Constitutive Activation of c-Met in Liver Metastatic B16 Melanoma Cells Depends on Both Substrate Adhesion and Cell Density and Is Regulated by a Cytosolic Tyrosine Phosphatase Activity*

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Serial selection in vivo for liver colonization of B16 murine melanoma cells consistently resulted in cell lines expressing elevated amounts of the hepatocyte growth factor/scatter factor receptor (c-Met), which is constitutively activated in the absence of its cognate ligand. In this paper we present evidence suggesting that c-Met constitutive activation in liver-specific B16 melanoma cells depends on both receptor concentration on the cell surface and a cytosolic tyrosine phosphatase activity. In fact, c-Met constitutive activation is suddenly lost upon detachment of the cells from the substrate and is dramatically decreased in adherent cells plated at low density. The loss of tyrosine phosphorylation of c-Met in suspension appears to depend, at least partly, on an increased cytosolic tyrosine phosphatase activity. Instead, lower activation of c-Met at low density mostly results from a decrease in receptor concentration on the membrane. Moreover, we show that c-Met activation does not occur homogeneously on the surface of adherent cells. In fact, receptor concentration and activation appear to be higher on the ventral surface (adherent to the substrate) than on the apical surface. Upon detachment, compartmentalization is lost, leading to a decrease in average receptor density on the plasma membrane and hence to a lower activation.

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic ligand able to trigger events as diverse as cell proliferation, cell motility and invasion, cell differentiation and morphogenesis, and angiogenesis (1). All of the biological effects of HGF/SF are transduced through its cellular receptor, the product of the c-met proto-oncogene, by the activation of its tyrosine kinase domain and the phosphorylation of a multifunctional docking site, which is responsible for the engagement of downstream mediators (2). Since tumor malignancy depends on events such as the ability of cancer cells to undergo uncontrolled cell proliferation, loss of differentiation, acquisition of a motile and invasive phenotype, and induction of neovascularization, the untimely activation of the met receptor might give a strong contribution to the metastatic phenotype, as indicated by both experimental models (3, 4) and clinical findings (5, 6).

We have recently reported that selection for liver-specific colonization in the B16 murine melanoma model system consistently correlates with an increased expression of constitutively active c-Met (7). The aim of the present study is to address mechanisms of constitutive c-Met activation in liver-specific B16-L59 cells. This cell line has been derived after nine passages through the liver and has the highest level of c-Met expression and activation, with no detectable production of the cognate ligand (7). Constitutive activation of the Met tyrosine kinase domain, in the absence of its natural ligand, may occur as a consequence of at least three different mechanisms: 1) loss of the extracellular and transmembrane domains and fusion with a strong dimerization motif, such as in the oncogenic form of met, tpr-met (8); 2) defective post-translational processing, leading to the transport to the plasma membrane of an uncleaved form of 190 kDa (9); and 3) amplification and overexpression of the proto-oncogenic form, as described in the gastric carcinoma cell line GTF-16 (10). Since B16-L59 cells produce a normally cleaved form of c-Met (7), the most likely explanation for its constitutive activation is the elevated density of receptor molecules on the cell surface, leading to their cross-phosphorylation and activation. In fact, we show here that the relative concentration of receptor molecules in different cell surface compartments has a strong influence on c-Met activation, which is high in dense, adherent monolayers, decreases in sparse monolayers, and almost disappears after cell detachment. Moreover, cell adhesion and density also affect cytosolic tyrosine phosphatase activity, which may be involved in regulating the activation state of c-Met. Results presented in this paper show for the first time in the same cell line the dramatic effects of both cell adhesion and density, plus the effects of temperature changes, on the activation state of a tyrosine kinase receptor, such as c-Met.

EXPERIMENTAL PROCEDURES

Cells, Extracts, and Antibodies—B16-L59 cells have been selected for liver-specific colonization from parental B16-F1 cells by means of repeated passages through the liver of syngeneic mice (11). They were routinely grown in DMEM with 10% FCS in a humidified incubator at 37 °C and periodically checked for mycoplasma infection.

