Cell Density Modulates Protein-tyrosine Phosphorylation*

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The growth of normal cells is arrested at saturating cell density in a process termed contact inhibition. An understanding of how cells communicate their contact with one another is critical for determining how cancers develop and spread. Because the molecular details of how fibroblasts communicate density changes are unclear, we examined cell density itself as a source of signaling events rather than examine specific receptors. A technique was developed to measure tyrosine phosphorylation acutely as a function of cell density. The tyrosine phosphorylation of a number of proteins was found to be modified in response to cell density. Three of these proteins were identified as Src, paxillin, and focal adhesion kinase (FAK), of which show an increase in their tyrosine phosphate levels with increasing density. All of these proteins are found in focal adhesions, and both FAK and paxillin are believed to be localized exclusively in focal adhesions. Thus, changing cell density alters tyrosine phosphorylation of focal adhesion components.

Cells need to sense their environment to make decisions. Environmental signals are sensed by receptors that bind ligands such as growth factors, extracellular matrix, or cell surface molecules on adjacent cells. These receptors in turn activate signaling pathways, which communicate the state of the environment to the nucleus so that decisions on growth, differentiation, motility, etc. can be made. The importance of these pathways is made evident by the hallmarks of transformation: loss of requirements for growth factors, extracellular matrix (ECM), and contact inhibition.

Signals from the environment can be positive or negative for growth. The study of positive signals such as growth factors has led to a general model where activation of the receptor by the ligand leads to autophosphorylation on tyrosine residues of the receptor. These phosphorylated residues serve as substrates to assemble an active signaling complex. The immediate result of the activation of these receptor-coupled enzymes is the activation of kinase cascades, which allow the amplification of the signal through the pathway. In each case, the end result of the kinase cascade is modified transcription of select genes in the nucleus. The extracellular matrix also serves as a positive signal for growth. The best characterized extracellular matrix receptors are integrins. Integrins are also important in cell migration and differentiation. Contact of fibroblasts with ECM proteins leads to the activation of multiple signaling pathways, including the activation of protein-tyrosine kinases. In fact, many different integrins can regulate protein-tyrosine kinases. In fibroblasts, the interaction of ECM and integrins results in the increased activity of the tyrosine kinase pp125 FAK (3). Increased FAK activity results in the tyrosine phosphorylation of FAK and the recruitment of signaling proteins such as Src and paxillin to FAK.

Although signals in response to growth factors and extracellular matrix have long been studied, the negative growth signals resulting from cell contact have received attention only more recently. Most of what is known about the signaling resulting form cell-cell contact comes from the study of epithelial cells. Epithelial cells have transmembrane receptors, cadherins, which are important for communicating signals generated at sites of cell-cell contact. The current model suggests that cadherins on one cell can interact with those on another cell and could serve as a molecular probe for cell density. Catenins form a complex with cytoplasmic tail of cadherins (4, 5) which can be phosphorylated by tyrosine kinases including MET, epidermal growth factor receptor, and Src (6, 7). Evidence that this signaling pathway plays a critical role in contact inhibition comes from studies of the Drosophila DLG protein. The DLG protein is found at cell-cell contacts and is homologous to ZO-1 and ZO-2 found in tight junctions (8). Mutations in the DLG protein result in overgrowth of the imaginal disc cells, indicating a role in tissue growth control and signal transduction (9). Although fibroblasts also exhibit contact inhibition, much less is known about the molecular signaling involved.

