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Induction and Regulation of Epithelial–Mesenchymal transitions

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ABSTRACT. Herein we discuss the factors that bring about the transformation of epithelial cells into cells of fibroblastic phenotype. This type of transformation, referred to as epithelium-to-mesenchyme transition (EMT), allows cells to dissociate from the epithelial tissue from which they originate and to migrate freely. EMT is therefore thought to play a fundamental role during the early steps of invasion and metastasis of carcinoma cells. Among biological agents which have been identified as inducers of EMT are a number of cytokines and extracellular matrix macromolecules. The coordinated changes in cell morphology, associated with the induction of cell motility and the disruption of intercellular junctions, are the consequence of a signaling cascade emanating from the plasma membrane and leading to changes in gene expression. Understanding the mechanisms regulating EMT of normal and transformed epithelial cells may offer new perspectives for designing therapies for the treatment of metastatic cancers of epithelial origin. BIOCHEM PHARMACOL **60**;8:1091–1099, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cell scattering; signaling pathway; cell adhesion; cell motility

Normal epithelia possess cell-to-cell contacts that account for both the proper development of these tissues during embryonic life and for the maintenance of homeostasis and architecture of epithelial structures during adult life. It has been proposed that epithelial tumor cells lose this restriction during the last steps of tumorigenesis, concomitantly with the loss of epithelial characteristics and the acquisition of motile behavior. Such phenotypic conversions are reminiscent of the so-called EMT† arising during the embryonic life at precise times and locations. EMT is an integral component of several morphogenetic and organogenetic processes. Gastrulation movements in many species, the emigration of neural crest cells from the neural tube, and the formation of cardiac valves proceed by EMT events [1–3]. Numerous cellular changes are associated with EMT. Fibroblasts that originate from epithelial cells through EMT events turn off genes coding for cell adhesion molecules and modify the type of intermediate filaments they express. In some cases, they start synthesizing extracellular matrix molecules such as fibronectin and certain types of collagen. They may also synthesize proteolytic enzymes involved in matrix degradation that contribute to cell motility and invasiveness. It should be stressed that not all EMTs exhibit the whole range of changes listed here. However, EMT is always associated with cell scattering, defined by the loss of intercellular junctions and acquisition of cell motility. Given the physiological and pathological importance of EMT, a considerable effort has been made during the past few years to characterize the mechanisms favoring cell dispersion. Several experimental models of epithelial cell scattering have been developed to investigate the mechanisms whereby epithelial cells can be transformed into fibroblast-like cells. We have previously characterized a rat bladder carcinoma cell line, NBT-II that is induced to scatter after stimulation by various growth factors (FGF-1, EGF, TGF-α) [4, 5‡], all of which bind to tyrosine kinase receptors. Under these conditions, cell dissociation can be monitored by the loss of desmosomes from the cell periphery, which correlates with the acquisition of active cell migration and scattering [6].

It should be emphasized that cell scattering consists of at least two biological events, which appear to occur simultaneously or synchronously in the cells:

(i) cell–cell dissociation, resulting from the breaking apart of intercellular complexes.
(ii) cell movement, driven by rearrangement of the cytoskeleton and formation of new cell–substratum contacts.

The two responses occur independently, as inferred from our own results, demonstrating that cell motility can be

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† *Abbreviations:* EMT, epithelial-mesenchymal transition; EGF, epidermal growth factor; FGF, fibroblast growth factor; TGF, transforming growth factor; HGF/SF, hepatocyte growth factor/scatter factor; RTK, tyrosine kinase receptor; NBT-II, Nara Bladder Tumor-II; SH, Src homology; and MAPK, Mitogen-activated protein kinase.

