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p38 mitogen-activated protein kinase interacts with vinculin at focal adhesions during fatty acid-stimulated cell adhesion

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Abstract

Arachidonic acid stimulates cell adhesion by activating $\alpha 2\beta 1$ integrins in a process that depends on protein kinases, including p38 mitogen activated protein kinase. Here, we describe the interaction of cytoskeletal components with key signaling molecules that contribute to spreading of, and morphological changes in, arachidonic acid-treated MDA-MB-435 human breast carcinoma cells. Arachidonic acid-treated cells showed increased attachment and spreading on collagen type IV as measured by electric cell-substrate impedance sensing. Fatty acid-treated cells displayed short cortical actin filaments associated with an increased number of $\beta 1$ integrin-containing pseudopodia whereas untreated cells displayed elongated stress fibers and fewer clusters of $\beta 1$ integrins. Confocal microscopy of arachidonic acid-treated cells showed that vinculin and phospho-p38 both appeared enriched in pseudopodia and at the tips of actin filaments, and fluorescence ratio imaging indicated the increase was specific for the phospho-(active) form of p38. Immunoprecipitates of phospho-p38 from extracts of arachidonic acid-treated cells contained vinculin, and GST-vinculin fusion proteins carrying the central region of vinculin bound phospho-p38, whereas fusion proteins expressing the terminal portions of vinculin did not. These data suggest that phospho-p38 associates with particular domains on critical focal adhesion proteins that are involved in tumor cell adhesion and spreading and that this association can be regulated by factors in the tumor microenvironment.

Keywords

p38 MAP kinase; Vinculin; Signal transduction; Arachidonic acid; Cell adhesion

Introduction

The adhesion of tumor cells to extracellular matrix (ECM) is a critical step in the process of cancer metastasis (Evans 1991; Weinberg 2007). Components of the tumor microenvironment modulate tumor cell behavior such as adhesion and invasion, often inducing cells to metastasize more readily [for recent reviews, see (Kenny and Bissell 2003; Kopfstein and Christofori 2006; Funasaka and Raz 2007; Kass et al. 2007; Li et al. 2007; Bidard et al. 2008; Le Bitoux and Stamenkovic 2008; Kim et al. 2011)]. Fatty acids are present in the body in significant amounts, originating both from dietary sources and from release by phospholipases from cell membranes (Perez-Chacon et al. 2009). Activation of platelets results in increased release of arachidonic acid from membrane lipid stores, implicating inflammatory responses in microenvironmental changes that can influence tumor cell behavior (Coussens and Werb 2002; Zou 2005; Erpenbeck and Schon 2010).

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The interaction between cells and the ECM involves cell adhesion receptors, the most ubiquitous of which are the integrins, a family of heterodimeric transmembrane surface glycoproteins (Hynes 1992; Lock et al. 2008; Barczyk et al. 2010; Rathinam and Alahari 2010; Shattil et al. 2010). The function of integrins is highly regulated (Desgrosellier and Cheresch 2010; Shattil et al. 2010; Kim et al. 2011). Previous work showed that *cis*-polyunsaturated fatty acids stimulated $\alpha_2\beta_1$ integrin-mediated adhesion of MDA-MB-435 cells to type IV collagen, without causing a detectably significant increase in the amount of cell surface β_1 or α_2 integrin subunits (Palmantier et al. 2001), suggesting that increased adhesion occurs in the absence of increased expression of cell surface integrins, and implicating other mechanisms.

Integrins are often clustered at focal adhesions, specialized sites of firm attachment of cells to the underlying ECM. Bundles of actin filaments often appear to terminate in focal adhesions, consistent with the notion that these sites provide a link between the actin cytoskeleton and the ECM (Chen et al. 2006; Geiger et al. 2009; Wolfenson et al. 2009; Yilmaz and Christofori 2009). Regulation of the activity of integrins potentially involves a number of signal transduction pathways plus key proteins that make up the cytoskeleton (Ziegler and Wolfgang 2008; Huvneers and Danen 2009; Guarino 2010). Structural and signaling proteins are aggregated in the focal adhesion sites on the cytoplasmic surface of the plasma membrane in association with actin stress fibers and integrin cytoplasmic domains. Several enzymes known to participate in signal transduction pathways have been identified within focal adhesions, including protein kinase C (PKC) isotypes and tyrosine kinases (Burridge et al. 1992; Kornberg et al. 1992). These enzymes may modify, or be regulated by, other proteins that are present in, or transiently associated with, the focal adhesion complex.

The actin cytoskeleton plays a critical role in regulating adhesive behavior in response to a variety of external stimuli (Brakebush and Fassler 2003). Arachidonic acid signaling can regulate F-actin formation and spreading of HeLa cells (Chun et al. 1997; Glenn and Jacobson 2002). However, the effects of arachidonic acid on the structural components of MDA-MB-435 cells during adhesion and spreading have not been previously characterized. Therefore, elucidating the molecular events involved in regulation of the adhesive phenotype in cancer cells could reveal targets for therapeutic prevention or treatment of various cancers (Dollé et al. 2006).

We have reported that 15-lipoxygenase-2 and PKC activities are involved in the rapid increased in adhesion of MDA-MB-435 cells to type IV collagen following addition of arachidonic acid (Palmantier et al. 1996; Palmantier et al. 2001; Nony et al. 2005). We also showed that the mitogen activated protein (MAP) kinase family protein p38 was necessary for arachidonic acid-mediated cell adhesion and that p38 activated its downstream substrate MAPKAPK2, and the small heat shock protein, HSP27 (Paine et al. 2000).

To unravel further the web of signaling pathways and protein interactions involved in fatty acid-mediated adhesion, we investigated the effects of arachidonic acid on the formation of focal adhesion complexes and the distribution of signaling and cytoskeletal proteins such as p38 MAP kinase, actin, α -actinin, talin and vinculin during cell attachment and spreading on collagen IV substrates. In this study we have used immunofluorescence microscopy to examine whether the rearrangement of actin filaments, focal adhesions, cytoskeletal proteins, and phosphotyrosine-containing proteins may be consistent with the arachidonic acid-induced increased adhesion of breast cancer cells to type IV collagen. Here we present the novel finding that the abundance of phosphorylated p38 appeared to increase in focal adhesion sites following treatment with arachidonic acid. In addition, immunoprecipitates of phospho-p38 MAPK from extracts of arachidonic acid-treated cells contained vinculin and

purified phospho-p38 MAPK was found to bind *in vitro* to a vinculin fragment corresponding to the central portion of this critical focal adhesion protein. These data suggest that phosphorylated p38 MAPK interacts with a specific domain on a cytoskeletal protein involved in the formation of focal adhesions during tumor cell adhesion and spreading and may provide insights into new targets for blocking specific steps in the metastatic cascade.