The complex signaling pathways involved in both positive and negative growth signals rely, in part, on tyrosine phosphorylation. Because of the critical role phosphorylation plays in communicating aspects of the cell’s environment, we examined whether cell density in culture could affect tyrosine phosphorylation. Because it is unclear how fibroblasts communicate density changes, we examined cell density itself as a source of signaling events rather than examining specific receptors and their downstream tyrosine-phosphorylated proteins. By plating cells at increasing densities, we could manipulate the amount of cell-cell contact and observe the phosphotyrosine profiles of cells plated at increasing densities. Using this approach, we have identified several proteins for which phosphorylation state is modified by cell density as a method to identify the phosphorylated proteins responsible for signaling cell contact. Here, we begin the identification and initial molecular characterization of some of the proteins for which phosphorylation changes in response to plating densities.
MATERIALS AND METHODS

Cell Culture—Balb 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells were grown to confluence. Confluent cells were trypsinized and replated in fresh medium (Dulbecco's modified Eagle's medium supplemented with 10% calf serum) at 0.5, 1, 3, or 5 x 10^6 cells/10-cm plastic tissue culture dish. Pre or P lanes on figures represent samples from confluent cells before trypsinization and replating. These cells have been treated with fresh medium to be consistent with the treatment of the replated cells. Cells were allowed to attach to the dish for 2.5 h. Gradual confluence studies were done by platting Balb 3T3 cells at 10% confluence and harvesting when cells were at different levels of confluence. Cell medium was changed at least 24 h before harvest to avoid serum effects.

Harvesting Cell Lysate—Cells were washed twice with ice-cold phosphate-buffered saline to remove medium. Cells were lysed at 0 °C on the plate with Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris, pH 8.0, 10% glycerol, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/μl aprotinin, 20 μg leupeptin, 1 mM Na_3VO_4, 10 mM NaF). Insoluble material was removed by a 3-min spin at 12,000 x g. The Nonidet P-40-soluble fraction was used for Western blots and immunoprecipitations. For the Src Western blot, the Nonidet P-40-insoluble fraction was re-extracted with RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), creating the Nonidet P-40-insoluble/RIPA-soluble fraction.

Western Blots—Equal microgram amounts of each lysate (generally about 25 μg) were separated by discontinuous SDS-PAGE. Transfer of proteins to nitrocellulose was accomplished by electrotransfer in transfer buffer (20 mM glycin, 48 mM Tris, 0.37% SDS) at 400 mA for 4 h at 4 °C. Nitrocellulose was blocked by 5% powdered milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Primary antibodies were diluted in 1 x TBST and incubated for 1 h at room temperature. Primary antibodies against FAK and paxillin were obtained from Transduction Laboratories and used according to the manufacturer. Anti-phosphotyrosine antibody (4G10) and anti-FAK antibody for immunoprecipitation were from UBI. Detection of Src was through anti-Src 327 antibody provided by Dr. Brugge, Harvard Medical School, and was diluted 1:5000. Proteins were detected using Amersham's ECL kit for Westerns. ECL was performed as suggested by the manufacturer.

Quantitative Western Blot—Precipitation of paxillin was accomplished by mixing 120 μg of lysate with 4 μg of anti-paxillin (Transduction Laboratories). Immunocomplexes were collected by the addition of protein A-Sepharose beads (Pharmacia Biotech Inc.). Washed complexes were separated by SDS-PAGE and transferred to nitrocellulose. The blot was stripped of antibody and reprobed with anti-phosphotyrosine antibody (4G10). Secondary antibodies coupled to peroxidase were from Amersham Life Science and were diluted 1:5000. Proteins were detected using Amersham's ECL kit for Westerns. ECL was performed as suggested by the manufacturer.

Immunoprecipitations—The precipitation of paxillin or tyrosine-phosphorylated proteins was accomplished by mixing 200 μg of lysate with either 4 μg of anti-paxillin (Transduction Laboratories) or 60 μg of 4G10. Immunocomplexes were collected by the addition of protein A-Sepharose beads (Pharmacia). Immunoprecipitations were washed twice with 0.5 M LiCl, 0.1 M Tris, pH 8, and once with phosphate-buffered saline. Immunoprecipitations in Fig. 3 were accomplished by mixing 240 μg of lysate with 16.5 μg of 4G10 or 4.5 μg of anti-FAK monoclonal antibody (UBI). Complexes were incubated with 1 μg of anti-mouse Sepharose beads (Zymed Laboratories Inc.). Beads were washed three times with lysis buffer.