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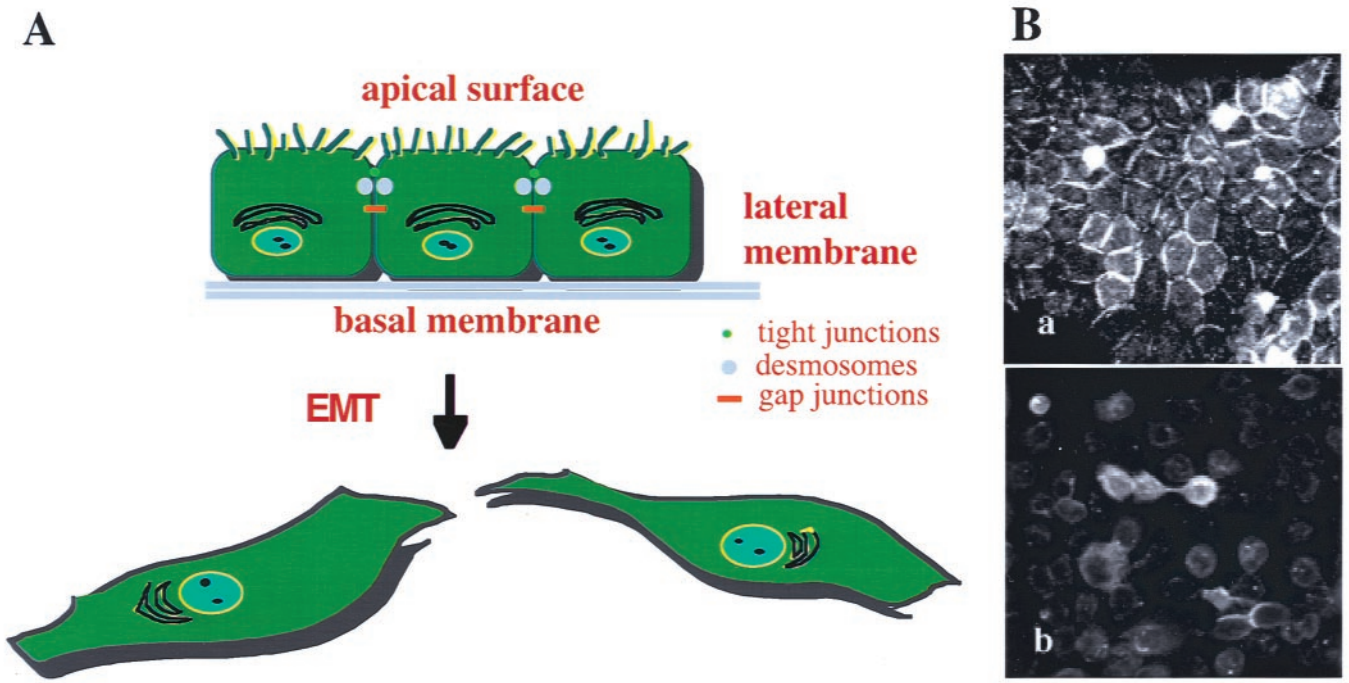

FIG. 1. EMT induces major changes in cell morphology and cell–cell contacts. (A) A schematic drawing of EMT highlighting the loss of intercellular connections and the changes in cell shape arising during EMT. (B) Immunofluorescence labeling of NBT-II cells before (a) and after (b) EGF induction showing the loss of desmosomes, as visualized with an antibody against desmoplakin.

blocked without perturbing cell–cell dissociation [6], and from those of Tsukamoto *et al.* [7], showing that cell–cell dissociation can be inhibited without affecting single cell migration (Fig. 1).

INDUCING FACTORS

In the majority of working models, cell scattering appears as a non-cell-autonomous process, requiring external stimuli to be activated. It should be stressed, however, that the most aggressive carcinoma exhibit a poorly differentiated phenotype, associated with an elongated morphology and loss of cell–cell interactions. It is tempting to postulate that, in these tumors, the process of EMT has been initiated by the constitutive activation of elements of the cellular pathway leading to EMT. When EMT is not triggered by oncogenic activation, the primary mode of induction of EMT is provided by specific growth factors or extracellular matrix components binding to their cognate cellular receptors. Polypeptide growth factors are multifunctional and are involved in a variety of responses, ranging from cell growth and survival to differentiation and migration processes. With the exception of TGF-β, which was demonstrated to be involved in EMTs during developmental morphogenetic events [8] as well as migration of normal [9] and cancer cells [10], the majority of growth factors exert their scattering effect through receptors with ligand-inducible intrinsic kinase activity (RTK). EGF and TGF-α, which interact with the same receptor, namely the EGFR, promote identical responses in most cell types, and activation of the EGFR can lead to both proliferation and epithelial cell scattering [4, 11–16]. We also demonstrated a similar role for FGF-1 on the rat bladder carcinoma cell line NBT-II. Accordingly, invertebrate homologs of FGF/FGFR have been shown to participate in developmental processes involving EMT-like changes [17, 18]. Interestingly, we found that FGF-2, which is devoid of scattering activity, acts as a scatter factor if NBT-II cells are transfected with the corresponding receptor [19]. This result suggests that any growth factor binding to a tyrosine kinase receptor is a potential scatter factor, provided that its cognate receptor is expressed and that the responding cell is equipped with the machinery needed for cell scattering. This idea is reinforced by the finding that a large number of growth factors that bind to RTK are endowed with scattering activity. Glial cell line-derived neurotrophic factor binding to Ret receptors [20] and neuregulin binding to ErbB-2 and ErbB-3 [21] can elicit EMT responses in certain cell types. The prototype of scattering factors is the HGF/SF binding to its receptor c-Met, a member of the RTK family. An exquisite feature of HGF/SF, as compared to other growth factors, resides in its ability to induce a more complex set of morphogenetic changes, including the formation of branching tubules in tridimensional collagen gels [22].