Cell extracts to be used for Western blot determination of c-Met and tyrosine phosphorylated proteins or for evaluation of tyrosine kinase and phosphatase activities were prepared with either Nonidet P-40 buffer (137 mM NaCl, 20 mM Tris/HCl, pH 8.0, 1% Nonidet P-40, 10% glycerol), or with CHAPS lysis buffer (5 mM MgCl2, 1 mM EGTA, 100 mM NaCl, 10% glycerol, 1% (w/v) CHAPS, 25 mM Hepes, pH 7.4) containing 1 mM orthovanadate (omitted for tyrosine phosphatase assay) and protease inhibitors (25 μg/ml each of aprotonin and leupeptin from Sigma; 1 μM AEBSF from Calbiochem). After incubation of cells for 20 min at 4 °C, extracts were clarified by centrifugation in an Eppendorf microcentrifuge, and proteins were determined by the DC-Lowry assay (Bio-Rad).

For Western immunoblot analysis, similar amounts of proteins were
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Separated by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinyliden difluoride membranes (Immobilon, Millipore Corp.), and treated with specific antibodies (anti-Met-peptide rabbit polyclonal antibodies were from Santa Cruz Biotechnology, anti-phosphotyrosine (anti-Tyr(P)) mouse monoclonal 4G10 was a kind gift of Kurt Ballmer, Friedrich Miescher Institute). Peroxidase-labeled secondary antibodies (Dako Corp.) were detected by the luminol reaction (Amersham Corp.) on Hyperfilm (Amersham Corp.).

Cytosol and Membrane Preparation—B16 cells were put in suspension in DMEM with 10% FCS, rinsed twice in PBS, resuspended in cold PBS plus protease inhibitors as above, and sonicated on ice to break the cells. After removal of nuclei by low speed centrifugation, membranes were pelleted by ultracentrifugation at 100,000 g for 30 min on ice. The extract, clarified by ultracentrifugation, and the postmembrane supernatant, taken as the cytosol fraction, were then used in the tyrosine kinase assay as described below.

Tyrosine Kinase and Phosphatase Activity—Cell extracts were prepared as described above from adherent cells plated at different densities as indicated in the figure legends or from cells in suspension. In the latter case, cells were detached from the dish by EDTA treatment, resuspended in complete DMEM, and incubated under different conditions, as described in the figure legends. After two rinses with PBS, the pellet was extracted in lysis buffer as above.

Tyrosine kinase activity was assayed in triplicate samples by incorporation of 2P in an artificial substrate (polyglutamyl tyrosine 4:1, Sigma) as described (7). Specific incorporation on tyrosine was calculated after subtraction of blanks (with no polyglutamyl tyrosine added) and expressed as specific activity (femtomoles of phosphate/min/μg of protein).

Alternatively, nonradioactive determination of tyrosine kinase or phosphatase activity was carried out with specific kits (Boehringer Mannheim), in which tyrosine phosphorylation of biotinylated synthetic peptides bound to an avidin-coated multwell plate was detected by ELISA after being labeled with a peroxidase anti-phosphotyrosine antibody. In this case, proteins from cell or membrane extracts or from cytosol were diluted in 20 mM Tris, pH 7.2 (tyrosine phosphatase assay), or 50 mM Hepes, pH 7.4 (tyrosine kinase assay), containing bovine serum albumin (1.0 mg/ml), β-mercaptoethanol (0.1%), and orthovanadate (100 μM; only tyrosine kinase assay). Tyrosine phosphatase activity is expressed as (T1 - T2)/T2 (Fig. 1, upper panel), where T1 and T2 and T2 are the optical reading of the control to which both proteins and an excess of orthovanadate were added and T2 is the optical reading at a certain time.