Kinase Assay—Src was immunoprecipitated from 650 μg of lysate with the addition of 3.5 μg of 32C Src antibody and protein A-Sepharose. The washes and kinase assay were performed as described (10).

Phosphatase Treatment—Paxillin immunoprecipitates were incubated with λ phosphatase buffer (50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 2 mM MnCl₂, 100 μg/ml bovine serum albumin) with or without 600 units of λ phosphatase for 30 min at 30 °C.

RESULTS

To gain insights into the signaling pathway used by fibroblasts to communicate cell-cell contact, we used a method in which confluent cells were trypsinized and replated at various densities, thus providing a method to control the amount of contact the cells have with each other. At the lowest density (0.5 x 10^6 cells/10-cm dish), cells exhibit little contact with other cells. At the highest density (5 x 10^6 cells/10-cm dish), each cell was in contact with several neighbors. Intermediate densities of 1 and 3 x 10^6 cells/10-cm dish yielded levels of contact consistent with their densities. Cells were allowed to interact for a fixed time (2.5 h) before they were harvested. This method reduces the number of variables such as medium replacement, which are necessary in experiments where cells grow together over several days, and allows for the detection of transient signals which could be lost over time. Because tyrosine-phosphorylated proteins have been shown to be important in many cell signaling pathways, we evaluated the phosphotyrosine pattern of the lysates obtained from cells in which the level of cell-cell contact was manipulated as a means to understand the molecular consequences of contact. Using this method, we have found differences in the phosphotyrosine pattern between lysates harvested from cells in which contact was minimal compared with cells that contacted many neighbors. Fig. 1 shows a typical phosphotyrosine pattern obtained from lysates prepared at 2.5 h after replating cells at increasing densities. The pattern can be divided into three categories: bands in which the phosphotyrosine signal increases with cell density, bands in which the phosphotyrosine signal seems to decrease with increasing cell density, and bands that do not respond to changes in cell density. Proteins of 120, 62, 45, and 20–30 kDa have increased phosphotyrosine levels in response to higher cell density. Other proteins such as a 68- and 58-kDa protein seem to have levels of phosphotyrosine that decrease with increasing cell density, and bands that do not increase with increasing cell density. Still other proteins do not appear to have changes in their phosphotyrosine content as a result of increased plating density (92 kDa).

Identification of Proteins—By comparing the molecular weight of the unknown protein to the molecular weights of known tyrosine-phosphorylated proteins, a list of candidates was generated. Antibodies against the candidates were ob-
Density-dependent Phosphorylation

Density-dependent Effects on pp60<sup>src</sup>-—Because we had observed changes in tyrosine phosphorylation as a function of cell density, it was reasonable to speculate that tyrosine kinases might be activated. Kinases known to be modulated by cell-cell and cell substrate contacts include pp60<sup>src</sup> and FAK.

As one of the bands that increased with density in the anti-phosphotyrosine (4G10) blot was about 60 kDa and aligned with the blot was reprobed with anti-Src antibodies, we examined the effects that different densities had on the activity and distribution of the tyrosine kinase pp60<sup>src</sup>. Several density-dependent effects were observed. First, replating cells at higher densities shifted pp60<sup>src</sup> to a higher apparent molecular weight (Fig. 2). Lysates from confluent cells, before replating, show a doublet when probed with antibodies against pp60<sup>src</sup>. Both bands of the doublet are of equal intensity. The Src antibody used in these experiments is directed to the SH3 domain of Src and recognizes c-Src as well as v-Src and has not been reported to show a preference for different forms. When cells were replated at low densities, Src remained as a doublet, but cells replated at high densities displayed mainly the upper band with only traces of the lower band (Fig. 2, compare the 0.5 lane to 5 lane of Nonidet P-40-soluble fraction). Second, only the slower migrating form was found in the Nonidet P-40-insoluble/RIPA-soluble fraction, and this form increased in abundance as cells were plated at increasing densities (Fig. 2, compare the 0.5 lane to 5 lane of Nonidet P-40-insoluble/RIPA-soluble fraction). Western blots on this fraction also indicate that the level of Src was high just before replating. Third, pp60<sup>src</sup> kinase activity increases with increasing cell density (data not shown). To determine if pp60<sup>src</sup> had increased kinase activity at higher densities, pp60<sup>src</sup> autokinase assays were done on the Nonidet P-40-soluble fractions. These results revealed that confluent cells and those plated at low density have low levels of kinase activity, whereas cells plated at high densities have high activity. In summary, when cells are replated, the amount of the slower migrating form as well as pp60<sup>src</sup>’s kinase activity increase with increasing density.