Materials and methods

Cell culture and reagents

MDA-MB-435 human cancer cells were obtained from Dr. Janet Price (Department of Cell Biology, M. D. Anderson Comprehensive Cancer Center, Houston, TX) and cultured as described (Price et al. 1990). Arachidonic acid (Cayman Chemical Co., Ann Arbor, MI) was obtained as a 328 mM solution in ethanol. Type IV collagen was from Becton Dickinson (Mansfield, MA). Versene and bovine serum albumin (BSA) fraction V were from Life Technologies (Carlsbad, California). Primary antibodies were obtained as follows: affinity-purified goat polyclonal anti- α -actinin (C-20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); monoclonal anti- α -actinin, (mouse ascites fluid clone BM-75.2) from Accurate Chemical & Scientific Corp. (Westbury, NY); monoclonal anti-human vinculin, (mouse ascites fluid, clone hVIN-1) from Sigma Chemical Co. (St. Louis, MO); mouse monoclonal IgG anti-phosphotyrosine, clone 4G10 and anti-human talin (mouse monoclonal IgG₁, clone TA205) from Upstate Biotechnology, Inc. (Waltham, MA). Rat anti-human β_1 integrin, monoclonal antibody 13, was prepared as described (Akiyama et al. 1989). Mouse monoclonal phospho-p38 MAPK (28B10) and rabbit polyclonal p38 MAPK were from Cell Signaling Technology (Danvers, MA). F-actin was localized using biotin-XX-phalloidin (Life Technologies/Invitrogen, Grand Island, NY). Secondary antibodies; Alexa Fluor 660-phalloidin; Image-iT FX Signal Enhancer and ProLong® Gold Antifade mounting medium with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) were from Life Technologies/Molecular Probes (Grand Island, NY). Gelatin and Triton X-100 were from Sigma. Paraformaldehyde was from Electron Microscopy Sciences (Ft. Washington, PA). Goat serum was from Zymed Laboratories, Inc. (San Francisco, CA). Dimethylsulfoxide (DMSO) was from American Type Culture Collection (Rockville, MD).

DNA vectors expressing glutathione S-transferase (GST)-fusion proteins with specific portions of vinculin were the kind gift of Drs. Kris DeMali and Keith Burrridge (University of North Carolina School of Medicine) and have been previously described (DeMali et al. 2002).

All procedures were carried out at room temperature (19 – 22°C) unless specified otherwise.

Cell harvesting and treatment

Subconfluent cells were harvested by a brief incubation with Versene and resuspended in complete minimum essential medium (MEM). Cells were washed twice with serum-free culture medium and resuspended in the same medium at a final concentration of 1.0×10^5 cells / ml. Cells were allowed to recover for 30 min at 37°C in a 5% CO₂ atmosphere before being treated with arachidonic acid. Arachidonic acid was prepared prior to addition to the cells either by adding an equal volume of 328 mM KOH to the arachidonic acid stock solution and diluting the mixture to 6 mM arachidonic acid in 0.9% NaCl, or by diluting the arachidonic acid stock solution in DMSO. Unless indicated otherwise, arachidonic acid was added to cells from concentrated stocks to yield a final concentration of 30 μ M.

Cell attachment and spreading

Cell attachment and spreading were assayed by an electric cell-substrate impedance sensing (ECIS) apparatus (model ECIS1600R, Applied Biophysics, Troy, NY). Applied Biophysics 8w10E+ arrays were washed overnight with serum free culture medium, then coated overnight at 4°C with 200 µl human collagen type IV at 3 µg/ml, diluted in calcium/magnesium-free phosphate buffered saline (137 mM NaCl, 1.7 mM KCl, 1 mM KH₂PO₄, 7.4 mM Na₂HPO₄) (PBS-) containing 10 mM acetic acid. Wells were washed twice with PBS- prior to addition of cells. Cells were plated at 50,000 cells per well in 200 µl of serum free MEM with either 30 µM arachadonic acid or an appropriate amount of vehicle. The array was placed into a 37°C incubator maintaining 5% CO₂ and impedance was monitored for 2 h, measuring the impedance across the surface of each well every 6.56 sec. Once the assay was complete, cells were washed twice in PBS-, fixed in 1% paraformaldehyde, and visually counted (three fields per well) to determine if there were differences in cell attachment between replicates at the end of the assay.

Cell treatment for immunofluorescence

For localization of stress fibers and focal adhesion components, MDA-MB-435 cells were harvested and allowed to recover as above. Cells were then suspended in serum-free medium and plated on type IV collagen-coated 18-mm diameter glass cover slips for 90 min in serum-free medium with or without arachidonic acid. At the appropriate time, the medium was removed, and cells were fixed and permeabilized using a modification of a previously described protocol (Chen et al. 1986).

Localization of specific proteins was carried out as follows. First, nonspecific binding sites were blocked by incubation of fixed cells for 20 min with 0.5% gelatin in phosphate buffered saline with calcium and magnesium (PBS- as above plus 1 mM CaCl₂ and 0.5 mM MgCl₂) (PBS⁺). Cells were then incubated for 20 min with primary antibody (45 min for the 4G10 antibody) in PBS⁺ containing 0.1% gelatin and 1% goat serum. Subsequent washes and antibody dilutions were carried out using PBS⁺ containing 0.1% gelatin and 1% goat serum. Cells were washed three times and incubated with the appropriate secondary antibody. The cover slips were then washed three times, mounted on glass slides with ProLong® Antifade with DAPI.

Cell treatment for fluorescence ratio imaging

Cells were harvested and treated for fluorescence ratio imaging as above with several modifications. Following permeabilization the coverslips were treated with Image-iT FX Signal Enhancer (Life Technologies/Molecular Probes, Grand Island, NY) for 20 min to reduce nonspecific interactions of fluorescent dyes with cell constituents. Mouse monoclonal anti-phospho-p38 (1:50), rabbit polyclonal anti-p38 (1:20), and biotin-XX-phalloidin (1:200) were applied to the cells first, then detected with goat anti-mouse IgG-Alexa Fluor 594, goat anti-rabbit IgG-Alexa Fluor 647, and streptavidin-Pacific Blue respectively. The coverslips were then incubated in mouse monoclonal anti-vinculin that was directly conjugated to FITC to identify focal contacts at the terminal ends of actin stress fibers. Coverslips prepared to account for any non-specific binding of secondary antibodies were treated as above, staining with biotin-XX-phalloidin and anti-vinculin-FITC, and either goat anti-mouse IgG-Alexa Fluor 594 or goat anti-rabbit IgG-Alexa Fluor 647 alone, omitting the anti-phospho-p38 and p38 primary antisera. All coverslips were mounted using Prolong Gold antifade mounting medium without DAPI.

