Abstract. Tyrosine phosphorylation of membrane-associated proteins is involved at two distinct sites of contact between cells and the extracellular matrix: adhesion plaques (cell adhesion and de-adhesion) and invadopodia (invasion into the extracellular matrix). Adhesion plaques from chicken embryonic fibroblasts or from cells transformed by Rous sarcoma virus contain low levels of tyrosine-phosphorylated proteins (YPPs) which were below the level of detection in 0.5-μm thin, frozen sections. In contrast, intense localization of YPPs was observed at invadopodia of transformed cells at sites of degradation and invasion into the fibronectin-coated gelatin substratum, but not in membrane extensions free of contact with the extracellular matrix. Local extracellular matrix degradation and formation of invadopodia were blocked by genistein, an inhibitor of tyrosine-specific kinases, but cells remained attached to the substratum and retained their free-membrane extensions. Invadopodia reduced or lost YPP labeling after treatment of the cells with genistein, but adhesion plaques retained YPP labeling. The plasma membrane contact fractions of normal and transformed cells have been isolated from cells grown on gelatin cross-linked substratum using a novel fractionation scheme, and analyzed by immunoblotting. Four major YPPs (150, 130, 81, and 77 kD) characterize invadopodial membranes in contact with the matrix, and are probably responsible for the intense YPP labeling associated with invadopodia extending into sites of matrix degradation. YPP150 may be an invadopodial-specific YPP since it is ~3.6-fold enriched in the invasive contact fraction relative to the cell body fraction and is not observed in normal contacts. YPP30 is enriched in transformed cell contacts but may also be present in normal contacts. The two major YPPs of normal contacts (130 and 71 kD) are much lower in abundance than the major tyrosine-phosphorylated bands associated with invadopodial membranes, and likely represent major adhesion plaque YPPs. YPP150, paxillin, and tensin appear to be enriched in the cell contact fractions containing adhesion plaques and invadopodia relative to the cell body fraction, but are also present in the soluble supernate fraction. However, vinculin, talin, and α-actinin that are localized at invadopodia, are equally concentrated in cell bodies and cell contacts as is the membrane-adhesion receptor β1 integrin. Thus, tyrosine phosphorylation of the membrane-bound proteins may contribute to the cytoskeletal and plasma membrane events leading to the formation and function of invadopodia that contact and proteolytically degrade the extracellular matrix; we have identified several candidate YPPs that may participate in the regulation of these processes.

Metastatic tumor cells interacting with the extracellular matrix exhibit cell-surface extensions that possess an aggressive function, namely extracellular matrix degradation, adhesion, and invasion (11, 20, 29). These cellular extensions identified in Rous sarcoma virus (RSV)-transformed chicken embryo fibroblasts (CEF[RSV]) cultured on matrix substratum are called invadopodia and are responsible for the invasion of transformed cells through the extracellular matrix (10, 34). Invadopodia possess two unique activities: an extracellular matrix-degrading proteolytic activity (11); and an intracellular, phosphokinase activity, pp60v-src (12). The v-src gene product pp60v-src is a tyrosine-specific kinase located primarily at the cytoplasmic face of the plasma membrane of transformed cells (5). Transformation of embryonic chicken cells by pp60v-src causes an extensive reorganization of contact patterns, which led to the classic observations of reduced adhesion (44, 48) and rounded morphology (46). The reduction of the number and size of adhesion plaques is concomitant with the formation of invadopodia, "rosettes" (13) or "podosomes" (41) after
Tyrosine-phosphorylation of proteins in target cells mediates signal transduction (43). Tyrosine-phosphorylation is also associated with proteins involved in regulating membrane protein trafficking through the cell such as in endocytosis (18) and in acrosomal exocytosis (39). Pp60^c 

is localized at the growth cone during neurite extension, a normal invasive process occurring during development (32). In addition, actin-organization factor (20) and growth factors such as bFGF (38) and transforming growth factor-α (16) can cause scattering of tumor cells and induce an increase in cell motility. Some of these motility factors bind to receptors that are tyrosine kinases (7), and thus potentially could modulate invadopodia function, since the activity of invadopodia requires cell-surface motility events (10).

A number of interesting tyrosine-phosphorylated substrates have been identified in recent years which are associated with the membrane and which may play a role in cellular invasion including the integrin-adhesion receptors and cytoskeletal-adhesion plaque proteins (8, 14, 27, 42). Integrin participates in the adhesion of invadopodia during cellular invasion (34) and its phosphorylation after transformation may alter cellular adhesion (3, 21, 23, 40). It is likely that integrins and membrane-associated proteases responsible for extracellular matrix degradation are localized together in the membranes in invadopodia (34). Adhesion to fibronectin would thus concomitantly facilitate degradation of fibronectin (4, 9) and its subsequent endocytosis (34). Furthermore, ligation and aggregation of integrin at the cell surface stimulates cellular production of matrix metalloproteinases (45). Integrin phosphorylated on tyrosine residues may interact with receptors or tensins containing SH2 domains which are localized at subcellular sites of invasion (8, 14, 28). These interactions might provide the means by which the adhesive functions of integrin could be coupled to nearby extracellular matrix-degrading proteases during migration and invasion. Furthermore, localized changes in integrin phosphorylation could promote transmembrane signals such as those stimulating the secretion of matrix metalloproteinases in fibroblasts (45).

We have established an in vitro model to identify the subcellular expression of invasiveness which uses a recently developed method of growing cells on fibronectin-coated, gelatin beads (36). Using this approach, it was demonstrated that cells exhibit dynamic responses in organization of the integrin-fibronectin receptor and talin upon initial contact (10 min) with fibronectin beads (36). After 1 h in culture, invadopodia extend from the transformed cells extracellularly into sites of bead matrix-degradation which are easily visualized (34). Here, we used specific antiphosphotyrosine antibodies to detect tyrosine-phosphorylated proteins (YPPs) in cells actively invading fibronectin-coated, gelatin beads.