FAK Phosphorylation Increases in Response to Cell Density—The anti-phosphotyrosine blot (Fig. 1) showed that, after 2.5 h of contact, a protein of about 120 kDa increased its level of tyrosine phosphorylation in density-dependent manner. This protein has increased tyrosine phosphorylation in both experiments in which cells grow together gradually and experiments in which wortmannin was used to inhibit spreading. Phosphorylation of this protein in response to density can be observed as early as 1 h (data not shown). This protein has been tentatively identified as focal adhesion kinase (pp125 FAK). This assignment is based on three facts. First, FAK is known to be one of the main tyrosine-phosphorylated bands in adherent fibroblasts. Second, blots probed with anti-phosphotyrosine (4G10), stripped of the antibody, and reprobed with a FAK antibody display a band at exactly the same location (data not shown). Third, immunoprecipitations with anti-phosphotyrosine (4G10) or FAK antibodies displayed a band of 125 kDa when probed with the anti-phosphotyrosine antibody 4G10 (Fig. 3). Caution should be used in evaluating the levels of tyrosine-phosphorylated FAK in the immunoprecipitation because the precipitating antibody does show different immunofinity for modified forms of FAK. The protein level of FAK does not change appreciably with changing densities (data not shown).

Tyrosine-phosphorylated Paxillin Increases in a Density-dependent Manner—Anti-phosphotyrosine blots of total lysates show a band at about 68 kDa (paxillin), which became less intense as cell density increased. Upon further study, it became clear that the level of tyrosine-phosphorylated paxillin actually increased with cell density. This fact was masked because paxillin undergoes heterogeneous modifications at higher cell density causing the phosphotyrosine signal to become diffuse. Upon review of the original anti-phosphotyrosine blots, one can see a 68-kDa band shifting into smear at that molecular weight. This only became clear after immunoprecipitations with 4G10 and anti-paxillin antibodies. Fig. 4A shows an immunoprecipitation with anti-paxillin antibody followed by a Western to detect tyrosine-phosphorylated proteins. At low density replating, most of the tyrosine-phosphorylated paxillin is in a single band with a light diffuse band above it, corresponding to other phosphorylated forms. When cells are replated at the highest density, the bottom band is missing and has been replaced by a much more intense smear above it. When the bottom band and the smear are taken as a whole, the overall phosphotyrosine signal increases as density increases. The reciprocal immunoprecipitation confirms this finding. In Fig. 4B, lysates were precipitated by anti-phosphotyrosine antibody (4G10) and then probed with anti-paxillin antibodies. At low density replating, the amount of tyrosine-phosphorylated paxillin is very low, as shown by the low levels recovered by precipitation with the anti-phosphotyrosine antibody 4G10 (Fig. 4B). When cells are replated at high density, the amount of tyrosine-phosphorylated paxillin recovered in a 4G10 immunoprecipitation is much higher (Fig. 4B). These experiments clearly show that the amount of tyrosine-phosphorylated paxillin increases with increased density, however, Western blots...
indicated that the amount of paxillin also increases with cell density (see below). To determine if the amount of phosphotyrosine per paxillin molecule was increasing or whether the increase in the phosphotyrosine signal was solely caused by the increase in protein levels, a quantitative Western was performed. The quantitative Western demonstrated that the level of tyrosine phosphate per molecule of paxillin increased more than 3-fold from the lowest to the highest density. In short, at low density replating, there are more paxillin molecules and they contain more tyrosine phosphate than the paxillin from cells replated at low density.