Cell Surface Biotinylation and Immunoprecipitation—106 cells were plated onto 0.4-μm pore filters of 35-mm transwell chambers (Costar Corp.) in DMEM with 10% FCS. The next day 100 mg/ml biotin-NHS (bismidoisocaprate N-hydroxysuccinimide ester; Sigma B-2643) from a 100 mg/ml stock solution in dimethyl sulfoxide was diluted to 1 mM in Ca2+/Mg2+-free PBS, and then to 0.5 mg/ml in serum-free DMEM. Apical or ventral surface labeling was achieved by adding 1.0 ml of biotin (0.5 mg/ml) to either the upper or the lower chamber of the transwell (whereas the opposite surface received the vehicle only) for 20 min at room temperature. After being rinsed three times with PBS, cells were extracted in CHAPS lysis buffer containing protease inhibitors and 1 mM orthovanadate for 20 min at 4°C as described above.

Immunoprecipitation was performed at 4°C. Aliquots of 350 μg of proteins in 500 μl of CHAPS buffer were preadsorbed with protein A-Sepharose (Bio-Rad) for 30 min. The supernatant then received either anti-Met (Santa Cruz Biotech) or anti-Tyr(P) (mouse monoclonal 4G10) and anti-mouse IgG (Dako Corp.) for 2 h. Immunocomplexes were then precipitated with protein A-Sepharose for 30 min, rinsed three times with CHAPS buffer, and solubilized in reducing Laemmli's sample buffer for further SDS gel electrophoresis and immunoblotting with avidin peroxidase (Sigma) to detect biotinylated surface proteins.

RESULTS

Both Adhesion and Density Control c-Met Tyrosine Phosphorylation—We have recently reported that the B16-L59 cell line, selected in vivo for liver-specific colonisation (11), expressed the c-met proto-oncogene at higher levels than the parental line B16-F1 and that as a consequence of its overexpression, c-Met was constitutively activated in B16-L59 cells (7). Data presented in Fig. 1 show that the difference between LS9 and F1 in tyrosine phosphorylation of c-Met and other proteins related to its kinase activity (7) is clearly visible only when cell extracts are prepared from adherent cells. On the contrary, in extracts obtained from cells incubated in suspension for 30 min, a pronounced decrease of tyrosine phosphorylation in both LS9 and F1 cells is detected, so that every difference has now disappeared (Fig. 1A, upper panel). Moreover, whereas the amount of c-Met in F1 cells does not seem to depend on their adhesion state, there is a net decrease in the amount of c-Met when LS9 cells are put in suspension (Fig. 1A, lower panel).

When we measured the tyrosine kinase activity detectable in LS9 cells (which we have shown to be mostly dependent on c-Met; Ref. 7) we found that it is decreased upon cell detachment by an amount that is directly proportional to the estimated decrease in c-Met expression (29% decrease in activity and 33% decrease in expression) (Fig. 1B). In contrast, since c-Met contributes only a minor fraction of the tyrosine kinase activity detectable in F1 cell extracts (7), no major changes in tyrosine kinase activity are detectable in cell extracts prepared from F1 either adherent or in suspension (Fig. 1B). It is, however, interesting to observe that, in spite of a comparable absence of tyrosine phosphorylation in both LS9 and F1 cell suspension extracts (Fig. 1A, upper panel), the amount of c-Met (Fig. 1A, lower panel) and its kinase activity (Fig. 1B) were still higher in LS9 than in F1, suggesting that the decrease in tyrosine phosphorylation that follows detachment is not entirely due to the decrease of c-Met tyrosine kinase activity.

