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The migratory behavior of avian embryonic cells does not require phosphorylation of the fibronectin-receptor complex

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When locomotory embryonic cells become stationary, they acquire new substratum-adhesion properties. In particular, the distribution of fibronectin receptors shifts from diffuse and highly mobile on the cell membrane to immobilized in close association with fibronectin molecules and cytoskeletal elements in focal contacts. Receptor phosphorylation has been proposed as a possible regulator of the interaction between the receptor and its intracellular and extracellular ligands. In the present study, we have compared the phosphorylation state of the fibronectin receptor in motile neural crest and somitic cells, in stationary somitic cells, and in Rous-sarcoma virus transformed-chick embryo fibroblasts, using immunoprecipitation following metabolic labeling. While no receptor phosphorylation was detected in motile embryonic cells, the beta subunit of the receptor was phosphorylated in stationary cells. This subunit was also highly phosphorylated in Rous-sarcoma virus-transformed chicken cells. These results suggest that phosphorylation of the fibronectin receptor cannot account for its distribution in the cell membrane and for the nature of the interactions between this receptor and its ligands in embryonic cells.

Neural crest; Cell motility; Fibronectin receptor; Phosphorylation; (Avian embryo)

1. INTRODUCTION

A number of migrating embryonic cell populations, including primordial germ cells, gastrulating cells, neural crest cells and lymphocyte precursors, utilize the extracellular matrix glycoprotein fibronectin as a substrate for their locomotion (review [1]). In vivo and in vitro experiments have shown that direct binding between fibronectin molecules and their corresponding 140 kDa receptor complex is required to provide cell movement [2–6]. However, nonmotile cells also depend on fibronectins for their anchorage to the substratum (review [7]). This apparent dual function of fibronectins (i.e. to promote cell anchorage and cell locomotion) results in part from differences in the modes of interaction between locomoting and stationary cells and fibronectin molecules. Stationary cells adhere to the substratum at restricted sites of the membrane, termed focal contacts and close contacts, and have large areas of the cell surface that do not come into contact with the substratum [5,8–10]. In contrast, locomotory cells interact more uniformly with the substratum at broad close contacts and develop only a limited number of focal contacts [5,9]. This difference is reflected in the cellular distribution of components that participate in cell-substratum adhesion.

In stationary cells, actin microfilaments are organized into prominent stress fibers that terminate at focal contact sites where α-actinin, vinculin and talin are concentrated (review [11,12]).

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In motile cells, actin microfilaments are not extensively bundled, and α-actinin, vinculin and talin distribute mostly as a diffuse pattern in the cytoplasm ([5,13,14]; and Duband, J.-L., Geiger, B., Burridge, K. and Thiery, J.P., unpublished). In addition, in stationary cells, the fibronectin receptor is concentrated in focal contacts where it is virtually immobile whereas, in motile cells, it is distributed as a nearly homogeneous pattern on the cell surface where it is highly mobile [5,15]. When locomotory cells become stationary, the fibronectin receptor becomes progressively immobilized and its distribution shifts from diffuse to concentrated into focal contacts [15].

These observations suggest that, in stationary cells, the fibronectin receptor is in close association with both extracellular and cytoskeletal structures, possibly providing firm anchorage to the substratum. This close interaction between fibronectin receptor, fibronectins and talin has also been described with the isolated proteins [16]. In motile cells, in contrast, receptor interaction with fibronectins and cytoskeleton is weak or only transient, allowing labile adhesion to the substratum. However, the possible regulatory mechanisms of the interaction between the fibronectin receptor and its intracellular and extracellular ligands are presently not known.

Transformed cells exhibit cell-substratum adhesion properties resembling those of motile embryonic cells. In particular, these cells lack typical focal contacts [17], and display a diffuse distribution of the fibronectin receptor [18,19] and a poorly organized actin-microfilament meshwork [20]. In addition, it has been shown that vinculin, talin and the fibronectin receptor are all phosphorylated in cells transformed by oncogenes encoding tyrosine kinases but not or only slightly in normal cells [19,21,22]. Phosphorylation of the receptor may result in a marked decrease of binding to talin and fibronectin molecules in vitro (Rohrschneider, L., Horwitz, A. and Burridge, K., personal communication), thus suggesting that it contributes to the regulation of the interaction of the receptor with talin and fibronectin molecules. Therefore, in order to approach the regulatory mechanisms of receptor interactions, we have compared the phosphorylation state of the receptor in motile and stationary cells using in vivo metabolic labeling followed by immunoprecipitation.

2. MATERIALS AND METHODS

2.1. *Embryos and immune reagents*

Japanese quail (*Coturnix coturnix* Japonica) embryos were used throughout the study. Eggs were incubated at 37 ± 1°C and staged according to the number of somite pairs. A rabbit polyclonal antibody directed against β-subunit of the avian fibronectin-receptor complex but immunoprecipitating the whole complex has been described elsewhere [23].