Confocal microscopy

Cells were evaluated with an LSM 410 UV inverted laser scanning microscope (Zeiss, Oberkochen, Germany), equipped with an Omnichrome Argon-Krypton laser. Nomarski differential interference contrast images were obtained with a Zeiss C- Apo 40X (1.2 na) water immersion objective. All other images were obtained with a Zeiss Plan-Apo 100X (1.4 na) oil immersion objective. Absorbance and emission wavelengths, respectively, were 405 nm and a 420-480 nm filter for DAPI; 495 nm and 519 nm for Alexa Fluor 488; 590 nm and 617 nm for Alexa Fluor 594; and 663 nm and 690 nm for Alexa Fluor 660.

Images of cells double stained for phospho-p38 and p38 were acquired with an inverted laser scanning LSM 710 microscope (Carl Zeiss, Oberkochen, Germany). The images were collected sequentially through frame switching using a 405 nm diode laser, 488 nm laser lines from the argon ion laser, a 561 nm diode laser, and a 633 nm helium neon laser as the light source. Images were obtained using a Zeiss Plan-Apo 63X oil (1.4 na) objective lens. For fluorescence imaging the two traditional photomultiplier tubes plus the 32 channel spectral photomultiplier tube were tuned to capture four channels of fluorescence emission in the following ranges: 415-495 nm for streptavidin-Pacific Blue; 498-579 nm for FITC; 584-642 nm for Alexa Fluor 594, and 645-735 nm for Alexa Fluor 647. The confocal pinhole was set to correspond to a z-resolution of 0.9 μ m. For quantitative purposes image acquisition was set for the condition where the fluorescence was expected to be the greatest in the focal contacts, which was in the MDA-MB-435 cells treated with arachidonic acid. All subsequent images, including the background control coverslips, were collected using identical settings.

The process for fluorescence ratio imaging of the relative amounts of phospho-p38 and p38 in focal contacts was a modification of the method published by Zamir et. al (Zamir et al. 1999). MDA-MB-435 cells treated with either vehicle or arachidonic acid were selected for imaging if 1) the cell had attached and begun to spread in the 90 min incubation period and 2) had well defined focal contacts identified by staining with anti-vinculin-FITC. Regions of interest were manually drawn around focal contacts that were identified by the vinculin-FITC antibody and were also localized at the terminal end of actin stress fibers made visible by phalloidin staining. Average pixel intensities from regions of interest identified in the secondary antibody only control coverslips were subtracted from the average pixel intensities of their respective channels (Alexa Fluor 594 for phospho-p38 and Alexa Fluor 647 for p38) from regions of interest identified in vehicle or arachidonic acid treated cells. The software used for image acquisition was the Zeiss Zen 2009 version 5.5 SP1. These values were exported to Microsoft Excel for further analysis using MetaMorph Offline (ver. 7.7.5).

Preparation of cell lysates from adherent cells

Cells were allowed to adhere to collagen type IV for 90 min at 37°C in the presence of 30 μ M arachidonic acid in serum-free MEM supplemented with 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 x MEM vitamins, then washed with 4°C PBS-. Cells were lysed in buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM sodium orthovanadate, 1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin and 2 mg/ml pepstatin (lysis buffer). The cell lysates were sonicated on ice four times for 5 sec each, cleared by centrifugation at 14000 X g for 10 min at 4°C, and subjected to 60 min of preclearing with ImmunoPure Plus (G) Immobilized Protein G beads (Pierce Biotechnology, Rockford, IL).

Immunoprecipitation of phospho-p38 MAPK

Phospho-p38 was immunoprecipitated from cell lysates with a mouse monoclonal IgG antibody (28B10) against phospho-p38 MAPK (Thr180/Tyr182) at a dilution of 1:50 overnight at 4°C with continuous mixing followed by a 3 h incubation at 4°C with 10% (v/v) final concentration of ImmunoPure Plus (G) Immobilized Protein G beads. The adsorbed immune precipitates were washed twice with 500 µl lysis buffer and then solubilized in 3x SDS gel electrophoresis sample buffer, boiled and analyzed by polyacrylamide gel electrophoresis.

Gel electrophoresis and immunoblotting

Precipitated proteins were resolved using the NuPAGE system (Invitrogen) under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membranes. Proteins were detected by incubating the blots with primary antibodies overnight at 4 °C, washing the blots three times for 5 min each in PBST [phosphate-buffered saline, 0.1% (v/v) Tween-20] or TBST [10 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween-20] and then incubating for 90 min with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody. Blots were probed with antibodies for vinculin (diluted 1:400), α -actinin (diluted 1:2000), actin (diluted 1:200), total p38 (1 µg/ml) or active p38 (Cell Signaling; #9211) (diluted 1:1000). Membranes were then blocked for 1 h and incubated with the appropriate secondary antibody, followed by washing as described. Primary antibodies (anti-vinculin, anti- α -actinin, anti-actin and anti-total p38) were used in PBST plus 5% (w/v) dry nonfat milk. The following secondary antibodies were used: HRP-conjugated sheep anti-mouse IgG (diluted 1:3000), HRP-conjugated donkey anti-rabbit IgG (diluted 1:2000), or HRP-conjugated goat anti-mouse IgG (diluted 1:3000) for detection of vinculin, α -actinin and actin, or phospho-p38 and total p38, respectively. Phospho-p38 was probed in TBST with 5% (w/v) BSA. All proteins were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce).

GST-vinculin fusion protein expression and pull-down assay with vinculin fragments

Competent BL21 *E. coli* (Invitrogen) were transfected with GST-vinculin fusion proteins expressing three different regions of vinculin (amino acids 1 to 398, 399 to 881, or 881 to 1066) and cultured in LB broth with ampicillin for 16 h at 37°C. Lysates were prepared by mixing the frozen bacterial cell pellets with NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM NaF, 1 mM Na₃VO₄) and sonicating. Following clarification of the bacterial lysates by centrifugation at 20,000 X g, glutathione-conjugated beads were added to the supernatant and incubated for 2 h at 4°C. The beads containing the GST-vinculin proteins were then collected by centrifugation at 20,000 X g and washed twice with NETN.

MDA-MB-435 cells were harvested, treated with 30 µM arachidonic acid, and lysed as described (Kornberg et al. 1992). The lysates were then added to the bead-glutathione-GST-vinculin complexes and incubated overnight at 4°C. The beads and any bound proteins were then collected by centrifugation at 500 X g and the pellet was washed 3 times with NETN. The final pellets were resuspended in SDS sample buffer and boiled for 5 min. The beads were collected by centrifugation at 200 X g and the released proteins were resolved by SDS polyacrylamide gel electrophoresis using a 4 – 12% gradient Bis-Tris gel (Invitrogen), transferred to a PVDF membrane and detected by immunoblotting.

