically with unesterified 3β-hydroxysterols (principally free cholesterol, in animal cells), forming in situ precipitates without disrupting other lipid interactions (9, 10). Here, we report that extracts prepared with digitonin from confluent, but not subconfluent endothelial cells, contained the tyrosine phosphatase SHP-2. SHP-2 was found in a complex with Ax II, a phospholipid-binding protein (11, 12) implicated in cholesterol trafficking (13), and both SHP-2 and Ax II were visualized at intercellular junctions in confluent endothelial monolayers. Our observations indicate that, as endothelial cells reach confluence, a complex containing SHP-2 and Ax II is recruited to intercellular junctions by a novel, cholesterol-dependent mechanism. These studies suggest that cell density regulates the tyrosine phosphorylation levels of membrane proteins through the cholesterol-dependent redistribution of tyrosine kinases and/or phosphatases.

MATERIALS AND METHODS
Antibodies and Reagents—Monoclonal antibodies to Ax II, SHP-2, and β-catenin were purchased from Transduction Laboratories (Lexington, KY). A monoclonal antibody to phosphotyrosine (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). A polyclonal antibody to actin was purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega (Madison, WI). Alexa fluor-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Digitonin was purchased from Calbiochem. All other chemicals were purchased from Sigma.

Cell Culture, Homogenization, and Fractionation—Subconfluent or confluent cultures of CPAE cells (ATCC, Manassas, VA) were grown, harvested, and homogenized in cytosol buffer (25 mM HEPES, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 0.2 mM sucrose, 1 mM dithiothreitol, 1 mM ATP, 0.01 mM mg/lamoguline methyl ester, 4 μg/ml leupeptin, 1 mM benzamidine, 1 mM 1,10-phenanthrene, 0.2 mM phenylmethylsulfonyl fluoride) as previously described (7) unless oth-

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The abbreviations used are: CPAE, cow pulmonary aortic endothelial; Ax II, annexin II; PTP, protein tyrosine phosphatase; PBS, phosphate-buffered saline; SH, Src homology; LDM, low-density membrane; HDM, high-density membrane.
FIG. 1. Dependence of tyrosine phosphorylation of CPAE membrane proteins on cell confluence and cholesterol. Subconfluent or confluent CPAE cells were incubated with or without 6% cyclodextrin for 20 min at 37 °C, harvested, and homogenized in cytosol buffer containing 1 mm ATP and 1 mm Na3VO4. Postnuclear supernatants were normalized for total protein, and equal volumes were centrifuged to yield membranes, which were analyzed on SDS gels, immunoblotted, and probed with an antibody to phosphotyrosine. Bands labeled as γ-catenin and pp120 were identified previously in antiphosphotyrosine immunoprecipitates (7).

To separate membranes into low- and high-density fractions, 0.5 ml of unfractionated membranes in cytosol buffer were layered on to 0.4 ml of a 35% sucrose cushion in cytosol buffer and centrifuged at 97,000 g for 20 min. Low-density or high-density membranes were collected from the 0/35% sucrose interface or the bottom of the 35% cushion, respectively. The membrane fractions were diluted 5-fold with cytosol buffer, with or without digitonin (0.01%), and centrifuged. Equal volumes of extracts were assayed for PTPase activity. Columns marked 'Δ' represent the difference in activities of the extracts prepared with or without digitonin. 1.0 unit of PTPase activity equals 1.0 nmol of phosphate hydrolyzed/min/mg of protein in membranes prior to extraction. In A, the PTPase activity of the unextracted membranes from confluent or subconfluent cells was 8.8 ± 1.0 or 7.3 ± 2.1 units, respectively (n = 3). In B, the PTPase activity of the unextracted membranes from untreated or cycloheximide-treated cells was 7.7 ± 2.6 or 9.0 ± 3.5 units, respectively (n = 3).