2.2. *Cell cultures and radioactive labeling*

Neural crest and somitic cell cultures were generated as described previously [5]. Cells were cultured in the presence of Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum in 35-mm tissue culture dishes that were previously coated with 25 μg/ml human plasma fibronectin in Dulbecco's phosphate-buffered saline (PBS; Gibco). Normal chick embryo fibroblasts (CEF) and Rous-sarcoma virus-transformed chick embryo fibroblasts (RSV-CEF) were obtained from 9-day-old embryos and cultured as described [24]. For radioactive labeling, migrating neural crest and somitic cells cultured for 15–20 h, stationary somitic cells cultured for 3 days, and subconfluent CEF and RSV-CEF were rinsed three times in DMEM and preincubated for 30 min either in phosphate-free DMEM or in methionine-free DMEM. Cells were then incubated for 4 h at 37°C either in phosphate-free DMEM containing 500 μCi/ml [^{32}P]orthophosphate and 50 μM sodium vanadate [25] or in methionine-free DMEM containing 500 μCi/ml [^{35}S]-methionine. The viability of cells during labeling was regularly confirmed with an inverted microscope.

2.3. *Immunoprecipitation and SDS-PAGE*

After radioactive labeling, cells were washed four times in Hank's balanced salt solution (Gibco) and were extracted for 10 min on ice with 250 μl/dish of 2% Triton X-100 in PBS supplemented with 3 mM phenylmethylsulfonyl fluoride. All subsequent steps were performed at 4°C. Lysates were clarified by centrifugation at 11000 × g for 15 min. An aliquot of each extract was precipitated with 5% trichloroacetic acid in the presence of bovine serum albumin, and radioactivity quantitated in a liquid spectrometer. Extracts containing equal amounts of acid-precipitable radioactivity were preabsorbed with a 50% suspension of protein-A-Sepharose with constant mixing for 20 min. After centrifugation to remove the Sepharose beads, extracts were incubated with constant mixing for 1 h in the presence of 20 μg/ml antibodies to the receptor or of control antibodies at the same concentration. Immunocomplexes were incubated with constant mixing for 30 min with an excess of a 50% suspension of protein-A-Sepharose. The beads were subsequently washed six times with 1% Triton X-100 in PBS, and extracted by boiling for 3 min in 2% SDS in 10 mM sodium phosphate, pH 7.0. Samples were then electrophoresed under nonreducing conditions on 7.5% polyacrylamide gels.

3. RESULTS AND DISCUSSION

Immunoprecipitates from [^{35}S]methionine-

labeled neural crest cells and somitic fibroblasts were prepared using polyclonal rabbit antibodies to the avian fibronectin receptor and analyzed by SDS-PAGE under nonreducing conditions (fig.1a, lanes 1–4). Three bands were specifically precipitated, corresponding to proteins of apparent molecular masses 120, 140 and 160 kDa. These bands corresponded respectively to the three distinct glycoprotein subunits termed β (or band 3) and α (bands 2 and 1, respectively) comprising the avian fibronectin receptor described previously with several different polyclonal and monoclonal antibodies [26,27]. No differences in pattern of receptor subunits could be detected between migratory neural crest cells (fig.1a, lanes 1,2) and stationary somitic cells (fig.1a, lanes 3,4). However, it cannot be excluded that modest differences in the relative concentration of each subunit may exist between the two cell types.

When immunoprecipitation was performed on cells that had been labeled in vivo with [^{32}P]orthophosphate in the presence of sodium vanadate, the β-subunit of the receptor appeared highly phosphorylated in RSV-CEF (fig.1b, lanes 7′–9′), and to a lesser extent in normal CEF (not shown), and stationary somitic fibroblasts (fig.1b, lanes 4′–6′). In contrast, no phosphorylation was detected on any of the receptor subunits in both motile neural crest cells (fig.1b, lanes 1′–3′) and motile somitic fibroblasts (not shown). It should be noted that, in contrast to a previous report [19], we did not find any phosphorylation of band 2 of the receptor in RSV-CEF, and we did obtain phosphorylation of the β-subunit in normal CEF, even though it was clearly weaker than in RSV-CEF.

In locomotory cells, the fibronectin receptor has been shown to interact only transiently with fibronectin molecules and with the cytoskeleton [5,15]. This situation is in striking contrast with that found in stationary cells, in which this interaction appears much stronger [5,15]. The nature of possible regulators of this association is not known presently. RSV-CEF, however, exhibit cell-substratum adhesion properties that resemble those of motile embryonic cells [17–20]. In particular, the fibronectin receptor displays a diffuse distribution [18,19]. In those cells, phosphotyrosine-containing proteins are preferentially accumulated in regions of cells that come into contact with the substratum, termed rosettes or podosomes [28,29]. Interestingly, elements that participate in anchorage of microfilaments to the cell membrane, i.e. vinculin, talin and the fibronectin receptor, are phosphorylated on tyrosine in transformed cells [19,21,22]. Rosettes are also the sites where pp60src, a protein with high tyrosine-kinase activity, is concentrated [30]. In addition, fibronectin-degrading activities have been localized coincident to the pp60src protein [31]. It has been proposed that the pp60src kinase exerts its effects through phosphorylation of various proteins, such as the fibronectin receptor, vinculin and talin. Such modifications may cause the disruption of receptor association with fibronectins and cytoskeleton (Rohrschneider, L., Horwitz, A. and Burridge, K., personal communication), and induce protease activity that locally degrades fibronectins [31].