When considering the multiplicity of responses growth factors can elicit, one wonders about the molecular basis on which cells choose the type of biological response to a given signal. We have brought a preliminary answer to this question by showing that the rat carcinoma cell line NBT-II cannot simultaneously enter the cell cycle and

undergo EMT after FGF-1 stimulation [23]. This property is shared by other cell types stimulated by other growth factors [14]. The choice between the two types of responses depends on the cell status and on the type of signaling pathway that follows the ligand-induced receptor stimulation.

THE SIGNALING PATHWAYS LEADING TO EMT

Activation of RTK by their cognate ligands generates cascades of cytoplasmic events initiated by the autophosphorylation of specific tyrosines on the activated receptors. With respect to growth factor-induced epithelial cell scattering, a major question concerns the specific transduction pathway involved in EMT, as opposed to other functions attributed to tyrosine kinase receptors (growth, survival, differentiation, etc.). In several cases, it has been clearly demonstrated that the biological function depends on particular sites on the activated receptor. For instance, only one of five C-terminal tyrosine residues of neu/erbB-2 is responsible for transmitting the mitogenic signal [24]. Ligand-dependent activation of RTK with morphogenetic activity has also been studied; for example, the C-terminal domain of let-23, an EGF receptor homolog of *Caenorhabditis elegans*, can be subdivided into elements with different cell type-specific functions [25]. However, defining the specific function of domains in the cytoplasmic portion of growth factor receptors is not always possible. The met receptor, for instance, comprises a short C-terminal sequence that serves as a docking site for several cytoplasmic effectors. Deciphering which phosphorylated tyrosine of the docking site is selectively implicated in cell scattering or tubulogenesis has led to conflicting results [26–29]. Another approach commonly used to identify signaling molecules involved in various cell functions consists of overexpressing wild-type or mutant (transdominant-negative or constitutively activated) forms of signaling molecules and analyzing the biological consequences of the overexpression. Using this strategy, we demonstrated that Src tyrosine kinase is a positive regulator of growth factor-induced cell scattering [30].

Src Involvement in EMT

Src belongs to a family of cytoplasmic tyrosine kinases. These enzymes have a pivotal role in the regulation of a variety of biological functions which are associated with changes in cell morphology, including malignant transformation [31], epithelial plasticity [32], and modulation of intercellular adhesion [33]. In addition, the Src family is required during mitogenesis induced by EGF, platelet-derived growth factor, and colony-stimulating factor-1 [34, 35]. The Src family is composed of nine members, all of which contain one SH2 and one SH3 domain. In this family, Src, Fyn, and Yes are ubiquitously expressed while the other members have a more restricted pattern of expression. The specificity of action of each individual member is not clear, as knockout experiments have pointed to the possible redundancy of function among the Src family during mouse embryogenesis [36]. We demonstrated that Src and Yes may also have redundant functions during cell scattering, probably by phosphorylating common substrates important for signaling [13]. Since the SH2 and SH3 domains, together with the kinase domain, are required for cell scattering, these data suggest that the SH2 and SH3 domains of Src and Yes, which are highly homologous, bind the same target proteins.