Furthermore, we have found that in addition to the adhesion state of the cells, cell density can also influence c-Met activation and the tyrosine phosphorylation pattern of adherent LS9 cells. At low cell density, both tyrosine phosphorylation and tyrosine kinase activity are lower than at high cell density (Fig. 1, C and D). Cell size may decrease with increasing cell density, and we have in fact found 50% less protein per cell in cell extracts at high cell density. This finding has prompted us to analyze tyrosine phosphorylation in these extracts by comparing similar amounts of proteins per lane (Fig. 1C, first two lanes) or different amounts of proteins derived from a similar number of cells (Fig. 1C, next two lanes). In both cases a decrease in tyrosine phosphorylation at the lower density could be detected. Interestingly, when comparing similar amounts of proteins, an apparent increase (about 33%) of c-Met at high density could be observed, which, however, disappeared when comparing proteins derived from the same amount of cells (Fig. 1C, bottom panel). These data indicate that the amount of c-Met per cell remains constant at the different densities, whereas its ratio to the total amount of proteins in cell extracts decreases at decreasing densities, leading to a lowering of its concentration on the cell surface.

The loss of tyrosine phosphorylation upon cell detachment appears to be a sudden event, independent of the method used for detachment (scraping in PBS shows the same effects; data not shown) and detectable as soon as LS9 cells are removed from the substrate (Fig. 2A). On the other hand, regaining tyrosine phosphorylation proceeds with slower kinetics, and little phosphorylation is achieved 30 min after replating, as compared to the normal phosphorylation pattern at 24 h (Fig. 2B).

Increased Activity of a Cytosolic Tyrosine Phosphatase in LS9 Cells in Suspension—The decrease in tyrosine phosphorylation that follows cell detachment could be partially prevented by the addition to the cell suspension of a general inhibitor of tyrosine phosphatase activity, such as orthovanadate (1 mM) (Fig. 3A), suggesting the involvement of a tyrosine phosphatase activity in the observed loss of tyrosine phosphorylation. The presence, however, of two different concentrations (10 and 50 μM) of phenylarsine oxide (Fig. 3A), which has been reported to be more specific for the inhibition of membrane-bound, receptor-type tyrosine phosphatases (12, 13), was not able to restore...
tyrosine phosphorylation in cell suspension (Fig. 3A). In agreement with these observations, measurement of tyrosine phosphatase activity in cell extracts derived from either adherent or suspended cells showed an increase of activity upon cell detachment (Fig. 3B). Interestingly, similarly to what observed for the tyrosine kinase activity, the tyrosine phosphatase activity also increased with the cell density. A proportional further increase upon cell detachment could be observed at both densities (Fig. 3B). Finally, the tyrosine phosphatase activity was much higher in the cytosolic than in the particulate fraction derived from resuspended cells (Fig. 3C), ruling out the involvement of a membrane-bound receptor-type tyrosine phosphatase in the observed events.

Temperature Dependence of the Tyrosine Phosphorylation Pattern—The dramatic reduction in tyrosine phosphorylation following cell detachment is also prevented by a temperature decrease to 4 °C and appears to be fully reversible, since further shifts of temperature between 37 and 4 °C of the cell suspension modified the tyrosine phosphorylation pattern as expected (Fig. 4A). One possible explanation for the recovery of tyrosine phosphorylation at 4 °C is that the phosphatase activity is more inhibited than the kinase activity by a temperature decrease. However, when we measured the rate of inhibition of the two activities when the temperature was lowered to 4 °C, we found the same amount of decrease for both the tyrosine kinase and phosphatase activities (Fig. 4B). A similar rate of decrease, however, does not necessarily mean that the absolute amounts of kinase and phosphatase activities remaining at 4 °C maintain the same balance as before. Therefore we also determined the kinetics of tyrosine phosphorylation of an artificial substrate at either room temperature or 4 °C, in the absence of the tyrosine phosphatase inhibitor orthovanadate, so to measure the actual balance of the kinase and phosphatase activities at the two temperatures (Fig. 4C). Results indeed show that, whereas at room temperature the tyrosine phosphatase activity is prevalent, slowly leading to a loss of tyrosine phosphorylation, the opposite is true at 4 °C. These results are further confirmed in the experiment illustrated in Fig. 4D. In this case, cell extracts from adherent cells have been prepared at either room temperature or 4 °C, in the presence or in the
absence of orthovanadate in the extraction buffer. When orthovanadate was present, and thus tyrosine phosphatase(s) inhibited, the amount of tyrosine phosphorylation was higher at room temperature than at 4 °C, because tyrosine kinase activity was obviously more efficient at the higher temperature. When, however, orthovanadate was omitted in the extraction buffer, so that the effect of both tyrosine kinase and phosphatase activities could be detected, the resulting tyrosine phosphorylation was higher at 4 °C than at room temperature, indicating again that at the higher temperature the balance of activities was in favor of the tyrosine phosphatase, whereas the balance was inverted at the lower temperature.