FIG. 2. Extraction of PTPase activity from CPAE membranes with digitonin. Subconfluent or confluent CPAE cells were incubated with or without 6% cyclodextrin for 20 min at 37 °C, harvested, and homogenized in cytosol buffer. Membranes were prepared from postnuclear supernatants, which were then normalized for total protein, extracted with cytosol buffer, with or without digitonin (0.01%), and centrifuged. Equal volumes of extracts were assayed for PTPase activity. Columns marked 'Δ' represent the difference in activities of the extracts prepared with or without digitonin. 1.0 unit of PTPase activity equals 1.0 nmol of phosphate hydrolyzed/min/mg of protein in membranes prior to extraction. In A, the PTPase activity of the unextracted membranes from confluent or subconfluent cells was 8.8 ± 1.0 or 7.3 ± 2.1 units, respectively (n = 3). In B, the PTPase activity of the unextracted membranes from untreated or cycloheximide-treated cells was 7.7 ± 2.6 or 9.0 ± 3.5 units, respectively (n = 3).

beads were solubilized in SDS gel sample buffer and analyzed by SDS-polyacrylamide gradient gel electrophoresis and immunoblotting with antibodies to SHP-2 and Ax II.

Immunofluorescence—Cells were plated on glass coverslips at the indicated densities and grown for 2 days. Where indicated, confluent cells were treated with 4% methyl-β-cyclodextrin for 3 min at 37 °C immediately prior to fixation. Cells were then fixed in ice-cold methanol for 15 min at −20 °C, washed three times with phosphate-buffered saline (PBS), incubated in blocking solution (fetal bovine serum (1.0%) plus Triton X-100 (0.5%) in PBS) for 15 min at room temperature, and incubated overnight at 4 °C with a monoclonal antibody to either SHP-2 or Ax II and a polyclonal antibody to actin, both diluted 100-fold in blocking buffer. Coverslips were washed three times for 5 min each in blocking buffer, incubated for 1 h at room temperature in Alexa 488-conjugated goat anti-mouse IgG and Alexa 594-conjugated goat anti-rabbit IgG, both diluted 1:400 in blocking buffer, washed as before, and mounted using Anti-Fade Mounting Medium (Molecular Probes). Cells were imaged using a Leica TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany), using the argon and krypton lasers to excite the anti-mouse and anti-rabbit antibodies respectively. Bleed-through was routinely eliminated by adjusting laser intensities and signal gain so that the cross-channel signal in the scanned images was zero.
immunoblotting.

Membranes were prepared and extracted with or without digitonin (0.1%). Supernatants were analyzed for SHP-2 and Ax II by immunoblotting. C, subconfluent or confluent CPAE cells were incubated with or without 6% cyclodextrin for 20 min at 37°C, harvested, and homogenized in cytosol buffer. Membranes were prepared and extracted with or without digitonin (0.1%). Supernatants were analyzed for SHP-2 and Ax II by immunoblotting. Membranes were prepared, extracted with either digitonin (0.01%) or phenyl phosphate (40 mM), and centrifuged. Supernatants were analyzed on 7.5–17.5% polyacrylamide gradient SDS gels. Proteins were either immunoblotted and probed for SHP2 and Ax II or visualized using Sypro Ruby protein stain (Molecular Probes). The band at the position of 66 kDa in the lane at the far left is an artifact.

**RESULTS**

**Dependence of Tyrosine Phosphorylation of Membrane Proteins on Cholesterol**—Earlier studies (3) had indicated that tyrosine phosphorylation of adherens junctions proteins decreased as endothelial cells formed confluent monolayers in culture. Recently (7) we reported that depletion of cholesterol by methyl-β-cyclodextrin induced tyrosine phosphorylation of several membrane proteins, including pp120 and γ-catenin. To determine the extent to which methyl-β-cyclodextrin reversed tyrosine dephosphorylations, which occur as endothelial cells reach confluence in culture, we directly compared tyrosine phosphorylation of adherens junction proteins determined in cytosol buffer. Membranes were prepared and extracted with or without digitonin (0.1%) or phenyl phosphate (40 mM), and centrifuged. Supernatants were analyzed on 7.5–17.5% polyacrylamide gradient SDS gels. Proteins were either immunoblotted and probed for SHP2 and Ax II or visualized using Sypro Ruby protein stain (Molecular Probes). The band at the position of 66 kDa in the lane at the far left is an artifact.