The mechanisms whereby Src kinases exert their functions are still unclear. Three hypotheses have emerged. First, Src may phosphorylate specific substrates which are mainly cytoskeletal-based components, and molecules localized in cell–cell and cell–substrate adhesion sites [37–40], the tyrosine phosphorylation of which could in turn alter the cellular architecture. Src may also participate in the entry into S-phase by inducing Myc via a specific transduction pathway [41]. Finally, Src activity could interact with other signaling pathways; for example, activated Src can bind Shc, an early element of the Ras cascade, leading to the activation of this pathway [42, 43]. The three possible modes of action of Src are not mutually exclusive and might depend on the cellular context. While Src affects epithelial cell scattering by phosphorylating cytoskeleton-linked proteins, it does not act upstream of the Ras/MAPK pathway and does not induce transcriptional activity [13] (Fig. 2). However, recent data obtained in *Drosophila* suggested that Src may act upstream of Jun kinase and Djun in epidermal closure, a process that implies epithelial remodeling [44]. The apparent discrepancy between this report and our results may be due to the fact that epithelial cell shape changes during epidermal closure are not comparable to EMT processes. Alternatively, since Jun kinase could only partially rescue the phenotype caused by Src loss-of-function mutations, this may indicate that activation of Jun kinase is an accessory pathway that could not be identified in our experimental system.

The Ras Pathway

Beside Src kinases, we demonstrated a role for the Ras signaling pathway in EGF-induced epithelial cell scattering, corroborating the implication of the Ras pathway in SF-induced signaling [45–47]. Accordingly, the SH2-SH3 domain-containing adaptor protein Grb2 that connects activated RTK to the Ras pathway participates in HGF-induced cell scattering [48]. The small GTP-binding protein Ras has multiple effector molecules, each of which defines a pathway with specific functions. Among the different Ras effectors, the best characterized is the serine/threonine kinase Raf that initiates a series of phosphorylations resulting in the activation of MAPK. The regulation by MAP kinases has been implicated in a wide variety of biological processes, including cell proliferation, integrin-mediated cell adhesion, secretion, neuronal cell differenti-

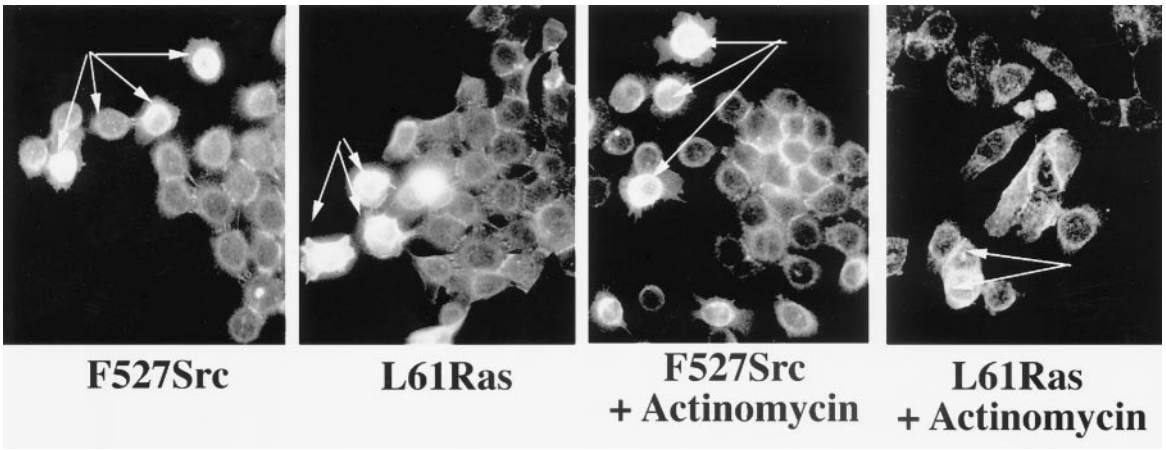

FIG. 2. Activated Ras but not Src needs transcriptional activity to induce EMT. NBT-II cells were cultured in the absence or presence of actinomycin in order to block gene transcription. Cells were microinjected with constructs coding for activated mutants of Src (F527Src) or Ras (L61 Ras), and cell scattering was monitored by the loss of desmosomal staining from cell–cell contacts. Injected cells are indicated by arrows. Note that activated Ras and Src induce cell dispersion. Actinomycin inhibits L61Ras- but not F527Src-induced cell scattering.