Serine/Threonine Phosphorylation and Stability of Paxillin Change in a Density-dependent Manner—Western blot analysis of total cell lysates with paxillin antibodies showed that, at lower densities, the paxillin migrated slower than paxillin from high density replating (Fig. 5). Tyrosine phosphorylation cannot explain this result, inasmuch as paxillin is less phosphorylated on tyrosine at low densities. One explanation was that at low density plating cells have high levels of Ser/Thr phosphate, which is not present when cells are plated at high density. Confluent cells have the faster migrating form of paxillin (Fig. 5), which is converted to a much slower form if cells are replated so they have little cell-cell contact. To confirm that these shifts were due to Ser/Thr phosphorylations and not other forms of modification, phosphatase treatments were done. Extracts were either treated or untreated with λ protein phosphatase. Treatment collapsed higher migrating species, as well as the slower migrating species, to a faster migrating doublet (Fig. 6). Although the λ phosphatase experiment indicates that the shifts are caused by phosphates, the data are consistent with the induction of a Ser/Thr kinase at low cell density or with the activation of a Ser/Thr phosphatase at high density.

To learn more about how paxillin behaves when cells are plated at different densities, lysates from the different densities were examined for their levels of paxillin by Western blot. The level of paxillin is high in confluent cells just before trypsinization and replating (Fig. 5). The amount of paxillin drops dramatically in cells replated at low density but remains high in cells plated at high density (Fig. 5). The loss of paxillin at low density replating is not caused by the movement of paxillin to a Nonidet P-40-insoluble fraction because addition of RIPA to the Nonidet P-40-insoluble fraction showed that Nonidet P-40 had extracted all the paxillin at each density examined (data not shown). To determine if the change in paxillin levels was the result of breakdown or increased synthesis, an [35S]methionine pulse-chase experiment was done (data not shown). Cells were radiolabeled before replating at the various densities, and lysates were harvested 2.5 h later. At low density, both the 35S signal as well as the signal generated by a Western blot were low; however, when cells were replated at high density, the 35S signal as well as the signal generated by a Western blot remained high. This result suggests that the change in paxillin levels is caused by breakdown at low density replating rather than increased synthesis at high density replating.

Next, we examined if the changes in paxillin levels and Ser/Thr phosphorylation could be detected at time points earlier than 2.5 h. At 1 h after plating, cells exhibited the paxillin shifting seen at the 2.5-h time point but did not exhibit as dramatic a level change as that seen at the longer time point (Fig. 7). The 1-h time point most likely represents the early phase of the paxillin turnover for several reasons. First, the paxillin levels at the lower cell densities are lower than the levels in the higher density cells indicating the beginnings of

**FIG. 4.** The level of tyrosine-phosphorylated paxillin increases with density. 200 μg of lysates obtained 2.5 h after plating, for each density, were immunoprecipitated with a mAb coupled to protein A-Sepharose beads. Immunoprecipitate was washed and separated by SDS-PAGE and transferred to nitrocellulose. A, lysates were immunoprecipitated with antibody against paxillin, and tyrosine-phosphorylated paxillin was detected with the mAb 4G10. Molecular weights are indicated to the right. The position of both paxillin and the heavy chain (H.C.) of the immunoprecipitating antibody are given. B, tyrosine-phosphorylated proteins were precipitated with 4G10, and the presence of paxillin in the precipitate was determined by Western blot analysis with a mAb against paxillin. The density of the Balb cells (× 10⁶) is given at the bottom. n = 3.

**FIG. 5.** Paxillin protein and phosphate levels change with density. Lysates from each density were separated by 10% SDS-PAGE and transferred to nitrocellulose. Paxillin was detected with a mAb against paxillin. The number of cells plated 2.5 h before harvest is given above each lane. B shows results from an independent experiment where the lysates were separated further, allowing the detection of several shifted paxillin forms. n = 7.
the turnover. Second, several smaller fragments are detected by the paxillin antibody in the low density lysates, suggesting that these may be breakdown products generated by the paxillin turnover.