We found that in transformed cells, high levels of YPPs were concentrated at the plasma membrane of invadopodia which degraded and subsequently invaded the fibronectin-coated, gelatin substratum. Low levels of YPPs were found in untransformed cell-adhesion sites. Both degradative and motile activities of the invadopodia were inhibited by genistein, an inhibitor of tyrosine-specific kinases (1, 2). These properties of the invadopodia, thus, distinguish them from other membrane extensions such as dorsal surface folds, ruffles, lamellipodia, or filopodia, as well as from membrane processes of the ventral surface that extend into preexisting niches of the substratum. In addition, we have designed a method for isolating the membranes that contact fibronectin-coated, gelatin substrata from both normal and transformed cells.

The molecular mass profiles of YPPs from adhesion plaques of normal cells are compared with YPPs from invadopodia of transformed cells by immunoblotting contact membrane fractions with anti-YPP antibodies. Four major YPPs were identified from isolated transformed cell contacts which may participate in invadopodia function. Our results suggest that the enhanced phosphorylation of tyrosine-containing proteins by pp60^c may directly contribute to the formation and function of the extracellular matrix-degrading structures, invadopodia.

Materials and Methods

Culture of Normal (CEF) and Transformed (RSVCEF) Cells on Gelatin Substratum

CEF and RSVCEF were cultured on rhodamine-conjugated fibronectin coupled to beads and fixed according to previously described methods (34, 36). Alternatively, cells were cultured on thick, hydrated gelatin films on coverslips, a modification of the method of Chen et al. (11). The gelatin films were hydrated in 150 mM NaCl/10 mM phosphate buffer, pH 7.4, PBS at 4°C for 10 min and briefly fixed in 0.5% glutaraldehyde. For biochemical experiments, cells were cultured overnight at 41°C on unlabeled, cross-linked gelatin films in tissue culture dishes. The films were prepared by coating dishes with PBS containing 2.5% gelatin and 2.5% sucrose, melted, and cooled to 50°C. Excess solution was aspirated, the dishes cooled, and the films then fixed by 2.0% glutaraldehyde in PBS for 15 min at room temperature. Films were washed in PBS or deionized water and quenched for at least 1 h at room temperature with DME (Cellgro, Mediatech, Inc., Herndon, Va.). Gelatin films were stored at 4°C with the addition of 2 mg/ml penicillin, and 20 U/ml streptomycin. Cells were seeded at subconfluent densities onto the beads (34,36) or onto films in dishes in culture medium consisting of DME, 1% chicken serum, 4% FBS, 10% tryptose phosphate broth, 200 µg/ml penicillin, and 2 U/ml streptomycin. In the case of incubation with 5 µg/ml genistein (ICN Biomedicals, Inc., Costa Mesa, CA), cells were allowed to attach to cross-linked gelatin beads or films at 41°C for the times indicated in the figure legends and then genistein was added to the culture medium.

Immunocytochemistry: Light and Electron Microscopy

Fixed cells were processed for either 0.1- or 0.5-µm thin, frozen sectioning using an Ultracut E43 ultramicrotome (Reichert Jung, Vienna) equipped with FC4D Low-Temperature Sectioning system as previously described (34). For immunofluorescent labeling of cells cultured on rhodamine-fibronectin cross-linked gelatin beads, 0.5-µm thin, frozen sections were labeled indirectly first with primary antibodies, either mouse monoclonal IgG directed against YPPs (Oncogene Science, Inc., Uniondale, NY) or mouse monoclonal IgG1 (Upstate Biotechnology Inc., Lake Placid, NY), and then with a fluorescein conjugate of goat antibody to mouse IgG. For demonstration of antibody specificity for phosphotyrosine residues of YPPs, sections were also labeled with monoclonal IgG2 or IgG1 in the presence of 1 mM O-phospho-L-tyrosine (Boehringer Mannheim Biochemicals, Indianapolis, IN).

CEF infected with wild-type or mutant ts68 RSV (12) were cultured on thick, fluorescent, fibronectin cross-linked gelatin films overnight and then extracted with 0.5% Triton X-100 following fixation (12). Fixed cells were labeled with anti-YPP antibodies as described above. Cells were also labeled with mouse mAb against paxillin (Zymed Laboratories, South San Francisco, CA) or rabbit antiactin (a kind gift from L.-B. Chen, Harvard University, Boston, MA). Primary antibodies were followed by rhodamine-
Subcellular Fractionation of Cells Cultured on Fibronectin–Gelatin Cross-Linked Matrix

Cell contact, cell body, and supernate fractions were prepared from just confluent monolayers of cells cultured overnight on gelatin films (10-cm dishes). After washing the monolayer three times with ice-cold 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, TBS, the cells were washed with an additional 10 ml of a buffer designed to stabilize the membranes and cytoskeleton, 10 mM MOPS, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 M sucrose, and 1 mM NaVO₃, 0.2 mM PMSF, and 0.02% NaN₃. Cell bodies were sheared away from the adherent cells in 5 ml of the same buffer (shearing buffer) using a 9-in. Pasteur pipette flamed to seal the end and produce an L shape. Shearing of cell bodies was accomplished by passing the 5-cm pipette rod over the surface of the gelatin using sweeping motions across and around the edges of the Petri dish for a total of 30 s. The sheets with adherent cell bodies were examined by phase-contrast microscopy and then washed and cultured overnight in the absence of genistein, were able to invade the beads in a manner described above for YPPs labeled using mouse mAb 4G10 antiphosphotyrosine antibody. Rabbit polyclonal antivinculin and rabbit polyclonal anti-α-actinin were visualized using previously described antibodies (36). Pannexin and tensin were visualized using the antibodies described above for immunocytochemistry. Rabbit anti-albumin was a kind gift from K. Burrage (University of North Carolina, Chapel Hill, NC). Immunoblotting of β₁ integrin was accomplished using rat mAb ES238 (35). Immunoblots were processed using goat anti-mouse, anti-rabbit or anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Corp., Arlington Heights, IL). The same or a duplicate nitrocellulose blot was sequentially labeled with a mouse and then a rabbit primary antibody. In between immunoblot experiments, the nitrocellulose blots were extensively washed and treated with 0.02% sodium azide to inactive the horseradish peroxidase moiety of previously blotted secondary antibodies. Particular antibodies were chosen for reblotting experiments based on their quality and the molecular masses of the blotted proteins to avoid overlapping reactions. Bands were visualized on film using the ECL detection system (Amersham Corp.) according to the manufacturer's instructions. Semiquantitative analysis of bands on immunoblots was carried out using an image analysis system (Carl Zeiss) with a Processor (Kontron Analytical). The image from an exposed film was digitized and the density of the bands within equal size squares was obtained. Background densities from adjacent areas were subtracted for each band. Since equal protein loadings from each fraction were run on a single gel, the relative specific activities of a given polypeptide can be compared for the fractions from both normal and transformed cells.