**Other Biochemical Methods**—Protein mixtures were analyzed on 7.5–17.5% polyacrylamide gradient SDS gels. Polypeptide bands were stained using undiluted Sypro Ruby protein reagent (Molecular Probes) overnight, rinsed in water, and imaged using a 300-nm UV transilluminator. Antibodies were diluted 1:2500–1:5000 for immunoblotting, horseradish peroxidase-conjugated antibodies were detected on immunoblots using a chemiluminescence reagent from PerkinElmer Life Sciences. Protein concentrations were determined using a Coomassie Blue-based reagent (number 500–0006) from Bio-Rad. Polyacrylamide gels and immunoblots are representative of at least three separate experiments.

**Results**

Dependence of Tyrosine Phosphorylation of Membrane Proteins on Cholesterol—Earlier studies (3) had indicated that tyrosine phosphorylation of adherens junctions proteins decreased as endothelial cells formed confluent monolayers in culture. Recently (7) we reported that depletion of cholesterol by methyl-β-cyclodextrin induced tyrosine phosphorylation of several membrane proteins, including pp120 and γ-catenin. To determine the extent to which methyl-β-cyclodextrin reversed tyrosine dephosphorylations, which occur as endothelial cells reach confluence in culture, we directly compared tyrosine phosphorylation of adherens junction proteins determined in cytosol buffer. Membranes were prepared and extracted with or without digitonin (0.1%) or phenyl phosphate (40 mM), and centrifuged. Supernatants were analyzed on 7.5–17.5% polyacrylamide gradient SDS gels. Proteins were either immunoblotted and probed for SHP2 and Ax II or visualized using Sypro Ruby protein stain (Molecular Probes). The band at the position of 66 kDa in the lane at the far left is an artifact.

**Cholesterol-dependent Binding of PTPase Activity to Membranes**—We sought to identify mechanisms by which cholesterol could regulate or localize tyrosine kinase or phosphatase activity. As noted earlier, digitonin, at very low (<0.02%) concentrations, binds specifically to membrane cholesterol (9) without disrupting other membrane lipids. Therefore, to determine whether any PTPases bound to membranes in a cholesterol-dependent manner, we measured the amount of PTPase activity that could be extracted by 0.01% digitonin from membranes prepared from confluent CPAE cells (Fig. 2). Extraction with buffer alone solubilized 0.2 ± 0.1 units (1 unit = 1 nmol of phosphate hydrolyzed/min/mg of membrane protein) of activity, whereas buffer containing digitonin solubilized 1.5 ± 0.2 units of activity. Thus, digitonin specifically extracted 1.3 ± 0.2 units of activity, representing 15% of the total membrane-bound activity (1.3/8.8; see legend to Fig. 2). In contrast, only 0.5 ± 0.2 units of activity could be specifically extracted with digitonin from membranes from subconfluent cells, representing just 7% (0.5/7.3; see legend to Fig. 2) of the total membrane-associated activity. Thus, 2.6-fold more PTPase activity was specifically extractable with digitonin from membranes from confluent rather than from subconfluent cells. Treatment of intact cells with methyl-β-cyclodextrin reduced the activity specifically extracted with digitonin from 1.4 ± 0.2 to 0.8 ± 0.2 units (Fig 2B), which represented, respectively, 18 and 9% of the total membrane-associated activity (1.4/77.7 and 0.8/9.0; see legend to Fig. 2), indicating that this activity did, in fact, associate with membranes in a cholesterol-dependent manner.

**Cholesterol-dependent Binding of SHP-2 to Membranes from Confluent Cells**—To determine the identity of the digitonin-extractable PTPase, we probed extracts from subconfluent or confluent cells for several known PTPases by immunoblotting.
A small but reproducible amount of SHP-2 was specifically extracted by digitonin from membranes from confluent, but not subconfluent, cells, although membranes from subconfluent and confluent cells contained almost equal amounts of the protein (Fig. 2). As reported previously (7), substantial amounts of Ax II could also be extracted from membranes from confluent cells with digitonin, and virtually no Ax II was detected in membranes from subconfluent cells, consistent with their low levels of cholesterol. Prior treatment of confluent cells with methyl-β-cyclodextrin substantially reduced the amounts of both SHP-2 and Ax II extracted by digitonin (Fig. 3B), confirming that both proteins were at least partially bound to membranes in a cholesterol-dependent manner.