ation, oocyte maturation, and activation of B-and T-cells. A role of the MAPK pathway has been demonstrated in HGF/SF-induced scattering [49–51]. In EGF-stimulated NBT-II cells, a robust activation of MAPK was observed, suggesting a role for this enzyme during cell scattering. Accordingly, the Jun/Fos complex, known to be activated by the Ras/MAPK pathway, was able to rescue the blockade of EGF-induced cell scattering induced by a transdominant-negative mutant of Ras [13]. MAPK may also act in cell scattering by activating transcription factors other than the Jun/Fos complex or by phosphorylating cytoplasmic substrates that remain to be identified. P38, another member of the MAPK family, has also been implicated in cell motility stimulated by platelet-derived growth factor and it is activated via a Ras-dependent pathway [52]. Ras is also likely to activate other signaling molecules involved in EMT. The phosphatidylinositol 3-kinase (PI3K) may well serve such a role, as its function in epithelial cell scattering has been demonstrated in several examples of substrate- or growth factor-induced cell migration [51, 53, 54]. However, as other studies failed to confirm the requirement of PI3K in cell motility, its function may depend on the experimental system [55]. Nevertheless, PI3K is likely to be involved in cell motility by activating the small GTase Rac [56–58], whose role in cell migration and invasiveness is well established [59–62]. Rac may in turn stimulate the p21(cdc42/Rac)-activated kinase Pak1, which could induce the phosphorylation of myosin light chain, thus linking the Rac-induced pathway to proteins directly affecting cell movement [63, 64]. In contrast to its positive role in cell motility, Rac has also been shown unambiguously to strengthen cell–cell adhesion, thereby preventing tumor cell invasiveness [65, 66]. Moreover, activities of both Rac and Rho are required for the formation of adherens junctions [66–68]. The apparent discrepancy between the two antagonistic roles attributed to Rac may be solved when considering that the type of response following Rac activation depends on the cell substrate. On substrates permissive for locomotion, under conditions preventing the formation of adherens junctions, expression of active Rac promotes motile behavior, whereas on substrates impeding cell motility, Rac-dependent cell–cell adhesion is favored [57]. Like Rac, Rho, another small GTPase of the Rho family, has complex functions in cell scattering, probably related to its attributed role in the assembly of focal contacts and actin stress fibers in fibroblast cells [69]. Rho plays a positive role in colony-stimulating factor-1-induced macrophage translocation [60] and in the migration and metastatic properties of human hepatocellular carcinomas [70]. The mechanism whereby Rho regulates cytokinesis and cell motility is partly accounted by its ability to stimulate the phosphorylation of the myosin light chain [71] and or adducin, an actin-binding protein [72]. In contrast to its positive role in cell motility, Rho activity may antagonize epithelial cell scattering signals [73], by reinforcing cell–cell adhesion sites.

THE GENETIC CONTROL OF EMT

Major signaling pathways end up in the nucleus where they act by regulating the transcriptional activity of specific genes. Because EMT events require the coordinate expression of several sets of genes, it is of pivotal importance to identify the molecular targets of signaling pathways which mediate the transcriptional regulation of these genes. Among them, several transcription factors can be qualified as master genes, capable of controlling the whole EMT process. Such a role may be attributed to the Fos transcriptional activator [74, 75], which induces an EMT process by activating a Wnt-like pathway initiated by β-catenin nuclearization [76]. In this respect, it is noteworthy that the Jun/Fos transcriptional complex lies downstream of the Ras pathway in NBT-II cells [13]. Jun could exert its effect in EMT by mediating the translocation of Rac to the plasma

membrane, which is a prerequisite for its activation and the subsequent cytoskeletal rearrangements required for cell motility [77].

Other transcription factors are also able to trigger EMTs. The Snail family has been implicated in EMTs during embryonic life and may also participate in tumor progression. Stable expression of Snail in Madin–Darby canine kidney cells induces a dramatic conversion from an epithelial to a mesenchymal phenotype, concomitantly with the loss of E-cadherin, and promotes the acquisition of invasive and migratory behavior [78, 79]. Another Snail family member, Slug, does not appear to participate in E-cadherin down-regulation, but may contribute to the maintenance of the mesenchymal phenotype [80]. Accordingly, we have found that Slug overexpression in NBT-II cells can overcome the blockade of EGF-induced EMT caused by the expression of a transdominant negative mutant of Ras [13]. However, Slug is unlikely to be the unique player in EMT of NBT-II cells, since its overexpression is not sufficient to promote EMT in the absence of EGF. Moreover, Slug overexpression in NBT-II cells triggers desmosome dissociation, but is unable to promote the expression of mesenchymal markers or the acquisition of cell motility [81].