Our present study shows that fibronectin-receptor phosphorylation does not occur in motile cells but rather is present when cells are immobile. This observation therefore questions the role of receptor phosphorylation in cells and of its influence on cell behavior, particularly with respect to transformed cells.

If phosphorylation does interfere with the association between the receptor and its corresponding ligands, our results would indicate that the processes of regulation of receptor binding differ considerably in embryonic motile cells and in transformed cells since, in both cases, most fibronectin receptors are freely mobile in the plasma membrane. Preliminary results in our laboratory suggest that fibronectin receptors are rapidly internalized in motile cells and not in stationary cells (Duband, J.-L., Yamada, K.M., Thiery, J.P. and Jacobson, K., unpublished). Receptor internalization rather than phosphorylation would be a possible alternative mechanism for disruption of complexes of fibronectin receptor and fibronectin or for removal of complexes from the surface of embryonic cells. This would result in the local detachment of the cell membrane from the substratum, a process necessary for cell locomotion.

Alternatively, if receptor phosphorylation is not involved in the regulation of fibronectin-receptor binding properties, our study would indicate that, even though RSV-CEF share common structural

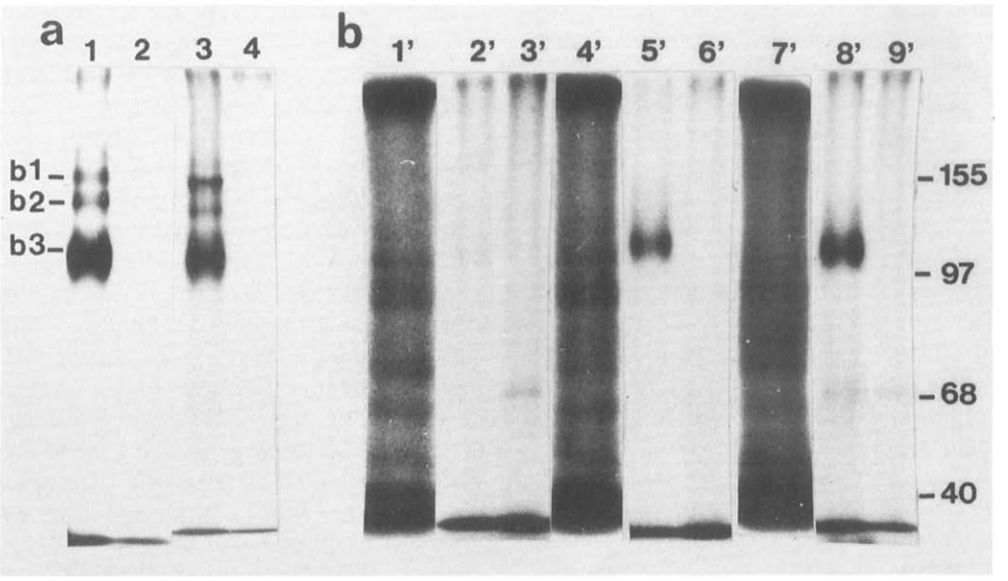

Fig.1. (a) Detection of the fibronectin-receptor complex in migratory neural crest cells (lanes 1,2) and in stationary somitic fibroblasts (lanes 3,4). (b) Phosphorylation of the receptor in motile neural crest cells (lanes 1′–3′), stationary somitic cells (lanes 4′–6′) and RSV-CEF (lanes 7′–9′). Cells were labeled in vivo for 4 h with [^{35}S]methionine or [^{32}P]orthophosphate, and Triton extracts containing equal amounts of acid-precipitable radioactivity were treated with polyclonal antibodies to the receptor (lanes 1,3,2′,5′,8′) or control antibodies (lanes 2,4,3′,6′,9′). Immunoprecipitates were electrophoresed on 7.5% polyacrylamide gels under nonreducing conditions. Lanes 1′,4′ and 7′ show total detergent extracts of neural crest cells, stationary somites and RSV-CEF, respectively, labeled in vivo with [^{32}P]orthophosphate. Numbers on the right represent the molecular mass × 10^{-3} of marker proteins. The three receptor subunits are indicated on the left.

characteristics with motile embryonic cells, they utilize adhesion mechanisms similar to those found in stationary embryonic cells; it is conceivable that rosettes present in RSV-CEF are the counterpart of focal adhesion sites in stationary embryonic fibroblasts. If this interpretation is correct, receptor phosphorylation occurring in focal contacts of normal cells and podosomes of transformed cells would be important in the stabilization of these adhesive plaques.

In summary, our data do not support the interesting hypothesis that phosphorylation of the fibronectin receptor is essential for the weakening of its interactions with talin and fibronectins in embryonic cell motility. Further studies will be necessary to define the functions of phosphorylation, particularly with respect to the formation of adhesion plaques; it may also be useful to determine the proportion of phosphorylated receptors among the total pool and their cellular localization in stationary and transformed cells.

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