Compartmentalization of c-Met in Adherent Cells—Since c-Met has been shown to be selectively exposed at the basolateral plasma membrane domain of polarized epithelial cells (14), an intriguing possibility was that in melanoma cells also, even though they are not polarized, the amount of c-Met on the ventral, adherent surface is higher than on the apical surface, creating different densities of receptor molecules in the two compartments, which in turn might influence constitutive activation and tyrosine phosphorylation. The distribution of c-Met on the cell surface of adherent LS9 cells was assessed by plating the cells on filters with pores of 0.4 μm, so that selective biotinylation of cell membrane proteins expressed on the ventral or the apical surface could be achieved by adding the biotin solution either in the lower or the upper chamber of the transwell ("Experimental Procedures" and Ref. 14). Cell extracts prepared after biotinylation were then immunoprecipitated with either anti-phosphotyrosine or anti-Met, and biotinylated proteins in the immunoprecipitate were detected on a Western blot by avidin peroxidase. The left panel of Fig. 5 reports a control showing that the tyrosine phosphorylation pattern of LS9 cells plated on filters and labeled from either side is comparable to what expected in adherent cells. In the further evaluation of the results, it should, however, be considered that, as a consequence of the peculiar shape of an adherent cell, the surface available for biotinylation might be higher on the apical side than on the ventral side. In fact, the total amount of

![Diagram](image-url)

**Fig. 2.** Kinetics of tyrosine phosphorylation loss upon cell detachment and regain of tyrosine phosphorylation after cell plating. A, B16-L59 cells were detached from a confluent plate by EDTA treatment, resuspended in a tube with 10 ml of DMEM with 10% FCS, and put on a rocking plate at 37 °C. At different times, as indicated, 2.5-ml aliquots were taken from the tube, rinsed in PBS by centrifugation, and extracted with CHAPS lysis buffer (SUSPENSION). For comparison, adherent cells were also extracted with CHAPS lysis buffer (ADH). 30 μg of proteins were analyzed by immunoblotting with anti-Tyr(P) (anti-PY) (upper panel) or anti-Met (lower panel). B, B16-L59 cells were detached from a confluent dish by EDTA treatment, resuspended in DMEM with 10% FCS, and incubated for 1 h at 37 °C. An aliquot of cells in suspension was rinsed with PBS and extracted with CHAPS (SUS), and the remaining cells were plated in six multiwell plates at 2 × 10^6 cells/cm^2. After 30 min or 24 h, adherent cells were rinsed with PBS and extracted with CHAPS lysis buffer. 15 μg of proteins were analyzed by immunoblotting with anti-Tyr(P) (anti-PY) (upper panel) or anti-Met (lower panel). The c-Met β subunit at 145 kDa is indicated on the left by an arrowhead.

![Diagram](image-url)