Results

Role of Tyrosine Phosphorylation in Invasion

In the present study, we cultured transformed cells on fibronectin–gelatin beads (34,36) and films (11) as described in the Materials and Methods. Cellular responses to contact with the beads were examined between 30 min to 3 d after culturing using Nomarski differential, interference-contrast microscopy of cell–bead aggregates (Fig. 1). Fig. 1 shows the invasion of transformed cells into the fibronectin-coated gelatin beads by means of optical sections through the middle of the beads. Invadopodia could be detected as membrane protrusions from the ventral surface of transformed cells within the matrix beads, with increasing numbers found after longer incubations (Fig. 1, 1 h, 1 d, and 2 d). The addition of 5 µg/ml of genistein (1,2), an inhibitor of tyrosine-specific kinases, to the media after 1 h of cell culture, inhibited the cell–surface mobility of the transformed cells as observed using video microscopy of living cells (not shown), and invadopodial extension into the bead (Fig. 1, 1 d + GT), suggesting a role for tyrosine phosphorylation during invadopodia formation. RSVCEF cultured on beads for 6 h with 5 µg/ml genistein after an initial period allowing for cell attachment, and then washed and cultured overnight in the absence of genistein, were able to invade the beads in a manner similar to control cells (not shown). Previously, we showed by thin, frozen section at the light...
Invasion of transformed cells into the fibronectin-coated gelatin beads and its inhibition by genistein. Each optical section achieved using Nomarski differential interference-contrast microscopy was taken through the middle of cell-bead aggregates. (8h) Transformed cells cultured for 1 h with beads showing their membranes trapped in the niches of the aggregated fibronectin-gelatin beads (arrowheads). These structures serve as a control for the extension of cell membranes in the absence of localized matrix degradation. (ld) Transformed cells cultured for 1 d with beads showing the active invasion of membrane extensions of spread cells into the bead (arrow). (ld + GT) An optical section of cell-bead aggregates cultured for 1 d in the presence of 5 µg/ml genistein after a period of 1 h for cell attachment is shown. Both ruffling activity of the dorsal surface and invadopodial activity of the ventral surface of transformed cells were inhibited. The shape of the cells remained unchanged during the incubation. (2d) An optical section of transformed cells grown on the beads for 2 d. Numerous invadopodia have penetrated the bead (arrows). Bar, 20 µm.

Figure 1. Invasion of transformed cells into the fibronectin-coated gelatin beads and its inhibition by genistein. Each optical section achieved using Nomarski differential interference-contrast microscopy was taken through the middle of cell-bead aggregates. (8h) Transformed cells cultured for 1 h with beads showing their membranes trapped in the niches of the aggregated fibronectin-gelatin beads (arrowheads). These structures serve as a control for the extension of cell membranes in the absence of localized matrix degradation. (ld) Transformed cells cultured for 1 d with beads showing the active invasion of membrane extensions of spread cells into the bead (arrow). (ld + GT) An optical section of cell-bead aggregates cultured for 1 d in the presence of 5 µg/ml genistein after a period of 1 h for cell attachment is shown. Both ruffling activity of the dorsal surface and invadopodial activity of the ventral surface of transformed cells were inhibited. The shape of the cells remained unchanged during the incubation. (2d) An optical section of transformed cells grown on the beads for 2 d. Numerous invadopodia have penetrated the bead (arrows). Bar, 20 µm.

and EM level that RSVCEF invaded fibronectin-coated, gelatin beads by extending invadopodia at sites of fibronectin-gelatin degradation and that internalized fibronectin occurred in vacuoles near invadopodia (34). Using mAbs against YPPs and low magnification views of sectioned cell-bead aggregates, we observe the striking localization of YPPs at the ventral surfaces of cells invading into fluorescent fibronectin cross-linked beads (Figs. 2-4). Degradation sites on the gelatin matrix where the rhodamine-labeled fibronectin was missing coincide with sites of invadopodia observed by phase-contrast microscopy and also with sites of YPP localization (Figs. 2 A, and 2 B, 3, 4 A, some examples are indicated by arrows). YPP labeling could be prevented in the presence of O-phospho-L-tyrosine (Fig. 2 C). Another internal control for specific YPP labeling was observed at preexisting niches in the bead where there was no YPP localization and the layer of fluorescent, fibronectin label was intact (Fig. 2 A, arrowheads). There was also no detectable YPP labeling in other sites within transformed cells including dorsal surface folds, ruffles, nucleus, and cytoplasm (Figs. 2-4). Normal CEF grown on the rhodamine-fibronectin beads did not have significant YPP labeling, or any detectable invadopodia or fibronectin degradation (Fig. 2 D).