Distinguishing Effects of Cell Density from Those Due to Cell Spreading—The changes in tyrosine phosphate seen at low cell density might in theory be due to cell spreading on the substrate, as the sparse cells did exhibit spreading at the 2.5-h time point. Notably, each of the molecules we have studied has been localized, at least in part, in focal contacts. To distinguish whether the tyrosine phosphorylations we observed were due to spreading, several approaches were taken.

First, we utilized an entirely different protocol to study the density effect. In this protocol, cells were allowed to become confluent gradually. We plated cells at a fixed density (10%) onto fresh dishes and harvested them over several days at different levels of confluence. Cells were harvested at 20%, 40%, and 80% confluence. In this method, cells have had an equal opportunity to spread at all points; thus, differences seen should be independent of spreading. As before, molecular consequences of contact were evaluated by the phosphotyrosine pattern of the lysates (Fig. 8). Although this method lacks the acute nature of our first protocol, it is apparent that the effects of density are visible with both protocols. This alternative approach also served to validate the methodology used.

Finally, we made use of the chance observation that cells treated with the PI 3-kinase inhibitor wortmannin showed a large defect in their ability to spread on plastic tissue culture dishes (Fig. 9). Wortmannin dramatically inhibited spreading at early time points (30–60 min). This inhibition partially recovered by 2.5 h but still showed a large defect in spreading (Fig. 9). Lysates were prepared at 2.5 h from cells plated at different densities in the presence of wortmannin. These lysates were examined for their phosphotyrosine content as before. The pattern obtained from these lysates is similar to patterns of obtained without wortmannin (Fig. 10). This would indicate that many proteins can become tyrosine-phosphorylated in response to density in a PI 3-kinase-independent manner.

DISCUSSION

Cells live in a milieu of signals that must be interpreted so the cell can respond correctly to its environment. Complex signals such as stress, growth factor availability, the presence of extracellular matrix, and the presence of other cells among others must be translated into actions such as proliferation, growth arrest, differentiation, and migration. Many of these
protein-tyrosine kinases such as MET and the epidermal growth factor receptor are thought to phosphorylate components of the cadherin-catenin complex, thereby effecting adhesion junctions and cell-cell contact in epithelial cells (12, 14). At the moment, however, we cannot determine whether the density-dependent activation of Src originates at sites of cell-cell contact or at other sites such as focal adhesions (see below).

Both paxillin and FAK, identified in this study as having increased tyrosine phosphorylation in response to increased cell density, are localized to focal adhesions. FAK, the tyrosine phosphorylation of which is generally associated with its activation, becomes tyrosine-phosphorylated in response to integrin engagement (11, 19, 20) and growth factors such as platelet-derived growth factor (21, 22). The increase in tyrosine phosphorylation that we observe can be seen at the 2.5-h time point as well as in the gradual confluence and wortmannin experiments, suggesting that this is the result of contact rather than spreading. Paxillin, a proximal signaling target for tyrosine kinases at the membrane, also shows increased tyrosine phosphorylation as cell density increases. Paxillin, like FAK, becomes phosphorylated in response to many extracellular events beside the one reported here, including integrin-mediated adhesion (11, 23–28). Tyrosine phosphorylation of paxillin and FAK are important for the assembly of functional signaling complexes. In fact, paxillin binds several known signaling factors, including FAK (29), CSK (30) Src (31), and Crk (32). Both paxillin and FAK can become tyrosine-phosphorylated in response to integrin engagement as well as growth factors such as platelet-derived growth factor. In fact, it now seems clear that signals from growth factor receptors and from integrins act synergistically in many signaling pathways. The data reported here suggest that cell density could modulate these signals by changing the phosphorylation state of proteins in focal adhesions. One would expect that signals such as contact with ECM, the presence of growth factors, and the density of cells would converge at some level so that a decision as to whether to divide or not could be reached.