SHP-2 contains two N-terminal Src homology (SH2) domains, which bind to numerous tyrosine phosphorylated proteins (5, 14–19). In addition, SHP-2 contains two tyrosine phosphorylation sites, one of which binds to the SH2 domain of GRB2 (20). To begin to evaluate the role of these sites in the cholesterol-dependent binding of SHP-2 to membranes, we extracted membranes from confluent cells with phenyl phosphate (40 mM), a competitive inhibitor of phosphotyrosine/SH2 interactions (Fig. 3C). In contrast to digitonin, phenyl phosphate extracted only trace levels of SHP-2 and Ax II, although SDS gels stained for total protein indicated that several other polypeptides were specifically extracted by phenyl phosphate. Thus, the cholesterol-dependent binding of SHP-2 to membranes appeared to occur by a mechanism involving other regions of SHP-2 besides its SH2 domains.

Identification of a Distinct Subcellular Pool of SHP-2, Dependent on Cell Confluence and Cholesterol—We reasoned that the digitonin-extractable SHP-2 in confluent cells might represent a separate pool of the protein. Since cholesterol-rich plasma membrane domains often migrate as low-density vesicles when centrifuged through sucrose density gradients, we separated membranes from confluent CPAE cells into low-density (LDM) and high-density (HDM) fractions and extracted them either with or without digitonin (Fig. 4A). SHP-2 was distributed approximately equally between the LDM and HDM fractions. However, while over half of the SHP-2 in the LDM could be extracted using digitonin, virtually none of the SHP-2 in the HDM fraction was solubilized by digitonin. Thus, the digitonin-extractable SHP-2 in confluent cells represented a distinct pool of the protein residing in a separate compartment. In contrast to SHP-2, β-catenin could not be extracted from either the LDM or HDM with digitonin, although SHP-2 has been reported to bind to β-catenin in vitro assays (5). As noted previously, virtually all the Ax II was found in the LDM, and a substantial amount of Ax II could be extracted with digitonin (7).

Since the digitonin-extractable SHP-2 in confluent cells was localized almost exclusively to the LDM, and subconfluent cells contained no digitonin-extractable SHP-2, it seemed plausible that SHP-2 was recruited to the LDM as cells reached confluence. In fact, in contrast to confluent cells, the LDM fraction from subconfluent cells contained virtually no SHP-2, while the HDM fraction contained substantial amounts of the protein. Thus, SHP-2 and Ax II were both recruited to membranes in the LDM fraction by a cholesterol-dependent mechanism as endothelial cells reached confluence.

Localization of SHP-2 and Ax II to Adhesion Bands and Stress Fibers—To identify the intracellular structures to which Ax II and SHP-2 were recruited, we visualized SHP-2 and Ax II in CPAE cells by confocal immunofluorescence microscopy (Fig. 5, A and B). In confluent cells, both SHP-2 and Ax II were visualized at sites of cell-cell adhesion, although diffuse, granular staining was also observed. Brief (3 min) treatments with methyl-β-cyclodextrin almost entirely dissociated Ax II and SHP-2 from the cell periphery. Generally, actin fibers were more readily visualized after treatment with methyl-β-cyclodextrin, suggesting that stress fibers were released from the cell periphery to the interior, although in some cells peripheral actin bundles were still seen. In many cells, retraction of the plasma membrane was evident, as noted previously (7). No bleed-through was detected in the SHP-2 or Ax II channels when cells were illuminated at the excitation frequency of the actin-bound fluorophore only (see Supplementary Fig. 1 at http://www.jbc.org).