Cell migration and invasion is a dynamic process, thus, sections reflect cells in various states of movement and invasion. In extensive observations of sections or whole transformed cells, we have observed that very small localized sites of fluorescent fibronectin removal are frequently coincident with YPP label. However, at large sites of fluorescent fibronectin removal or at areas where extensive degradation has taken place, YPP label is sometimes poorly coincident or the cell is nowhere to be found (also see Fig. 12 below). Therefore, bright punctate sites of labeling for YPP at the membrane may localize at active invadopodia or at putative sites of invadopodial formation which can be observed prior to obvious signs of matrix removal by the cell. These bright patches of membrane labeling are observed at early stages of invasion, 1 h after seeding onto the gelatin
Figure 2. Specific localization of YPPs at sites of fluorescent fibronectin removal by transformed cells. For each 0.5-μm thin, frozen section of cell–bead aggregates, the left panel shows the composite phase contrast and fluorescent image of rhodamine-stained, fibronectin-coated gelatin beads; the right panel shows the immunofluorescent image of fluorescein-labeled antibody directed against YPPs. Arrows indicate examples where the invadopodia penetrated the matrix at foci, where fluorescent fibronectin coated on the bead’s surface had been removed extracellularly, and where YPPs were concentrated intracellularly. (A) Transformed cell–bead aggregate cultured for 1 d showing invasive cells at the lower right and their invadopodia (arrows) as well as noninvasive cells to the left and upper right whose membranes extended into preexisting niches (arrowheads). Cell membranes at these niches were distinct from invadopodia and showed no YPP localization. (B) A section from the periphery of a cell–bead aggregate showing localization of YPPs at the invadopodia (arrows). Note that numerous membrane extensions on dorsal cell surfaces are free of YPP labeling. (C) A section of a cell–bead aggregate similar to that shown in panel A illustrating the specificity of YPP labeling. Immunofluorescent labeling for YPPs at the invadopodia was competitively inhibited by the addition of 1 mM of O-phospho-L-tyrosine during the immunolabeling procedure. (D) A normal CEF grown on an intact fibronectin bead shows the absence of invadopodia, fibronectin degradation, and YPP labeling. Bar, 10 μm.
substratum when matrix removal is first detectable (Fig. 3, cell at left). Most cells at this early stage, however, do not show matrix removal or the localized bright patches of YPP labeling (Fig. 3, cell at right). Invadopodia that extend into the bead far enough to be detected by phase-contrast, light microscopy typically have YPPs associated with their tips, as shown in a higher magnification view of a 0.5-μm frozen section (Fig. 4, A and A’). Immunostaining of pp60^{c-src} occurs more broadly over the invadopodia, as shown in another high magnification view (Fig. 4, B and B’).

To give a measure of the correlation among matrix degradation, presence of invadopodia, the pp60^{c-src} tyrosine kinase, and YPP immunolabeling, we determined the percentage of cells that were associated with matrix degradation (fluorescent fibronectin removal), and that were immunostained for pp60^{c-src} (mAb EB7), invadopodia (mAb CPI), and YPP (mAb 1G2) localization in thin, frozen sections of cell–bead aggregates (Table I). The majority (81%) of RSVCEF contained detectable staining for pp60^{c-src}, although in ~40% of the cells, the labeling was diffuse rather than localized at the membrane. Approximately 56% of cells contained YPP label, and 38% CPI staining. CPI monoclonal antibody, although not adequate for immunoblotting, is an excellent morphological marker for invadopodia (10). Fibronectin degradation was observed in ~48% of RSVCEF. In ~7% of normal cells, YPP labeling could be detected in the frozen, thin sections, whereas no degradation or invadopodia was detected. Together, these results suggest a role of the pp60^{c-src} tyrosine kinase in tyrosine phosphorylation of membrane-associated proteins and in the formation of invadopodia.

To obtain en face views of the YPP labeling pattern on ventral membranes of intact cells including adhesion plaques, we used YPP labeling of detergent-extracted cells that were cultured on fluorescently labeled fibronectin. Cells infected with a temperature-sensitive mutant of pp60^{c-src} (12) were used to localize YPPs in cells grown at the nonpermissive (Fig. 5 A) or permissive temperature for transformation (Fig. 5 B). The cells were cultured for 4 h at the nonpermissive temperature (41°C) on thick, cross-linked gelatin films coated with rhodamine–fibronectin and some cultures were shifted to the permissive temperature (36°C) for cell transformation overnight. Immunostaining of coverslips containing equal cell numbers was used so that levels of staining would be roughly comparable. A low level of YPP staining can be detected in adhesion sites of cells that appeared normal or transformed (Fig. 5 A and B, arrowheads). However, invasive cells were detected at the temperature permissive for transformation, and in these cells, more intense YPP labeling co-localizes with sites of fluorescent fibronectin removal in a rosette pattern (Fig. 5 B, arrow). The intense labeling for YPP at sites of degradation correlates with the YPP labeling of invadopodia detected in thin, frozen sections.

In similar experiments to those shown in Fig. 5, cells infected with the temperature-sensitive mutant for transformation (ts68) were cultured for 4 h at the nonpermissive temperature, and then cultured overnight in the presence or absence of genistein at the permissive temperature for trans-
Image analysis was carried out to determine the relative amounts of YPP labeling in invadopodia versus adhesion plaques of individual transformed cells as shown in Figs. 5 and 6. The data from three representative cells are shown in Fig. 7. In a noninvasive transformed cell, adhesion plaque labeling represents 100% of total ventral cell-surface labeling for YPP. In two invasive cells, invadopodia represents 81% and 45%, respectively, of total ventral cell-surface YPP labeling. The two invasive cells in these examples each have approximately sevenfold higher ventral YPP labeling than the noninvasive cell. This example serves to illustrate that invadopodial YPPs would be a significant component of the YPPs isolated from transformed cell contacts.

**Identification of YPPs in Invadopodia**

Considering the increased expression of YPPs in cells containing invadopodia and the fact that 56% of transformed cells contain invadopodia, we sought to isolate invadopodia from transformed cells and adhesion plaques from normal cells to compare their YPP composition on immunoblots using anti-YPP antibody. The identification of differences in the composition of YPPs associated with these structures might indicate those molecules that are involved in the function of invadopodia and uniquely associated with them. Cells were cultured on fibronectin, cross-linked gelatin films in tissue culture dishes, and the tenacity of cells adhering to gelatin films made it possible to shear away cell bodies, leaving behind invasive and noninvasive cell contacts.