In addition to the above-mentioned transcription factors, members of the Ets family are also candidates for playing a role in EMTs. The expression of the Ets family of transcription factors has been observed in situations involving extensive cell migration and remodeling [82, 83]. The Ets family members may participate in EMT by controlling urokinase-type plasminogen activator (uPA) activity, which facilitates cell migration by degrading extracellular matrix components [84]. Ets may also regulate the transcription of other genes that remain to be identified.

THE STRUCTURAL TARGETS OF EMT SIGNALS

As already stated, two distinct processes take place during epithelial cell scattering. Growth factor-induced cell–cell dissociation precedes acquisition of cell motility, and these two processes are likely to be regulated independently. Dissociation of cell–cell contacts may occur through distinct mechanisms. Down-regulation of E-cadherin, the transmembrane adhesion molecule that participates in the establishment of adherens junctions, is well documented and may account for a number of EMTs [85–87]. A clear example of E-cadherin down-regulation is provided by the emergence of mesodermal E-cadherin negative cells originating from E-cadherin-positive epithelial progenitors during mouse gastrulation [88]. In this case, E-cadherin down-regulation may directly result in mesoderm migration [89]. E-cadherin down-regulation may also account for certain types of cancers [85–87]. In the majority of cases, the absence of E-cadherin can be correlated to either silencing mutations in the E-cadherin gene or down-regulation of its expression, occurring at the transcriptional level [90]. However, in a number of experimental EMTs and in a significant percentage of invasive and metastatic tumors, E-cadherin is still expressed at the cell periphery. In these cases, the genes coding for E-cadherin and for its associated cytoplasmic partners, the catenins, are not mutated, and repression of the transcription of the genes cannot be evidenced. As an example, the NBT-II carcinoma cell line undergoes a growth factor-induced EMT without any modulation in E-cadherin and catenins expression [91]. Similarly, v-src transformation of Madin–Darby canine kidney cells [92] and RSV transformation of chick lens cells [33] lead to disruption of cell–cell contacts without inhibiting E-cadherin expression. In the latter cases, tyrosine phosphorylation of catenins was proposed to be responsible for the loss of E-cadherin-dependent cell adhesion, but the mechanism whereby catenin phosphorylation triggers cell dissociation remains to be clarified. Likewise, Shibamoto *et al.* reported on the tyrosine phosphorylation of catenins following EGF or HGF treatment of carcinoma cells [93]. Cell dissociation occurring in EMT processes in which E-cadherin-dependent adhesion is not a direct target of EMT signals may result from the perturbation of intercellular junctions other than adherens junctions. In this respect, we have observed that EGF-driven signals result in the dissociation of desmosomes, resulting from the internalization of desmosomal components, concomitantly with their down-regulation [6]. Accordingly, the transcription factor Slug does not induce E-cadherin down-regulation [79], whereas it participates in desmosome breakdown in NBT-II cells [81].

As observed by Cano *et al.* [79], disruption of cell junctions may not be sufficient to promote the entire set of morphological changes, including acquisition of cell motility, that accompany EMT processes. Therefore, even though intercellular junctions appear as primary targets in epithelial cell scattering, other cellular events, independent of cell dissociation, may contribute to the initiation and completion of the process. For instance, cytoskeletal remodeling and simultaneous acquisition of cell motility are not likely to directly depend on cell dissociation. They are rather governed by cell–substrate adhesion sites, in which integrins represent the transmembrane receptors for extracellular components [94]. In this respect, we have found that EGF-induced cell scattering of NBT-II cells involves cell motility, which relies primarily on the engagement of $\alpha 2\beta 1$ integrins by a substrate permissive for cell locomotion [95]. Moreover, activation of $\alpha 2\beta 1$ integrins by collagen can be sufficient to drive a growth factor-independent EMT process, during which cell dissociation appears as a consequence of the mechanical forces exerted on cell–cell adhesion sites by cells in motion [96]. The mechanism by which the EMT signal is converted into cell motility probably involves the actin/myosin motility machinery, which can be a direct target of the growth factor-induced signaling pathway. The Rho family of small G proteins may be the link between the growth factor signal and the cytoskeletal reorganization which is necessary for cell motility. An alternative yet not exclusive hypothesis is that