Could events other than cell-cell contact be the cause of phosphotyrosine changes observed here? Spreading could affect our results inasmuch as cells plated at high densities are less able to spread then those plated sparsely. To address whether some of the changes described may have been caused by spreading rather than cell-cell contact, we have examined the density effects as the cells become confluent over several days. This method removes the effect of spreading and gave us results very similar to our original method (compare Figs. 1 and 8). Finally, we noticed that the drug wortmannin dramatically inhibits spreading and we have begun to use it to inhibit spreading. These different methods allow us to rule out a causal role for spreading in most of the observed tyrosine phosphorylations. A second mechanism other than cell-cell contact which could affect our results involves the secretion of extracellular matrix components by the newly plated fibroblasts. At high cell densities, more matrix might be laid down by the cells; this in turn might lead to higher integrin engagement, resulting in the activation of FAK etc. However, our cells were plated in medium containing serum and therefore ECM should have been present. Furthermore, we found that our results were unchanged when we plated cells directly onto fibronectin coated plates (data not shown). Thus, although it remains possible that factors other than cell-cell contact play a role in our observations, cell-cell contact remains the simplest explanation given the data.

Although the tyrosine phosphorylations that we see are likely due to cell density, they could be caused by cell-cell contact and the signaling generated by this contact or by other
phenomenon related to density. Several models can be envisioned for how cells communicate their density. One way in which this information could be transmitted is through cell surface receptors. This system might consist of receptors that could interact with a ligand on other cells. Interaction of these “density” receptors would start a tyrosine phosphorylation cascade and serve as a molecular probe for cell density. The activation of the density receptor would activate Src and FAK and result in the tyrosine phosphorylation of paxillin and other proteins. Alternatively, receptors at points of cell-cell contact may be modulating signaling molecules in the focal adhesions. We have not yet identified many of the tyrosine-phosphorylated proteins shown in Fig. 1, and it remains possible that one of these proteins is the density receptor. In human fibroblasts, a density-dependent receptor has been reported (33). Human fibroblasts have a receptor called contactinhibin receptor, which is important in contact inhibition (33). The receptor binds to contactinhibin, its membrane-bound ligand, located on adjacent cells and mediates growth inhibition. The contactinhibin receptor can be serine/threonine-phosphorylated, and this modification results in a lower affinity for its ligand (33). Neither the receptor nor the membrane-bound ligand were reported to have kinase activity.

Density might be sensed by gradual loss of substrate contact area or cell shape changes as the cell becomes confluent. As cells become more confluent, the area of substrate each cell has to interact with decreases. Cell density, therefore, could be sensed by the gradual loss or modification of integrin-extracellular matrix interactions as the area of substrate per cell decreases. Our data, however, indicate that both FAK and paxillin have increased tyrosine phosphorylation in response to increased density. Because decreased area might be expected to lead to fewer focal contacts, one might naively expect that tyrosine kinase activity at focal adhesions would decrease. However, higher cell density may result in hyperactive signalling from the remaining focal adhesions. Finally, cell density could affect signals emanating from focal adhesions by altering the cells shape (34, 35). As density increases, cells are forced to share the surface area forcing them to adopt a tall and narrow shape. Shape changes can affect DNA synthesis (34, 35) and might affect tyrosine phosphorylation in some manner currently not understood.

Normal cells must integrate many aspects of their environment, chief among them whether they are in contact with substrate and or cells. To characterize contact inhibition on a molecular level, we have mapped the overall tyrosine phosphate pattern as cell density increases and have begun to identify those proteins for which pattern changes in response to density. To our surprise, the first three proteins that we have identified are generally thought of as focal adhesion proteins.

One interpretation of our data is that cell-cell contacts modify the relation between cells and the substratum by altering the signaling from focal adhesions. Identification of the remaining proteins should help understand some of the molecular events involved in contact inhibition.

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