Subconfluent layers of CEF or RSVCEF grown on gelatin films were sheared in the presence of 10 mM MOPS, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 M sucrose, 1 mM Na₃VO₄, 0.02% NaN₃ at 4°C as described in the Materials and Methods. Immunofluorescent microscopy of membranes after shearing in shown in Figs. 8 and 9. B and C. In the case of contact membrane fragments from normal
Figure 5. Low levels of YPPs are present in adhesion plaques of normal and transformed cells, but high levels are present in invadopodia. Whole cells grown on rhodamine–fibronectin-coated, thick, cross-linked gelatin films were fixed and permeabilized with Triton X-100. ts68 mutant RSV-infected CEF grown overnight at the nonpermissive temperature (row A) or the permissive temperature for cell transformation (row B) were photographed using phase-contrast microscopy (left). The fluorescent fibronectin-coated gelatin substratum was visualized using direct epifluorescence microscopy (ECM), and YPPs were visualized using indirect immunofluorescence microscopy of anti-YPP mAb staining (YPP). Cells at the nonpermissive temperature or permissive temperature contained YPP labeling of adhesion plaques (arrowheads in A, and cell at right in B, YPP), whereas transformed cells had bright labeling of invadopodia membranes associated with sites of fibronectin degradation (arrows in B, ECM, and YPP). Note lower level of YPP labeling at adhesion sites in a transformed cell (arrowhead, B). Bar, 10 μm.

After shearing away of cell bodies, the contact membranes and the gelatin matrix were vigorously scraped from the surface of the plastic dish using a cell scraper. This material was extracted with Triton X-114 into soluble and insoluble fractions, and the Triton X-114–soluble fraction was partitioned into aqueous and detergent phases. An amount from each fraction representing an equivalent amount of the crude extract was compared for transformed cell contacts by immunoblotting with mouse monoclonal anti-YPP antibody. Figure 10 illustrates that the majority of YPPs can be extracted from contact membranes in Triton X-114 and that they are mostly hydrophilic; they partition to the aqueous phase. Normal and transformed cell contacts were then compared on the same immunoblot (Fig. 11). Fig. 11, A and B are identical except that in B, 2 mM O-phospho-L-tyrosine was included in the incubation with anti-YPP mAb to demonstrate the specificity of the labeling patterns observed in A. Comparing 20 μl of normal cell contacts (Fig. 11, lane N 20) with 2 or 20 μl of transformed cell contacts (Fig. 11, lanes T 20 and T 2) reveals a marked increase in YPPs from transformed cell contacts compared with normal contacts. Figure 11 C illustrates differences between normal (100 μl) and
Figure 6. Inhibition of fluorescent fibronectin removal and membrane localization of YPP in invadopodia by genistein. Cells were cultured as described in Fig. 5. ts68 mutant RSV-infected cells were cultured for 4 h at the nonpermissive temperature, 36°C, overnight in the presence or absence of genistein. Cells were photographed using phase-contrast microscopy (left). In row A, cells were treated with 5 μg/ml genistein (see Materials and Methods), and in row B the cells were treated with the solvent dimethyl sulfoxide in the absence of genistein. The fluorescent fibronectin-coated gelatin substratum was visualized using direct epifluorescence microscopy (ECM), and YPPs were visualized using indirect immunofluorescence microscopy of anti-YPP mAb staining (YPP). Cells, following treatment with genistein, did not remove the fluorescently labeled substratum, and YPP labeling of invadopodia at the membrane was reduced, although bright diffuse YPP labeling remained. Adhesion plaque staining in streaks was still apparent at the membrane (arrowheads in A, ECM, and YPP). In the absence of genistein, cells degraded the matrix and invadopodia were labeled with anti-YPP (arrow in B, YPP). No degradation was associated with adhesion plaques either in the presence or absence of genistein (arrowheads in A and B, ECM). Bar, 10 μm.

Figure 7. Distribution of YPP labeling in contacts of invasive and noninvasive cells. The total amount of YPP labeling attributable to invasive (cells A and C) versus noninvasive (cell B) contacts within a given cell was determined from a light micrograph negative of a given transformed cell (20 μl) cell contacts when more protein was run in the normal cell contact lane and the film exposure for transformed cell YPP was reduced. The major YPPs of normal contacts run as a smear at ~120-130 kD and at 71 kD

transformed (20 μl) cell contacts when more protein was run in the normal cell contact lane and the film exposure for transformed cell YPP was reduced. The major YPPs of normal contacts run as a smear at ~120-130 kD and at 71 kD in lanes where protein is somewhat overloaded (Fig. 11 C, N 100). At lower protein loadings, bands at 130 and 71 kD (Fig. 11 A, N 20) are detected. Transformed cell contacts have four major YPPs, at 150, 130, 81, and 77 kD. YPPs at 150, 81, and 77 kD appear to be absent in normal contacts, whereas YPP130 may be present in both normal and transformed cell contacts.

Tyrosine Phosphorylation of Membrane-Associated Proteins in Invadopodia

Paxillin and tensin, two cytoskeletal, tyrosine-phosphorylated proteins that are localized in adhesion plaques, were immunolabeled in whole, detergent-extracted cells cultured on fibronectin-gelatin cross-linked matrix. Tensin (Fig. 12, A and B) and paxillin (Fig. 12, C and E) labeling was found in patterns similar to those observed for YPPs at sites of localized degradation by transformed cells (some examples are indicated by arrows). Paxillin and tensin staining could be seen variously at invadopodia in rings at the center of cells (Fig. 12 A-E, for example). Paxillin staining was detectable in all cells and its absence in Fig. 12 D indicates the absence of a cell, as confirmed by Nomarski differential, interference-contrast microscopy (not shown). Thus, paxillin and tensin are two additional cytoskeletal proteins that together with a growing list of YPPs (25) would be isolated with adhesion plaque as well as invadopodial fractions.