At the lowest cell density, Ax II immunofluorescence was generally faint, consistent with the low levels of particulate Ax II in these cells (Fig. 3A), although some co-linear staining with actin could be detected, and SHP-2 exhibited clear co-localization with actin. At somewhat higher densities, both Ax II and SHP-2 were visualized along actin bundles at the cell periphery, as well as along stress fibers, although some diffuse granular staining was also observed. Generally, confluent cells showed a somewhat lower level of stress fiber staining, suggesting that actin filaments were redistributed from the cell interior to the periphery as intercellular junctions formed.

Identification of a Membrane-bound Complex Containing SHP-2 and Ax II—Our studies indicated that, as endothelial cells reached confluence, SHP-2 and Ax II are coordinately recruited to a low-density membrane fraction, apparently derived from cell-cell adhesion sites. We reasoned, therefore, that SHP-2 and Ax II might be physically associated in confluent...
FIG. 5. Localization of SHP-2 and Ax II to the actin cytoskeleton. CPAE cells were plated at the indicated cell densities on glass coverslips. After 2 days of growth, only cells plated at the highest density were confluent. One set of confluent cultures was treated with 6% methyl-2-cyclodextrin for 3 min immediately prior to fixation. Cells were fixed with methanol at −20°C for 15 min and processed for immunofluorescence microscopy using a monoclonal antibody to either SHP-2 (A) or Ax II (B) and a polyclonal antibody to actin. Images shown are confocal sections in the plane of adherens junctions, i.e. approximately one-third the distance from the basolateral to the apical surface. Scale bars equal 40 μm.
FIG. 5—continued

Regulation of SHP-2 by Cholesterol and Cell Confluence
washed extensively and immunoblotted for SHP-2 and Ax II. The beads were then incubated with or without a monoclonal antibody to SHP-2. The beads were washed extensively and immunoblotted for SHP-2 and Ax II.

CFAE cells. To test this hypothesis, we prepared immunoprecipitates from membrane extracts using a monoclonal antibody to SHP-2 and probed them for Ax II (Fig. 6). A crude membrane fraction from confluent CFAE cells was extracted with 0.2% Nonidet P-40 and centrifuged, and the supernatant was immediately diluted 20-fold with cytosol buffer and incubated overnight at 4 °C with anti-mouse IgG-agarose beads, which had previously been incubated with or without a monoclonal antibody to SHP-2. The beads were then washed extensively and immunoblotted for SHP-2 and Ax II.

DISCUSSION

We have found that the tyrosine phosphorylation levels of membrane proteins in endothelial cells appear to be regulated by cholesterol levels: as endothelial cells reached confluence, tyrosine phosphorylation levels decreased as cholesterol levels increased (7), and partial depletion of cholesterol with methyl-β-cyclodextrin restored tyrosine phosphorylation levels to those seen in subconfluent cells (Fig. 1). While other studies have also indicated inverse relationships between tyrosine phosphorylation and cholesterol (21–24), our observations represent the first instance in which physiological changes in cholesterol are linked both to physiologically significant tyrosine phosphorylation events and to the subcellular relocalization of a tyrosine kinase or phosphatase. Our observations also provide a mechanism for the attenuation of signaling pathways at higher cell densities in the presence of high concentrations of activating ligands.

Our observations indicate that SHP-2 is recruited to sites of cell-cell contact as endothelial cells reach confluence, apparently as a function of increasing cholesterol. While SHP-2 has been found to associate with numerous signaling proteins via its SH2-domains, this recruitment appears to be at least in part independent of phosphotyrosine/SH2 interactions, since almost none of the membrane-bound SHP-2 in our preparations was extractable with phenyl phosphate (Fig. 3C) and the extraction of SHP-2 with digitonin was only seen at high cell densities, where membrane proteins were tyrosine phosphorylated at very low levels (Fig. 1; cf. Refs. 3 and 7). In endothelial cells, the SH2 domains of SHP-2 have been reported to interact with phosphotyrosines in both platelet endothelial cell adhesion molecule-1 (18) and β-catenin (5), both of which have been localized to sites of cell-cell contact. However, these interactions were observed when intact cells were treated with orthovanadate, which induces tyrosine phosphorylation of membrane proteins, and destabilizes adherens junctions. Thus, it seems plausible that the binding of SHP-2 to platelet endothelial cell adhesion molecule-1 and β-catenin occurs in response to signals initiated by growth factors, e.g., vascular endothelial growth factor, which leads to the breakdown of intercellular junctions and the resumption of the cell cycle. In contrast, the cholesterol-dependent binding of SHP-2 to membranes that we report here was observed in the absence of orthovanadate and may represent a mechanism for maintaining junctional stability.