Statistical Analysis

Student's T-test was used to analyze the data in Figures 4 and 9. The statistical analyses used in Table 1 compared integrated intensities of phospho-p38, total p38 and the ratio of phospho-p38 to total p38 integrated intensity, as well as the area of the region of interest,

between regions from arachidonic acid-treated and vehicle-treated cells. The intensities, their ratios, and the areas of interest were not properly distributed, and log-transformations did not adequately improve this. Therefore, Mann-Whitney tests were used to compare regions of interest from arachidonic acid-treated cells with regions of interest in vehicle-treated cells; p-values from these tests are one-sided; averages shown are geometric means. In addition, two-sided Fisher's exact tests were used to compare the proportions of regions of interest having intensities of 0 between arachidonic acid-treated and vehicle-treated cells.

Results

Arachidonic acid increases attachment and spreading of cells on type IV collagen

Previously published studies showed that arachidonic acid stimulated MDA-MB-435 human metastatic carcinoma cells to attach more readily to type IV collagen by inducing signal transduction pathways that resulted in activation of integrin cell adhesion receptors (Palmantier et al. 1996; Palmantier et al. 2001). In this study, we asked whether arachidonic acid affected longer-term cell adhesive processes such as spreading, focal adhesion formation, and cytoskeletal rearrangements on type IV collagen substrates. We examined cell spreading with an electric cell-substrate impedance-sensing assay. Because the impedance across the surface depends on the amount of the surface covered by cells, both attachment and spreading contribute to the measured signal. Our control experiment used cells treated only with vehicle (ethanol in most experiments) on collagen IV, instead of poly-L-lysine, to get the best possible conditions to examine the effect of arachidonic acid in the presence of authentic integrin-based cell adhesion and spreading. Arachidonic acid-treated cells generated dramatically increased impedance compared to cells untreated with fatty acid (Fig. 1). The change in impedance reached a plateau between 1.5 and 2 h with these cells on collagen IV in the presence of 30 μ M arachidonic acid. Our previous studies on cell adhesion showed that the majority of arachidonic acid-induced cell attachment occurred within 45 minutes (Palmantier et al. 2001). Furthermore, results from multiple spreading experiments in which spreading was monitored by microscopy (not shown) indicate that the overwhelming majority of MDA-MB-435 cells that attach to collagen IV substrates are spread within 90 min. Thus, in order to observe events that are occurring in a significant number of cells as they spread, we chose 90 min of incubation on type IV collagen for the characterization of the cytoskeleton and focal adhesions in these tumor cells.

Arachidonic acid induces a reorganization of the cytoskeleton in MDA-MB-435 cells

Filamentous actin (F-actin) has been shown to play a critical role in the adhesion of cells to extracellular matrix (Higgs and Pollard 2001; Blystone 2004; Wiesner 2005; Olson and Sahai 2009; Vicente-Manzanares et al. 2009). We examined changes in F-actin stress fibers in cells treated with arachidonic acid for 90 min on type IV collagen substrates using an Alexa Fluor 660-labeled phalloidin. Stress fibers of vehicle-treated cells (Fig. 2A) were compared with those of arachidonic acid-treated cells (Fig. 2B) by laser scanning confocal microscopy. Vehicle-treated cells displayed prominent elongated stress fibers that contained striking, long actin filaments that appear to approximately span the length of the cell. Arachidonic acid-treated cells displayed fewer elongated actin stress fibers but we observed a substantial increase of short cortical filaments localized largely at or near the periphery of the cell. These changes of F-actin structures were accompanied by an increase in the number of pseudopodia per cell and an overall change in cell morphology from spindly to flat.

Arachidonic acid alters localization of vinculin and β 1 integrins

Vinculin is a key protein involved in the formation of focal adhesions (Hüttelmaier et al. 1998; Ziegler et al. 2006; Mierke 2009). Thus, we examined whether the distribution of β 1 integrins was altered at focal adhesion sites in arachidonic acid-treated cells that had spread

on type IV collagen substrates. Cells were allowed to spread with or without arachidonic acid, fixed, and probed for vinculin, to reveal focal adhesions, and for β_1 integrins. Untreated cells displayed a punctate staining pattern for vinculin (Fig. 3A) and fairly diffuse β_1 integrin staining (Fig. 3C) with relatively few well-defined focal adhesions. Occasional clusters of β_1 integrins were found in what appeared to be pseudopodia of untreated cells (Fig. 3C). β_1 integrins were relatively infrequently colocalized with vinculin-containing focal adhesions on untreated cells (Fig. 3E and 3G). In contrast, arachidonic acid-treated cells displayed strong vinculin (Fig. 3B) and β_1 integrin (Fig. 3D) staining at the cell periphery, near or at the tips of pseudopodia, in patterns that were highly reminiscent of focal adhesion sites. Prominent colocalization of β_1 integrins with focal adhesions, as identified by vinculin staining, was frequently observed in arachidonic acid-treated cells (Fig. 3F and 3H).

When we quantitated the number of vinculin- and β_1 integrin-containing focal adhesions, we observed a 3.7-fold increase of vinculin-containing focal adhesions and a similar 4-fold increase of β_1 integrin sites in arachidonic acid-treated cells when compared to untreated cells (Fig. 4). Similarly, a 4-fold increase in vinculin-containing focal adhesions that colocalized with β_1 integrins was observed when cells were treated with arachidonic acid.

Talin is a major protein component of focal adhesions that contains binding sites for the cytoplasmic domain of integrin β -subunits, F-actin, vinculin, and at least two signaling proteins, including focal adhesion kinase (Ingber 1997; Cram and Schwarzbauer 2004; Critchley 2009; Moser et al. 2009). As judged by confocal immunofluorescence microscopy, arachidonic acid-treated cells showed many more vinculin-containing foci around the periphery of the cells than did untreated MDA-MB-435 cells (compare Fig. 5A with 5D). Furthermore, untreated cells expressed talin primarily in the cytoplasm (Fig. 5B) whereas arachidonic acid-treated cells displayed increased talin staining in pseudopodia (Fig. 5E). Merging these talin images with micrographs of vinculin staining (Fig. 5A and 5D) revealed colocalization of vinculin and talin at few focal adhesions in the untreated cells (Fig. 5C) but at a larger number of focal adhesions in cells treated with arachidonic acid (Fig. 5F), consistent with the initial finding that arachidonic acid treatment leads to significant redistribution of critical focal adhesion proteins such as vinculin and talin (Franco et al. 2006).

α -Actinin, another protein implicated in coupling integrins to the actin cytoskeleton, has been shown also to stimulate the actin-binding activity of vinculin (Bois et al. 2006). Thus, we examined the effect of arachidonic acid treatment of MDA-MB-435 cells on the localization of α -actinin. As shown in Figure 6, the localization of α -actinin after arachidonic acid treatment appeared similar, but not identical, to those of F-actin (compare Fig. 6A and B with Fig. 6C and D). α -Actinin appeared to have a broader, slightly more diffuse localization than F-actin in both untreated (Fig. 6C) and treated cells (Fig. 6D), with slightly greater accumulation around the periphery of treated cells.