**Fig. 3.** A cytosolic tyrosine phosphatase is further activated upon cell detachment. A, B16-L59 cells were detached from a confluent plate by EDTA treatment, resuspended with 25 ml of DMEM 10% with FCS and aliquoted in five tubes (5 ml/tube). Control cells remained untreated, whereas the other tubes received orthovanadate, phenylarsine oxide, or dimethyl sulfoxide alone at the indicated concentrations. After 30 min of incubation on a rocking plate at 37 °C, cells were rinsed with PBS by centrifugation and extracted with CHAPS lysis buffer (SUSPENSION). For comparison, adherent cells were also extracted with CHAPS lysis buffer (ADH). 20 μg of proteins were analyzed by immunoblotting with anti-Tyr(P) (anti-PY) (upper panel) or anti-Met (lower panel). The c-Met β subunit at 145 kDa is indicated on the left by an arrowhead. B, cell extracts were prepared with CHAPS lysis buffer from LS9 cells monolayers at different densities (low density, 3.5 × 10^4 cells/cm^2; high density, 2 × 10^6 cells/cm^2) or after detachment by EDTA treatment of companion monolayers plated at the same densities. Tyrosine phosphatase activity was quantitated in 30 μg of proteins by a nonradioactive ELISA as described under "Experimental Procedures." C, B16-L59 cells detached by EDTA treatment were fractionated in a soluble, cytosolic fraction and a particulate, membrane fraction by sonication and ultracentrifugation. After extraction of the membrane fraction with CHAPS lysis buffer, 10 μg of proteins were assayed for tyrosine phosphatase activity by nonradioactive ELISA.
biontylation detectable in the two cell extracts was much higher when biotin was added in the upper chamber (thus labeling the apical surface) than when biotin was added to the lower chamber (thus labeling that portion of the ventral surface that is accessible to the solution through filter pores) (Fig. 5).

**DISCUSSION**

Evidence has been presented in this paper that constitutive activation of c-Met in B16-L59 cells may depend upon spontaneous dimerization and cross-phosphorylation of receptor molecules (in the absence of the specific ligand, which normally triggers this event) as a consequence of their density in the plasma membrane, as suggested for the GTL-16 cell line (10). However, the level of protein tyrosine phosphorylation in a cell is the result of the competing activities of both tyrosine kinases and phosphatases. We have already shown that the main tyrosine kinase activity detectable in B16-L59 cells comes from the HGF/SF receptor, the product of the c-met proto-oncogene, which is constitutively active in these cells (7).

We now show...
that a cytosolic enzyme accounts for most of the tyrosine phosphatase activity (Fig. 3C). The balance between these two activities appears to depend on conditions such as adhesion to a substrate, compartmentalization of c-Met, cell density, and temperature.

Upon cell removal from the substrate, there is a sudden loss of tyrosine phosphorylation (Fig. 1A), which is not fully accounted for by the decrease in c-Met and its kinase activity (Fig. 1, A and B). Indeed, in spite of the fact that both c-Met expression and tyrosine kinase activity are still higher in B16-L59 than in B16-F1 cells in suspension, the amount of tyrosine phosphorylation in cell extracts is now at comparable levels in the two lines. Activation of a tyrosine phosphatase after cell detachment might help to explain this observation. A similar event has been described in chick embryo fibroblasts, in which release from the substrate activated a tyrosine phosphatase activity associated with both the cytosolic and membrane fraction (15). In the case of L59 cells, we clearly show that it is a cytosolic tyrosine phosphatase activity that is enhanced after cell detachment (Fig. 3C), and thus is a likely candidate for tyrosine phosphorylation control. However, unlike what described in the GTL-16 cell line (16), the cytosolic tyrosine phosphatase here described is not found in association with c-Met (results not shown).

Cell density, like what was reported in other systems, also had some relevance on tyrosine phosphorylation levels observed in adherent cells (Fig. 1C). Tyrosine phosphorylation and cellular distribution of the epidermal growth factor receptor in A431 human carcinoma cells are modulated by cell-cell contact (17). However, while in our system we report increased tyrosine phosphorylation with increasing density, the response of A431 cells to epidermal growth factor-induced phosphorylation was more pronounced at low density (17). This observation is likely explained by an increase in a soluble tyrosine phosphatase activity in A431 cells grown as either dense monolayers or spheroid cultures (18). Similarly, fibroblasts in which growth arrest was induced by confluence showed an elevation of a membrane-associated tyrosine phosphatase activity, which might represent a regulatory event in the control of cell proliferation caused by cell contact (19). To date, several receptor-type protein tyrosine phosphatases have been described, the expression and/or activity of which is regulated by cell density and cell-cell contact (20).