To compare the specific enrichment of YPPs and known
Figure 8. Actin and integrin are retained in isolated invadopodial membranes. Normal (A-C) and transformed cells (D-F) were cultured on cross-linked gelatin substratum overnight, cell bodies were sheared away, and then cells were fixed and stained with rat mAb ES238 followed by fluorescein-conjugated goat anti-rat antibody and rhodamine-phalloidin as described in Materials and Methods. Immunofluorescence microscopy of anti-β1 integrin labeling (In) is shown in the top two panels (A, and D), and the rhodamine epifluorescence images of phalloidin labeling are shown in the middle panels (B, and E) revealing the distribution of actin (A). The bottom panels (C, and F) are the Nomarski differential interference-contrast images of the cellular material adhering to the surface of the gelatin cross-linked film. In normal cells, the majority of the cell body was sheared away from well-spread cells leaving behind membrane fragments at cell contact sites that were labeled by anti-integrin β1 antibody (arrow, A) but only minimally by phalloidin (arrow, B). In contrast, profiles of ventral membranes of transformed cells showed diffuse integrin distribution (D), and actin localization in a rosette pattern (arrow, E) which coincides with the mottled Nomarski differential interference-contrast image. Bar, 10 μm.

cytoskeletal and membrane proteins in the invadopodia fraction, three subcellular fractions were prepared from cells cultured on the fibronectin–gelatin cross-linked matrix: supernate, cell body, and cell contact fractions (see Materials and Methods). These fractions from normal and transformed cells were first characterized with respect to the total protein recovered from each fraction and the percent total protein in each fraction (Table II). The differences in the distribution of protein between the supernate and cell body fractions and between normal and transformed cells is probably due to the fact that transformed cells are rounded, whereas the normal cells are well spread. The normal cells may fragment more after shearing, thus releasing an increased amount of soluble cytosolic proteins into the supernate.

Equal protein loadings (22 μg) of each fraction were compared after SDS-PAGE and immunoblotting using mouse monoclonal antiphosphotyrosine antibodies and antibodies against β1 integrin, α-actinin, talin, vinculin, tensin, and paxillin (Fig. 13). Specific labeling of YPPs and these membrane-associated polypeptides in the subcellular fractions was estimated by densitometry and the ratios between the supernate or cell contact fraction and cell body obtained (Table II). Immunoblotting using mAb ES238 recognizing β1 integrin was performed to follow the distribution of the plasma membrane since immunofluorescence microscopy demonstrates its localization at the plasma membrane of cells cultured on fibronectin–gelatin cross-linked matrices (34). Integrin β1 present in the transformed cell supernate fraction (Fig. 13) may represent soluble proteins or alternatively, proteins associated with nonpelleting membrane vesicles. Talin, vinculin, and α-actinin are not enriched in the contact fractions or supernates relative to the cell bodies, however, tensin and paxillin are concentrated in the cell contact fraction and supernate, and are less prominent in the cell.
Invadopodia are labeled by the marker mAb CPI. (A) A transformed cell, cultured overnight on a cross-linked gelatin bead, has extensively invaded the bead as seen in this 0.1-μm thin, frozen section. Antiintegrin labeling (10 nm gold) is found over the membranes of some invadopodia (arrow). A 3× enlargement of an area immediately to the right of the inset shows the labeling of the invadopodial marker CPI mAb (5 nm gold) which can be found over and near the membranes of invadopodia. Frozen sections were double immunolabeled with polyclonal rabbit anti-140K integrin complex antibody and rat monoclonal CPI, followed by 10 nm gold-conjugated anti-rabbit and 5 nm gold-conjugated anti-rat secondary antibodies. (B and C) Sheared ventral membranes from RSVCEF cultured overnight on cross-linked gelatin films were prepared for immunolabeling as described in Fig. 8. The films were immunolabeled with CPI, followed by fluorescein-conjugated goat anti-rat antibody. Immunofluorescence microscopy of the fluorescein image of CPI labeling (C, CP) shows bright labeling of the welts seen by Nomarski differential interference-contrast microscopy (B). Bars, (A) 50 μm; (B and C) 20 μm.

The specific activity of the total YPPs isolated from RSVCEF contacts was ~38-fold higher than that of YPPs from CEF contacts (<1 versus 38, Table II; Fig. 13 bottom panel). YPPs were also enriched in the transformed cell contact fraction ~1.7 times more than YPPs from the transformed cell body fraction (Table II; Fig. 13 bottom panel). Comparison of the specific activities of YPP81/77 and YPP150 in cell contacts, cell bodies, and supernates, however, reveals a modest enrichment of the YPP81/77 in the cell contact fraction which paralleled the behavior of total YPPs (1.5- versus 1.7-fold enrichment over cell body), but a marked enrichment of the YPP150 in the cell contact fraction (3.6-fold over cell body) and also a twofold enrichment in the supernate fraction (Table II). Thus, the subcellular distribution of the tyrosine-phosphorylated YPP150 is similar to the distribution of these proteins in the cell body fraction (Fig. 13). In contrast to cytoskeletal proteins, the specific activity of the total YPP81/77 and YPP150 in cell contacts, cell bodies, and supernates, however, reveals a modest enrichment of the YPP81/77 in the cell contact fraction which paralleled the behavior of total YPPs (1.5- versus 1.7-fold enrichment over cell body), but a marked enrichment of the YPP150 in the cell contact fraction (3.6-fold over cell body) and also a twofold enrichment in the supernate fraction (Table II). Thus, the subcellular distribution of the tyrosine-phosphorylated YPP150 is similar to the distribution of these proteins in the cell body fraction (Fig. 13).
The Triton X-114-soluble material from the original extract. Most of the YPPs are Triton X-114-extractable and partition to the aqueous phase. Cell contacts were prepared from transformed cells cultured overnight on gelatin material (TX), the Triton Xoll4-insoluble material (IN), and from transformed cell cultures (adhesion plaques plus invadopodia). The identity of the isolated contact sites adhering to the gelatin was determined by immunofluorescence microscopy using mAb CPI recognizing invadopodia and anti-β1 integrin antibody that labels the membrane fragments associated with invadopodia and adhesion plaques. Data on the YPP profiles of the isolated cell contacts, together with the results on the immunolocalization of YPPs in cells cultured on gelatin cross-linked matrix lead us to conclude that the YPP130 and YPP71 observed in immunoblots of CEF contacts represent the major membrane-associated YPPs present in adhesion plaques. We have achieved this without prior treatment of cells with vanadate or other reagents to enhance the recovery of the minor component of YPPs since we selectively enriched for membrane contact sites using cell fractionation. The increased amounts of YPPs in RSVCEF contacts compared to CEF contacts is consistent with the 10-fold overall increase in YPPs which was previously observed in whole cell extracts after transformation (24). We found that the specific activity of YPPs determined from immunoblots was estimated to be ~38-fold higher in cell contacts isolated from transformed cell contacts relative to contacts from untransformed cells. Image analysis of YPP immunolabeling of contacts within a transformed cell population demonstrates that contact YPP labeling from cells containing invadopodia may be as much as sevenfold higher than amounts localized in cells containing only adhesion plaques. Image analysis also resulted in an es-