Focal adhesions typically contain protein tyrosine kinases (Schaller 2001; Wozniak et al. 2004)(Vuori 1998). Thus, we examined MDA-MB-435 cells for arachidonic acid-induced changes in the location of tyrosine-phosphorylated proteins. The most prominent sites of phosphotyrosine staining were at the ends of stress fibers in untreated cells, mostly along the plasma membrane (Fig. 6E). This staining was qualitatively similar in untreated control and arachidonic acid treated cells (compare Fig. 6E and Fig. 6F), but appeared to be quantitatively increased in the arachidonic acid-treated cells, perhaps due to the apparent larger surface area present in these cells resulting from a more spread morphology. Colocalization of α -actinin with F-actin was observed primarily at the ends of stress fibers in untreated cells (Fig. 7A) but was found predominantly at areas rich in cortical actin

filaments in arachidonic acid-treated cells (Fig. 7B). Colocalization of phosphotyrosine and F-actin appeared at the ends of stress fibers in untreated cells (Fig. 7C), but at the midsections of cortical fibers in arachidonic acid-treated cells (Fig. 7D). Examination of α -actinin and phosphotyrosine localization yielded a similar pattern, with co-staining occurring at ends of stress fibers in untreated cells (Fig. 7E) and midway along cortical fibers in arachidonic acid-treated cells (Fig. 7F). When all three images were merged, there appeared to be an increase in the number of sites at the cell periphery simultaneously containing α -actinin, F-actin, and phosphotyrosine residues when cells are treated with arachidonic acid (compare Fig. 7G with Fig. 7H).

p38 MAPK associates with vinculin in arachidonic acid-treated cells

We and others have shown that arachidonic acid stimulates p38 MAPK phosphorylation (Hii et al. 1995; Paine et al. 2000) and that this activation is required for concomitant activation of cell adhesion to ECM proteins. p38 MAPK is usually found primarily in the cytoplasm, but its location in cells after activation is not well defined. Since activated p38 MAPK can activate MAPKAP kinase 2, which in turn can phosphorylate Hsp27, a protein that interacts with cytoskeletal components (Landry and Huot 1995; Hino and Hosoya 2003) and lead to activation of RhoA in these cells (Garcia et al. 2009), we examined the MDA-MB-435 cells for possible association of p38 with cytoskeletal proteins after arachidonic acid treatment. Concomitant with a striking increase in the number of vinculin-containing focal adhesions at the periphery of arachidonic acid-treated cells (Fig. 8A vs. 8B), we also observed an increased amount of the active form of p38 appearing in the periphery of arachidonic acid-treated cells (Figs. 8C and 8D), in regions similar to those containing increased α -actinin, F-actin, and phosphotyrosine (see Fig. 7). The merged images suggest that there is increased phospho-p38 and vinculin colocalization at sites of focal adhesions after arachidonic acid treatment and that these areas of colocalization also appear to become larger after treatment (Fig. 8E vs. 8F, and 8G vs. 8H for enlarged views). Quantitative analysis of multiple micrographs suggested a significant increase in active p38 at sites of focal adhesions following arachidonic acid treatment (Fig. 9).

One possible explanation for the apparent increase in the active, phospho-form of p38 MAPK at focal adhesions is that arachidonic acid induces a general increase in association of p38 proteins at these sites. We tested this hypothesis directly by applying fluorescence ratio imaging (Zamir et al. 1999) to cells that were treated with vehicle or with arachidonic acid, then fixed and stained with specific antibodies to vinculin, p38 MAP kinase and phospho-p38 MAP kinase, or with phalloidin (Fig. 10). Vinculin stained areas at the ends of actin filaments (Fig. 10A and D) were used to generate regions of interest encompassing focal adhesions, and the ratio of signal from phospho-p38 specific antibodies (Fig. 10B) to that of total p38 antibodies (Fig. 10C) within those regions was compared (Table 1). The results indicate that while the relative amount of phospho-p38 increased in vinculin-staining focal adhesions upon treatment with arachidonic acid, the total p38 actually decreased. Thus, the ratio of phospho- to total p38 increased significantly, indicating that the increased association of active p38 was not due to a general increase in p38 at these structures.

These results suggested that vinculin and p38 MAPK can form a complex that is appropriately located to regulate focal adhesion formation. To test more directly whether such a complex can form, we used immunoprecipitation followed by immunoblotting to determine if there is an association between vinculin and p38. As shown in Fig. 11A (bottom), there is a small amount of phospho-p38 immunoprecipitated from untreated cells. The amount of immunoprecipitated phospho-p38 increased greatly after treatment of the cells with arachidonic acid, consistent with our previously published results (Paine et al. 2000). This increase in precipitated phospho-p38 is accompanied by a striking increase in the amount of vinculin in the immunoprecipitates (Fig 11A, top), suggesting that a complex

of focal adhesion components exists that contains both vinculin and phospho-p38 in arachidonic acid-treated cells.

p38 MAPK forms a complex with a specific GST-vinculin fusion protein in vitro

To define more precisely the interaction between phospho-p38 and vinculin, we expressed three different fragments of vinculin as GST fusion proteins, which together span the entire vinculin molecule, and which have been used previously to determine the binding site on vinculin for other proteins, such as talin, that are critical for focal adhesions (DeMali et al. 2002). Complexes of the GST-vinculin fusion proteins, bound to glutathione-conjugated beads, were mixed with lysates from cells that were untreated or treated with 30 μ M arachidonic acid prior to lysis.

Consistent with previous reports (Bakolitsa et al. 2004), an immunoblot for talin indicated that only the vinculin fragment carrying the head portion of vinculin (residues 1 – 398) was able to bind specifically and pull down talin from the cell extracts, confirming that this domain carries the talin binding site (Fig. 11B, upper panel). There was no observable difference in the amount of talin recovered from untreated or arachidonic acid-treated cells (compare lanes 3 and 4, Fig. 11B, upper panel), suggesting that the vinculin-talin interaction is constitutive in these cells under the conditions used. On the other hand, phospho-p38 was detected only in the precipitates with the vinculin construct containing the central section of the protein, amino acids 399 through 881 (lane, 6, Fig. 11B, lower panel). Furthermore, there was a marked increase in the amount of phospho-p38 in precipitates of the 399 - 881 vinculin-GST construct from arachidonic acid-treated cells (compare lanes 5 and 6, Fig. 11B, lower panel). Neither talin nor phospho-p38 was detected in significant amounts in pull downs of the 881-1066 vinculin fragment containing residues 881 – 1066 (lane 8, Fig 11B, lower panel), that is, the C-terminal domain of vinculin, suggesting that the p38 binding is specific for a particular region of vinculin and is not due to nonspecific interactions with either the beads or the GST moiety present on all three vinculin constructs. Thus, it appears that the active form of p38 specifically interacts with the central domain of vinculin in cells that are stimulated by arachidonic acid.