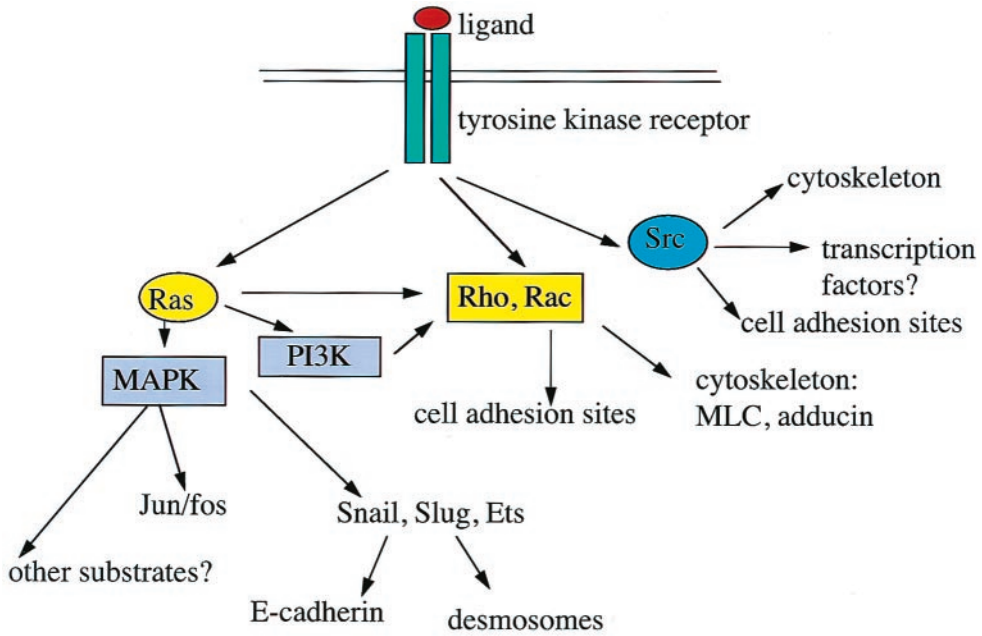

FIG. 3. Schematic representation of the signaling pathways induced during EMT. Ligand-induced dimerization of RTK promotes the activation of several signaling pathways, leading to changes in cell adhesion, cytoskeletal remodeling, and induction of specific genes. Structural molecules and transcription factors, thought to belong to these pathways, are indicated. MLC: myosin light chain.

cell motility is triggered by integrins activated by the EMT signal. Integrin activation by extracellular signals is known to initiate a series of intracellular events that result in various biological responses, including cell motility [97–99] (Fig. 3). Several effector molecules of the integrin-mediated signal have been demonstrated to affect the actin cytoskeleton [100, 101]. For example, in NBT-II cells, the docking protein paxillin, which is associated with the actin cytoskeleton, plays a central role in $\alpha 2\beta 1$ integrin-mediated cell motility [102].

Although an interplay between the signals affecting cell–cell adhesion and those affecting cell–substrate adhesion and cell motility should exist, it remains to be identified.

CONCLUDING REMARKS

The multiplicity of distinct pathways and molecules, each of which can lead independently to EMT-like events is astonishing. It is therefore tempting to speculate on several important properties of signaling pathways in the context of EMT. First, in normal cells, activation of one signaling molecule produces a signal that is not sufficient to trigger the whole array of modifications observed during EMT. It is only by the summation of individual signals elicited by distinct molecules that the biological response can take place. The EMT response is therefore dependent on the additional activities of distinct and specific signaling molecules. This mechanism could account for the fact that EMTs are highly controlled and spatio-temporally regulated processes that do not occur under usual circumstances. Cancer cells, on the other hand, may generate a cell-autonomous EMT-like process via oncogenic activation of signaling molecules. The oncogenic activation provides a signaling intensity similar to the summation of the individual signals existing in normal cells and may therefore lead to EMT without additional stimulations. The situation of cancer cells may be reproduced in experimental systems in which active forms of signaling molecules are overexpressed above the threshold level needed to achieve EMT.

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