Figure 10. YPPs from transformed cell contacts are mostly Triton X-114-extractable and partition to the aqueous phase. Cell contacts were prepared from transformed cells cultured overnight on gelatin films in the presence of vanadate and extracted with Triton X-114 as described in Materials and Methods. The Triton X-114-soluble material (TX), the Triton X-114-insoluble material (IN), and the aqueous (AQ), and detergent phases (DT) were separated by SDS-PAGE and immunoblotted with mouse mAb 4G10 to identify the YPPs. Each lane represents an aliquot representing an equal volume from the original extract. Most of the YPPs are Triton X-114 soluble (TX) and partition to the aqueous phase (AQ). Molecular masses (kD) are given at left.

Discussion

Invadopodia, visualized using immunofluorescent and immunoelectron microscopy, are membrane protrusions which extend beneath the cell into the substratum after degradation of the exogenous fibronectin and gelatin matrix. They may also be involved in the internalization of digested matrix material (34). By using immunomicroscopy, we demonstrate that YPPs are localized in adhesion plaques from noninvading normal or transformed cells, but find that they are highly elevated in association with invadopodia, another class of contact, which are involved in substrate degradation and invasion. These results are consistent with previous morphological studies (31,37), and extend these observations to in-

of tensin and paxillin, although the subcellular distribution of the unphosphorylated form of the YPP150 is unknown. Taken together, the fractionation and immunolocalization data are consistent with the interpretation that tyrosine-phosphorylated YPP150 is localized in invadopodia.
Figure 11. Identification of YPPs from transformed cell contact membranes that are absent from adhesion plaques of untransformed cells. Cell contacts from normal (N) or transformed (T) cells grown overnight on gelatin films were isolated in the presence of a vanadate-containing buffer as described in Materials and Methods. 20 μl of the total Triton X-114-soluble fraction from normal (lane N 20) and transformed (T 20) cells were compared, as well as 2 μl of RSVCEF contact membranes (T 2) (A). All samples were reduced and boiled. Immunoblots were probed using mouse mAb 4G10 in the absence (A) or presence of 2 mM O-phospho-L-tyrosine (B), followed by secondary goat anti-mouse conjugated to horseradish peroxidase. In (C), a lane containing 100 μl of ethanol precipitated contacts from untransformed cells (N 100) is shown. Lanes N 100 and T 20 were cut from the photograph of the gel and compared side-by-side. A smear of protein is seen at 120–130 kD in the N 100 and another band at 71 kD. In the transformed cell contacts, lane T 20, four major bands are observed, at 150, 130, 81, and 77 kD. Molecular masses (kD) are shown.

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The estimation that more than 41% of the YPPs are contained in invadopodia and would thus significantly contribute to the YPP profile from isolated transformed cell contacts.

Comparison of cells cultured on plastic with cell bodies and cell contacts from cells cultured on matrix in the last three lanes of Fig. 13 demonstrates that cell contacts adhering to the matrix contain the highest specific activity of YPPs (Table II). To test whether genistein treatment grossly alters the specific activity of YPPs contained in isolated invadopodia, transformed cells were cultured overnight in the presence or absence of 5 μg/ml genistein after a period of 1 h for cell attachment. However, changes in the pattern of YPPs in subcellular fractions were not detectable on immunoblots (data not shown).

Differences in immunoblotting patterns were observed for talin, tensin, and paxillin that may reflect differences in the proteolytic milieu between cell body and cell contact fractions of normal and transformed cells or differences in secondary modifications of polypeptides. Comparison of talin between cell body and cell contact fractions, for example, reveals changes in the stoichiometry of the upper and lower immunoreactive bands of talin (Fig. 13, talin). The lower band likely represents a proteolytic fragment of talin, but it is not known if these differences arise before or after subcellular fractionation. Likewise, the multiple bands observed in the tensin blot may be due to the proteolytic sensitivity of the protein. With respect to paxillin, transformed cell fractions contain additional immunoreactive bands compared to fractions from normal cells. In addition, the profile of cell bodies and cell contacts is different. The significance of this is unknown, but these alterations may reflect changes in secondary modifications of paxillin in transformed cells and/or proteolytic variations in cell bodies versus cell contacts.