Discussion

We have shown that treatment of MDA-MB-435 cells with arachidonic acid results in increased spreading of these cells on collagen type IV substrates. Exposure to this *cis*-polyunsaturated fatty acid also results in the formation of a complex consisting of the active form of p38 MAPK and vinculin, an essential component of cell-matrix adhesions; a striking rearrangement of actin filaments; and an increase in focal adhesions that contain β 1 integrin and the key structural proteins, α -actinin and talin. These cytoskeletal alterations are accompanied by an increase in phosphotyrosine-containing proteins, specifically phospho-p38 MAPK, that co-localize with α -actinin and the tips of F-actin microfilaments at the cell periphery.

The movement of tumor cells from their site of origin and their entry into secondary sites requires regulated cell adhesion that underlies the process of cell migration (Evans 1991). Therefore, adhesion molecules must bind and release their ligands on the adhesive substrate in a controlled manner that permits locomotion (Matsumoto et al. 1995). These same molecules must also interact through focal adhesions with the F-actin cytoskeleton. Proteins such as vinculin, paxillin, talin and α -actinin, have been identified as some of the cytoplasmic proteins that can be involved in cell-ECM adhesions (Chen and Singer 1982; Burridge and Chrzanowska-Wodnicka 1996), helping to make this link. Our results suggest that activated p38 MAPK can be part of focal adhesions. We hypothesize that active p38

may play a role in regulating cytoskeletal proteins and the formation of focal adhesions that are important for cell adhesion and migration after arachidonic acid treatment.

Cell locomotion, mitosis, cytoplasmic organization, intracellular movement of organelles, and maintenance of cell shape are all processes that also require the participation of the cytoskeleton (Alberts et al. 2002) in concert with signaling and adaptor proteins (Berrier and Yamada 2007; Pullikuth and Catling 2007). Thus, exposure of tumor cells to exogenous growth factors, cytokines and nutrients can induce significant phenotypic changes through modification of cytoskeletal structures. Our results are consistent with previous studies with HeLa cells, which demonstrated that exposure to phorbol esters, leading to PKC activation and release of arachidonic acid, stimulates spreading on collagen substrates and increases the relative content of F-actin in these cells (Chun and Jacobson 1992; Chun et al. 1997).

In our hands, arachidonic acid-stimulated cells exhibited reorganization of transverse actin filaments into more cortical actin filaments. Redistribution of both the microfilaments and microtubules are involved in the change of cell shape and locomotion (Li et al. 2005). Tang et al. showed rearrangement and disruption of adhesion plaques in the cytoskeleton of endothelial cells can result from 12-(S)-HETE treatment (Tang et al. 1993), and observed that 12-(S)-HETE, a lipoxygenase metabolite of arachidonic acid, rearranged microfilaments from typical thick elongated filaments to thinner or cortical actin filaments. We have recently demonstrated that 15-(S)-HETE appears to be the active metabolite of arachidonic acid metabolism that activates the signal transduction pathways in these breast cancer cells (Nony et al. 2005), leading to increased adhesion to the type IV collagen. This product of the 15-lipoxygenase is a prime candidate to be responsible for the cytoskeletal rearrangements that we have observed in this study.

Changes in the actin cytoskeleton of cells in response to microenvironmental stimuli has been linked to the regulation of other cellular structures such as membrane ruffles, microspikes, and lamellipodia at the edge of motile cells (Hall 1994; Le Clainche and Carlier 2008). Many actin-binding proteins are involved in the regulation of actin filament formation (Higgs and Pollard 2001) and at least 24 proteins have been described to interact directly with the intracellular domain of integrins, regulating cytoskeletal rearrangements and interactions with the extracellular matrix (Brakebush and Fassler 2003; Zaidel-Bar and Geiger 2010). We have examined several of these proteins in the present study.

Talin is a major scaffold protein at focal adhesions, with binding sites for vinculin, integrins, focal adhesion kinase, phosphatidylinositol phosphate kinase, and actin (Chen et al. 1995; Ingber 1997; Cram and Schwarzbauer 2004; Critchley 2009; Moser et al. 2009). It is clear that talin is required for integrin clustering in *Drosophila* (Brown et al. 2002), but other data also suggest that talin is recruited to focal complexes by integrin clustering (Di Paolo et al. 2002), where it binds the phosphatidylinositol phosphate kinase in an integrin-dependent process (Ling et al. 2002). Furthermore, talin appears to be critical, in combination with F-actin, for the activation of vinculin (Chen et al. 2006). We show here that fatty acid-treatment of MDA-MB-435 breast tumor cells induces increased β_1 integrin clustering and an associated increase in talin staining in pseudopodia. However, it is not yet clear whether the integrin clustering in these cells occurs first, due to signals generated by the signaling pathways activated by the fatty acid (Paine et al. 2000; Palmantier et al. 2001; Kennett et al. 2004), or whether talin is being regulated by these initial fatty acid induced signals, leading to enhanced integrin clustering and focal adhesion stability.

Our observation of increased α -actinin staining at the periphery of arachidonic acid treated cells is consistent with a dramatic increase in focal adhesions and potentially increased signaling through integrins. It is interesting that α -actinin can recruit the MEKK1 kinase, a

regulator of calpain, which in turn cleaves several cell-matrix adhesion components thereby regulating the assembly of adhesion molecules (Cuevas et al. 2003), because we previously reported that calpain activity is increased in arachidonic acid-treated cells (Kennett et al. 2004). It will be very important to determine if there is a concomitant increase in “outside-in” signaling dependent on the fatty acid-induced changes in interaction with the extracellular matrix. It has been proposed that alterations in the adhesion molecule-mediated signaling may play a critical role in tumor progression (Christofori 2003); our data support this possibility, since MDA-MB-435 cells are more metastatic in animals that have been fed diets rich in n-6 polyunsaturated fatty acids (Rose et al. 1994).

Vinculin is a highly conserved cytoskeletal protein that is found at both cell-cell and cell-extracellular matrix type junctions (Geiger et al. 1980; DeMali 2004; Ziegler et al. 2006). Vinculin plays a critical role in the regulation of focal adhesion function (Saunders et al. 2006) and also appears to be a target of certain signal transduction pathways (Perez-Moreno et al. 1998), consistent with it functioning as a controlling point for much of the interaction at focal adhesions. Thus, vinculin appears to be acting not only as a structural component of focal adhesions, but also as an adaptor protein, bringing multiple actin-organizing proteins into close proximity with regulatory kinases.