The finding that Ax II co-immunoprecipitates with SHP-2 from membrane extracts from confluent endothelial cells suggests that the two proteins are recruited as a complex to the cell periphery with increasing cell density and membrane cholesterol. Ax II has also been localized to adhesions between confluent Madin-Darby canine kidney cells (25). Ax II has been found to bind directly to anionic liposomes (11, 12), and cholesterol has been reported to enhance this binding (26), suggesting that an Ax II/SHP-2 complex could bind directly to membrane lipid domains enriched in cholesterol. Membrane anchoring could also occur through an intrinsic membrane protein such as CD44, which was recently found to anchor Ax II to detergent-resistant, cholesterol-rich membranes (rafts) in mammary epithelial EpH4 cells (27). Both Ax II (27–30) and SHP-2 (31) have been identified in rafts in cultured cells, and cholesterol-sequestering reagents have been found to dissociate Ax II from both chromaffin granules (26) and endosomes (32). Alternatively, it is conceivable that the localization of either Ax II or SHP-2 to sites of cell-cell attachment could occur through direct binding to actin, since both Ax II (33, 34) and SHP-2 (35) have been reported to bind to actin filaments in vitro, and both proteins were found along stress fibers in subconfluent endothelial cells (Fig. 5).

The nature of the plasma membrane domains to which SHP-2 and Ax II are recruited with increasing cell confluence is not clear. Ax II has been found not to co-purify with caveolae isolated from rat lung blood vessels (36), and caveolin did not localize to cell junctions in endothelial cells,2 in contrast to Ax II (Fig. 6). Our findings that much of the membrane-bound Ax II in confluent cells is extractable with low levels of digitonin and that Ax II is predominantly localized to intercellular junctions argue that these domains are also found at intercellular junctions. Nevertheless, we have found that confluent 3T3L1 fibroblasts, which are contact-inhibited, but do not elaborate defined junctional structures, also contain both elevated levels of cholesterol and Ax II and SHP-2, which can be extracted with low levels of digitonin.3 In addition, we have found that, in growing endothelial monolayers, cholesterol and membrane-bound Ax II increased prior to the formation of cell junctions (7). These observations strongly suggest that the domains to which Ax II and SHP-2 are recruited are not uniquely targeted to intercellular junctions but might arise more generally in contexts where the attenuation of tyrosine kinase-based signaling is required. Further studies will be needed to clarify the precise nature of these domains and the range of signaling processes which they regulate.