Comparison of normal contacts with transformed contacts not only reveals quantitative differences, but also qualitative differences in the detectable YPPs. In particular, a YPP migrating at ~150 kD appears to be transformation specific, occurring only in invasive cell contact fractions. Its three- to fourfold enrichment in the cell contact fraction compared with the cell body fraction of transformed cells and its similarity to the distributions of paxillin and tensin, two cytoskeletal proteins that are localized at normal and invasive cell contacts, is consistent with the interpretation that the phosphorylated form of this polypeptide is uniquely associated with invasive contacts. Recently, mAbs have been prepared against the transformation-sensitive YPPs from chicken cells (17,22,26,47). A YPP150 has not been described to our knowledge. Another molecule with similar molecular mass, phospholipase C-γ, that is involved in signal transduction mechanisms associated with tyrosine phos-
**Figure 12.** Paxillin and tensin localize in invadopodia at sites of matrix removal. (A-E) Cells were cultured on fluorescein-fibronectin beads, and indirectly labeled with mouse mAb antipaxillin or rabbit polyclonal antitensin as described in the Materials and Methods, followed by a rhodamine conjugate of goat anti-mouse or goat anti-rabbit antibodies, respectively. Tensin labeling of invadopodia in cells invading into the cross-linked substratum (A and B) co-localizes with sites of fluorescent fibronectin removal (A' and B', arrows). Paxillin labeling of invadopodia (C and E) also co-localizes at sites of fluorescent fibronectin removal (arrows, C' and E'). Migrating invasive cells often leave behind "tracks" of fluorescent fibronectin removal (A', C', and D'), and occasionally the cell becomes detached at sites of extensive matrix removal (D') as evidenced by the lack of cellular paxillin staining (D). The absence of a cell was confirmed by Nomarski differential, interference-contrast microscopy. Bar, 10 μm.
The distribution of YPPs, β₁ integrin, paxillin, tensin, talin, α-actinin, and vinculin in subcellular fractions from normal and transformed cells. Cell contacts from CEFs (Normal) or RSV-CEFs (Transformed) grown overnight on plastic tissue culture dishes (P) or gelatin films (G) were isolated in the presence of 10 mM MOPS, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 M sucrose, and 1 mM Na₃VO₄, 0.2 mM PMSF by shearing away cell bodies as described in the Materials and Methods. The cell bodies were pelleted at top speed in a microfuge and a supernate (S) and cell body fraction were obtained. The contacts and cell bodies were extracted with Triton X-114 and the soluble fractions from the cell body (CB) and contact membrane (C) fractions were obtained. (*) highlights the major band for tensin and paxillin. 22 μg of protein was loaded in each lane. Immunoblotting of proteins was as described in the Materials and Methods.

Some of the major YPPs from transformed cell contacts may be related to those from normal contacts. For example, the pI 30 of transformed cells might be related to similarly migrating bands present in normal cell contacts. The YPP81/77 bands of transformed cells might be related to the YPP71 band from normal cell contacts, although the YPP81/77 bands consistently migrate more slowly than the band from normal cell contacts. YPPs are greatly enriched in transformed cell contacts compared to their specific activities in normal contacts. This increase may influence the adhesive and invasive properties of transformed cells. Future experiments will be directed towards determining the identity of the YPPs from transformed and normal cell contacts using existing and newly obtained mAb reagents against transformation-specific polypeptides, including testing the phosphorylations, does not co-localize with sites of fluorescent fibronectin removal associated with invadopodia of transformed chicken cells (Mueller S. C., and W. T.-I. Chen, unpublished data).

### Table II. Distribution of Cell Contact-Associated Polypeptides within Subcellular Fractions: Determination of Specific Activities by Image Analysis of Immunoblots

|          | Normal | Transformed |
|----------|--------|-------------|
|          | S CB C | S CB C      |
| Integri-n-β₁ | 73(1.0) 59(1.0) 62(1.1) | 29(1.1) 27(1.0) 20(0.7) |
| α-Actinin  | 98(0.8) 129(1.0) 156(1.2) | 140(0.8) 187(1.0) 141(0.8) |
| Talin     | 76(1.5) 50(1.0) 91(1.8) | 71(1.0) 69(1.0) 59(0.9) |
| Vinculin  | 4(1.3) 3(1.0) 38(12.7) | 3(1.0) 5(1.3) |
| Tensin    | 40(2.2) 18(1.0) 83(4.6) | 49(4.9) 10(1.0) 41(4.1) |
| YPP150    | -(-) -(-) -(-) | 48(2.0) 24(1.0) 87(5.6) |
| YPP81/77  | -(-) -(-) -(-) | 34(0.7) 48(1.0) 74(1.5) |
| Total YPP | -(-) -(-) <1(-) | 13(0.6) 22(1.0) 38(1.7) |

* The densities of the first 6 lanes of each immunoblot shown in Fig. 13 were determined semiquantitatively using an image analyzer as described in the Materials and Methods section and the legend to Fig. 13.  
† Mg of total Triton X-114-extractable protein in each fraction.  
‡ The percent of the total Triton X-114-soluble protein contained within each fraction is given.  
** Results are given in arbitrary units representing specific activities since each lane that was immunoblotted contained 22 μg protein. These specific activities are comparable for normal and transformed cell fractions for each polypeptide.  
†† Specific activities are given as a ratio relative to the specific activity in the cell body for normal or transformed cells.  
§§ No detectable bands were observed or they were too low for densitometry.

Figure 13. The distribution of YPPs, β₁ integrin, paxillin, tensin, talin, α-actinin, and vinculin in subcellular fractions from normal and transformed cells. Cell contacts from CEFs (Normal) or RSV-CEFs (Transformed) grown overnight on plastic tissue culture dishes (P) or gelatin films (G) were isolated in the presence of 10 mM MOPS, pH 6.8, 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 0.3 M sucrose, and 1 mM Na₃VO₄, 0.2 mM PMSF by shearing away cell bodies as described in the Materials and Methods.  

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possibility that the YPP71 band of normal cell contacts or YPP81/77 bands from transformed cells is related to paxillin, a recently described YPP (42), and that YPP30 may be related to previously described molecules with molecular masses of ~120 kD (19,26). Although the YPP150 comigrates with one of the major breakdown products of tensin and the YPP81/77 doublet comigrates with the two uppermost bands recognized by antipaxillin in transformed cell fractions, their identities are as yet unknown.

In conclusion, the ability to isolate invadopodia and adhesion plaques via a novel cell fractionation procedure has allowed identification of a YPP150, which is enriched in transformed cell contacts ~3.6-fold over its concentration in the cell body fraction. This polypeptide may represent an invadopodia-specific YPP that is functionally important during transformed cell invasion into the extracellular matrix. Future studies on the formation, function, and subcellular composition of these structures will be greatly facilitated using this combined biochemical and morphological approach to identify unique components of invadopodia.

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