Previous work has demonstrated the importance of p38 MAPK (Han et al. 1994) in response to stress, the regulation of apoptosis, and the expression of pro-inflammatory cytokines (Lee et al. 1994). Furthermore, the regulation of p38 is complex, involving phosphorylation at both tyrosine and threonine residues (Rangaud et al. 1995). These characteristics suggest that p38 is in a key position to control cell activities. Our own work previously demonstrated that p38 phosphorylation is critical to the stimulation of cell adhesion by fatty acids in human carcinoma cells (Paine et al. 2000). Other researchers have shown that arachidonic acid stimulates phosphorylation of FAK in MDA-MB-231 human breast cancer cells, and increases the adhesion of these cells to laminin through a pathway that depends on Src, ERK1/2, and lipoxygenase (Navarro-Tito et al. 2008; Villegas-Comonfort et al. 2012). Our current finding that p38 MAPK is also present in focal adhesions, and that it associates with vinculin when phosphorylated, is consistent both with its role in signaling to key focal adhesion components and with the role of vinculin as a signal transduction adaptor protein and provides a novel mechanism by which signals transduced by the MAPK family of proteins can modulate adhesive interactions.

p38 MAPK regulates F-actin formation through phosphorylation of Hsp27 (Rouse et al. 1994; Guay et al. 1997; Schäfer and Williams 2000). *In vitro*, Hsp27 can function as an actin cap-binding protein that inhibits actin polymerization (Landry and Huot 1995). This function is consistent with the protective function of Hsp27 *in vivo* that appears to be exerted primarily at the level of microfilaments. Furthermore, this function is regulated by phosphorylation, which is both necessary and sufficient for a change in Hsp27 structure, from oligomeric complexes to homotypic dimers (Lambert et al. 1999). Thus, it is reasonable that we find that arachidonic acid treatments results in increased phospho-p38 associated with focal adhesion sites found where F-actin fibers appear to terminate. We hypothesize that this increased localization of phospho-p38 at focal adhesion sites may be related to the increase in cell attachment and spreading.

Protein phosphorylation is a key element in the formation and maintenance of focal adhesions and also plays a role in the regulation of cell spreading and migration (Zamir and Geiger 2001; Wozniak et al. 2004; Zhao and Guan 2009). p38 MAPK is a Ser/Thr kinase, the activity of which is clearly critical for adhesion, since inhibition of the p38 kinase completely blocks the ability of arachidonic acid to induce cell adhesion to the type IV collagen (Paine et al. 2000). Our demonstration of a direct interaction between purified p38

MAPK and the central region of vinculin *in vitro* suggest that there may be targets of this MAPK at sites of focal adhesions. It will be important to determine if vinculin is a direct substrate for p38 MAPK phosphorylation or whether vinculin is acting as a scaffold for the attraction of p38 to focal adhesions, where the kinase then phosphorylates other targets, and the identity of those targets.

A significant increase in focal adhesions containing β_1 integrin and vinculin was apparent in the human breast carcinoma cells that were exposed to arachidonic acid. This colocalization of vinculin with β_1 integrin is consistent with its role as an integrin binding protein (Ezzell et al. 1997) and with our previous results indicating that $\alpha_2\beta_1$ is required for the arachidonic acid-induced adhesion to type IV collagen (Palmantier et al. 2001) and other previously published studies showing that integrins in focal adhesions are important for cellular signaling and adhesion (Massia and Hubbell 1991; Kornberg et al. 1992; Haimovich et al. 1993). The increased β_1 integrin localization in the focal adhesions may explain the mechanism of the increase in cell adhesion after arachidonic acid treatment either by altering affinity for the type IV collagen ligand or by inducing clustering, thus providing the basis for multivalent interactions with the substrate.

Our results suggest that there may be multiple signaling pathways associated with the regulation of critical cytoskeletal transformations necessary for metastasis, and that a better understanding of these targets and their interactions will both shed light on the process of cell adhesion and allow us to suggest mechanisms for reducing the efficiency of the metastatic process and improve cancer patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Arachidonic acid increases attachment and spreading of cells on type IV collagen
MDA-MB-435 cells were treated with 30 μ M arachidonic acid in ethanol (brown, green, dark blue and pink lines) or with ethanol alone (orange, red, light blue and light green lines) and assayed at 37°C / 5% CO₂ for altered impedance across a surface coated with type IV collagen. The different lines represent the pized impedance measured in separate wells. The curves show impedance from 4 treated and 4 untreated wells in the same experiment and is representative of 3 independent experiments.

Fig. 2. Arachidonic acid induces alterations of the organization of F-actin filaments

MDA-MB-435 carcinoma cells were plated on type IV collagen-coated cover slips in the absence (Panel A) or presence (Panel B) of 30 μM arachidonic acid for 90 min at 37°C. Cells were fixed, permeabilized, and stained with Alexa Fluor 660-phalloidin and DAPI as described in Materials and Methods. Cells were examined either by confocal fluorescence microscopy using excitation/emission wavelengths of 663 nm / 690 nm, respectively, for phalloidin. All panels are shown at the same magnification and the bar in panel B is equivalent to 50 μm .

Fig. 3. Arachidonic acid induces increased focal adhesion formation and colocalization of β_1 integrin with vinculin

The distribution of β_1 integrins and vinculin was determined by dual labeled indirect immunofluorescence confocal microscopy. Untreated (panels A, C, E and G) or arachidonic acid-treated (panels B, D, F, and H) MDA-MB-435 cells were cultured on type IV collagen substrates for 90 min at 37°C. Cells were then fixed, permeabilized, and stained for vinculin (panels A, B) or β_1 integrin (panels C, D). Merged images showing colocalization of β_1 integrin and vinculin are shown in panels E and F, with enlarged regions, outlined by the dotted-line boxes, shown in panels G and H. Vinculin was localized with mouse anti-human vinculin and Alexa Fluor 594-conjugated goat anti-mouse IgG and is displayed as green. β_1 integrin subunit was localized with rat anti-human β_1 integrin and Alexa Fluor 488-labeled goat anti-rat IgG and is displayed as red. Prominent areas of colocalization are indicated with arrows and shown as yellow. Bar = 10 μ m.

Fig. 4. The number of focal adhesions increases in arachidonic acid treated MDA-MB-435 cells Vinculin containing focal adhesions and β_1 integrins were detected in control, untreated, cells (white bars) and in arachidonic acid treated cells (black bars) as described in Fig. 3. Bars represent mean focal adhesions per cell from 10 cells chosen at random $\pm$ SD. *indicates $P < 0.001$ compared to the untreated cells.

Fig. 5. Arachidonic acid-treated cells show increased colocalization of vinculin and talin in focal adhesions

Double immunofluorescent localization of vinculin and talin in MDA-MB-435 cells treated with arachidonic acid for 90 min at 37°C. A representative untreated cell is shown in A, B, and C; an arachidonic acid-treated cell is shown in D, E, and F. Vinculin (A, D) and talin (B, E) were localized simultaneously with directly labeled antibodies (mouse anti-human vinculin directly labeled with Alexa Fluor 594 and mouse anti-human talin directly labeled with Alexa Fluor 488, respectively). Colocalization of vinculin and talin is shown in panels C and F. Bar = 10 μ m.