3 A. Burkart, S. Corvera, and H. S. Shpetner, unpublished observations.

2 H. S. Shpetner, unpublished observations.
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REFERENCES

1. Sorby, M., and Ostman, A. (1996) J. Biol. Chem. 271, 10963–10966
2. Rahimi, N., and Kastlauskas, A. (1999) Mol. Cell 10, 3401–3407
3. Lampugnani, M. G., Corada, M., Andrionpoulos, P., Eiser, S., Rizzu, W., and Dejana, E. (1997) J. Cell Sci. 110, 2065–2077
4. Shubamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeuchi, M., and Iti, F. (1994) Cell Adhes. Commun. 1, 295–305
5. Ukropec, J. A., Hollinger, M. K., Salva, S. M., and Wolkalis, M. J. (2000) J. Biol. Chem. 275, 5983–5986
6. Matsumiya, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S., and Takeuchi, M. (1992) J. Cell Biol. 118, 703–714
7. Carvera, S., DiBonaventura, C., and Shpetner, H. S. (2000) J. Biol. Chem. 275, 31414–31421
8. Harder, T., Kellner, R., Parton, R. G., and Grueenberg, J. (1997) Mol. Biol. Cell 8, 533–545
9. Elias, P. M., Goerke, J., Friend, D. S., and Brown, B. E. (1978) J. Cell Biol. 78, 577–596
10. Esparis-Ogando, A., Zurzolo, C., and Rodriguez-Boulan, E. (1994) Am. J. Physiol. 267, C166–C176
11. Drust, D. S., and Creutz, C. E. (1988) Nature 331, 88–91
12. Johnstone, S. A., Hubaishy, I., and Waisman, D. M. (1992) J. Biol. Chem. 267, 25976–25981
13. Uittenbogaard, A., Eversen, V. W., Matveev, S. V., and Smart, E. J. (2002) J. Biol. Chem. 277, 4925–4931
14. Case, R. D., Piccinine, E., Wolf, G., Bennett, A. M., Lechleider, R. J., Neel, B. G., and Shoelson, S. E. (1994) J. Biol. Chem. 269, 10467–10474
15. Hausberger, S. F., Bennett, A. M., Neel, B. G., and Birnbaum, M. J. (1995) J. Biol. Chem. 270, 12865–12868
16. Siminovitch, K. A., and Neel, B. G. (1998) Semin. Immunol. 10, 329–347
17. Hu, Y., Zent, J. M., and Gimbrone, M. A., Jr. (2001) J. Biol. Chem. 276, 48549–48553
18. Masuda, M., Osawa, M., Shigenatsu, H., Harada, N., and Fujiiwa, K. (1997) FEBS Lett. 408, 331–336
19. Noguchi, T., Matozaki, T., Fujinaka, Y., Yamae, T., Tsuda, M., Takada, T., and Kasuga, M. (1996) J. Biol. Chem. 271, 27652–27658
20. Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7385–7389
21. Furuichi, T., and Andersson, R. G. (1998) J. Biol. Chem. 273, 21099–21104
22. Visconti, P. E., Galantino-Homer, H., Ning, X., Moore, G. D., Valenzuela, J. P., Jorgon, C. J., Alvarez, J. G., and Kopf, G. S. (1999) J. Biol. Chem. 274, 3235–3242
23. Kabouridis, P. S., Janzen, J., Magee, A. L., and Ley, S. C. (2000) Eur. J. Immunol. 30, 954–963
24. Barabe, F., Pate, G., Fernandes, M. J., Bourgoin, S. G., and Nacache, P. H. (2002) J. Biol. Chem. 277, 13473–13478
25. Harder, T., and Gerke, V. (1993) J. Cell Biol. 123, 1119–1132
26. Ayala-Santmartin, J., Henry, J. P., and Pradel, L. A. (2001) Biochim. Biophys. Acta 1510, 18–28
27. Oliferenko, S., Paiba, K., Harder, T., Gerke, V., Schwarzler, C., Schwarz, H., Beug, H., Gunthert, U., and Huber, L. A. (1999) J. Cell Biol. 146, 843–854
28. Harder, T., and Gerke, V. (1994) Biochim. Biophys. Acta 1233, 375–382
29. Babichuk, E. B., and Draeger, A. (2000) J. Cell Biol. 150, 1113–1124
30. Sasag, I., Regnouf, F., Henry, J. P., and Pradel, L. A. (1997) FEBS Lett. 410, 229–234
31. Lacalle, R. A., Mira, E., Gomez-Mouton, C., Jimenez-Baranda, S., Martinez, A. C., and Manes, S. (2002) J. Cell Biol. 157, 277–289
32. Zeuschaer, D., Stoerndahl, W., and Gerke, V. (2001) Eur. J. Cell Biol. 80, 499–507
33. Jones, P. G., Moore, G. J., and Waisman, D. M. (1992) J. Biol. Chem. 267, 12993–12997
34. Filipenko, N. R., and Waisman, D. M. (2001) J. Biol. Chem. 276, 5310–5315
35. Xu, F., Zhao, R., Peng, Y., Guerriera, A., and Zhao, Z. J. (2001) J. Biol. Chem. 276, 29479–29484
36. Stan, B. V., Roberts, W. G., Predescu, D., Iida, K., Saucon, L., Ghitesu, L., and Palade, G. E. (1997) Mol. Biol. Cell 8, 595–605