We report here an increase of both the tyrosine kinase and phosphatase activities with increasing cell density (Figs. 1D and 3B), unlike all the known systems. Therefore, the decrease in tyrosine phosphorylation observed at low density cannot be accounted for by a higher tyrosine phosphatase activity. Cell size, however, can be affected by cell density, and B16-L59 cells appeared to be smaller at higher densities, whereas the amount of c-Met per cell remained stable (Fig. 3C). These results indicate that the same amount of c-Met is distributed on the surface of cells of different size at different densities, thus attaining a concentration and activation that is higher in dense monolayers than in sparse monolayers.

Expression of c-Met in adherent B16-L59 cells appears to occur predominantly on the ventral surface (Fig. 5). It is known that some growth factor receptors are expressed at the basolateral surface in polarized epithelial cells, as shown for c-Met itself (14) and for the epidermal growth factor receptor both in vitro (21) and in vivo (22). However, polarization of epithelial cells requires cell adhesion to a substrate and is rapidly lost upon detachment (23, 24). Compartmentalization of c-Met on the ventral surface may help explain observations such as the dependence of c-Met constitutive activation on cell density and receptor concentration on the membrane, loss of activation after cell detachment, and the ability of B16-L59 cells to further respond to HGF/SF stimulation (7), in spite of the presence of a constitutive activated receptor. In fact, increased c-Met expression (and therefore concentration) on the ventral, adherent surface of melanoma cells might account for the most part of constitutive activation. Receptor molecules expressed at the apical surface would be available for ligand binding and further triggering of the motility (7) and growth (not shown) responses. Upon cell detachment, compartmentalization is lost, and receptor molecules become homogeneously distributed on the cell surface, leading to a decrease in their relative concentration and a consequent decrease of their constitutive activation.

In conclusion, we have presented evidence indicating that constitutive c-Met activation in B16-L59 cells depends on overexpression and density of receptor molecules on the cell surface. This density is reduced in subconfluent cell cultures, resulting in a decreased tyrosine phosphorylation and activation of c-Met. Compartmentalization of c-Met on the ventral surface of adherent B16-L59 cells locally increases the density of receptor molecules, leading to a more efficient dimerization and cross-phosphorylation. This favorable distribution is lost upon cell detachment, which results both in an absolute decrease of c-Met on the cell surface and in a reduced density on the membrane. Moreover, a soluble tyrosine phosphatase activity becomes more active when B16-L59 cells are released from the substrate. The combination of these two events leads to a dramatic decrease of tyrosine phosphorylation in B16-L59 cells in suspension. This decrease can be partially prevented by orthovanadate (but not by phenylarsine oxide) and fully prevented by low temperatures (Fig. 4), which appear to shift the balance between tyrosine kinase and phosphatase activities much more in favor of the former.

Progression of different tumor types toward a more malignant phenotype has been linked in many cases to overexpression of c-Met (5, 25–28), which may thus become constitutively activated, and confer a higher responsiveness to HGF/SF, as we have shown in the case of B16-L59 cells (7). However, results presented in this paper indicate that even high levels of overexpression are not a necessary and sufficient condition for c-Met constitutive activation, and other factors, such as adhesion and density, may strongly influence the delicate balance between tyrosine kinase and phosphatase activities, which finally result in the activation state of c-Met. Adhesion is also a critical event in the process of cancer metastasis that may affect targeting, invasion, and growth of malignant cells. It is therefore tempting to speculate that the activation state of c-Met may depend on the specific ability of different organ environments to support adhesion (leading to c-Met compartmentalization and a decrease in tyrosine phosphatase activity) and further growth (enhancing c-Met density on the cell surface) of metastatic cells. As a consequence, more c-Met may become constitutively activated at those sites where malignant cells adhere and grow efficiently, starting a positive feedback, since c-Met itself may contribute to the malignant phenotype.

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