Fig. 6. Arachidonic acid induces changes in the distribution of F-actin, α -actinin, and phosphotyrosine

Cells were cultured on type IV collagen-coated cover slips in the absence (panels A, C, and E) or presence (panels B, D, and F) of 30 μ M arachidonic acid for 90 min at 37°C. Cells were fixed, permeabilized, and stained as described in the Materials and Methods. A triple-labeling technique was used to document the location of F-actin, α -actinin, and phosphotyrosine. F-actin (A, B) was visualized with Alexa Fluor 660-labeled phalloidin. α -Actinin (C, D) was localized with goat polyclonal anti- α -actinin IgG as the primary antibody and Alexa Fluor 594-labeled rabbit anti-goat IgG as the secondary antibody. Phosphotyrosine (E, F) was visualized with mouse monoclonal anti-phosphotyrosine IgG as the primary antibody and Alexa Fluor 488-labeled rabbit anti-mouse IgG as the secondary antibody. Boxes indicate areas that are enlarged in Fig. 7. Bar = 10 μ m.

Fig 7. Phosphotyrosine shows increased colocalization with cytoskeletal components after arachidonic acid treatment

Each panel shows an enlarged area from pictures in Fig. 6. F-actin and α -actinin images are merged in panels A and B, with F-actin shown as red, α -actinin shown as green, and areas of colocalization shown in yellow. Phosphotyrosine and F-actin images are merged and displayed in panels C and D, with F-actin shown as red, pTyr shown as dark blue, and areas of colocalization shown as pink. Phosphotyrosine and α -actinin images are merged and shown in panels E and F, with pTyr shown as dark blue, α -actinin shown as green, and areas of colocalization shown as light blue. Merged images from all three stains are shown in panels G and H, using the same colors as above, except that colocalization of F-actin, α -actinin, and phosphotyrosine is shown in white.

Fig. 8. Phospho-p38 MAPK and vinculin colocalize at focal adhesions in arachidonic acid-treated human carcinoma cells

Cells were cultured on collagen type IV-coated coverslips in the absence (A, C, E and G) or presence (B, D, F and H) of 30 μ M arachidonic acid for 90 min at 37°C. Cells were then fixed and stained for phospho-p38 and vinculin as described in Materials and Methods. Vinculin staining is shown in panels A and B, phospho-p38 is shown in panels C and D, and the merged images showing vinculin in red and phospho-p38 in green are shown in panels E, F, G and H with areas of colocalization shown in yellow. The boxes in panels E and F indicate the enlarged areas shown in panels G and H. Arrows indicate examples of sites of co-localization of phospho-p38 and vinculin. Bar = 10 μ m.

Fig. 9. Vinculin and phospho-p38 MAPK show increased colocalization after treatment with arachidonic acid

Cells were treated and visualized as in Fig. 8. Twenty untreated (open bars) and 20 arachidonic acid-treated (solid bars) cells were examined. Focal adhesions, identified as distinct spots of vinculin staining, and areas of distinct colocalization of vinculin and phospho-p38 (as shown by arrows in Fig. 8G and 8H) were counted and plotted as number per cell. Error bars represent the standard deviation. Both total number of focal adhesions per cell and number of focal adhesions with phospho-p38 increased significantly ($p < 0.0001$ by Student's t-test) following treatment with arachidonic acid (solid bars compared to paired open bar).

Fig. 10. Fluorescence ratio imaging demonstrates a specific increase in phospho-p38 at focal adhesions in cells treated with arachidonic acid

Cells were untreated or treated with arachidonic acid and plated on type IV collagen-coated slides for 90 min, then fixed permeabilized, and stained with primary antibodies to vinculin (A), phospho-p38 (B), total p38 (C) or with phalloidin to identify F-actin (D). The cell shown here is an example of an arachidonic acid-treated cell from cells observed in 3 independent experiments, with over 200 regions of interest collected from untreated cells and over 700 regions of interest collected from treated cells. Panel E shows a merged image of panels A, B, C and D. All micrographs are portrayed at the same magnification; the bar in panel E represents 50 μm . Following image collection, data was collected into an excel file (Supplemental Table 1) and analyzed as described.

Fig. 11. Vinculin immunoprecipitates with phosphorylated p38 MAPK

(A) Cells were treated with arachidonic acid and cultured on type IV collagen-coated dishes for 90 min, then harvested and lysed. Phospho-p38 was immunoprecipitated, and the precipitates were analyzed by polyacrylamide gel electrophoresis and immunoblotting for either vinculin (top) or phospho-p38 (bottom). C – total cell lysate control (not precipitated); U – immunoprecipitate from untreated cells; EtOH – immunoprecipitate from ethanol-treated cells, done as a control to rule out any possible effects of the addition of the vehicle in which AA is administered; AA – immunoprecipitate from arachidonic acid-treated cells. Data are representative of three independent experiments. (B) Glutathione S-transferase, either alone (lanes 1 and 2) or fused to one of three sections of the vinculin gene, was expressed in bacteria, precipitated with glutathione-conjugated beads, and used to bind proteins from MDA-MB-435 cell extracts that were either left untreated (-) or treated (+) with 30 μ M arachidonic acid. The precipitated proteins were subjected to SDS-PAGE and then immunoblotted for either talin (top) or phospho-p38 (bottom). The positions of molecular weight markers are indicated on the left. The figure is representative of experiments carried out at least than four times with independent preparations of vinculin-GST fusion proteins and cell extracts.

Table 1

Fluorescence Ratio Imaging of p38 and Phospho-p38 in Focal Adhesions

	Regions of Interest Examined	Fluorescence Intensity ($\times 10^{-5}$) Phospho-p38	Fluorescence Intensity ($\times 10^{-5}$) p38	Ratio of Phospho-p38:p38
		mean $\pm$ s.e.	mean $\pm$ s.e.	mean $\pm$ s.e.
Vehicle-treated	271	8.6 $\pm$ 0.7	17.6 $\pm$ 1.4	10.3 $\pm$ 2.6
Arachidonic Acid-treated	793	13.7 $\pm$ 0.7	5.8 $\pm$ 0.4	43.9 $\pm$ 4.4
p value		0.0004	< 0.0001	< 0.0001

MDA-MB-435 cells were treated with arachidonic acid or vehicle as a control, plated onto collagen Type IV coated slides, and allowed to adhere for 45 min. Cells were then stained with specific antibodies, and regions of interest were manually drawn around focal contacts, identified by staining with an anti-vinculin antibody as described. After staining for p38 and phospho-p38, average pixel intensities from regions of interest were quantitated as described (materials and methods). Mann-Whitney tests were used to compare regions of interest from the two treatment groups; p-values are one-sided; averages shown are geometric means. A second experiment yielded similar statistically significant changes in phospho-p38 and total p38 fluorescence intensities, with p38 decreasing and phospho-p38 increasing, after arachidonic acid treatment.