Fibronectin Receptor Exhibits High Lateral Mobility in Embryonic Locomoting Cells but Is Immobile in Focal Contacts and Fibrillar Streaks in Stationary Cells

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Abstract. The dynamic process of embryonic cell motility was investigated by analyzing the lateral mobility of the fibronectin receptor in various locomotory or stationary avian embryonic cells, using the technique of fluorescence recovery after photobleaching. The lateral mobility of fibronectin receptors, labeled by a monoclonal antibody, was defined by the diffusion coefficient and mobile fraction of these receptors. Even though the lateral diffusion coefficient did not vary appreciably (2×10^{-10} cm²/s $\leqslant D \leqslant 4 \times 10^{-10}$ cm²/s) with the locomotory state and the cell type, the mobile fraction was highly dependent on the degree of cell motility. In locomoting cells, the population of fibronectin receptors, which was uniformly distributed on the cell surface, displayed a high mobile fraction of $66 \pm 19\%$ at 25°C ($82 \pm 14\%$ at 37°C). In contrast, in nonmotile cells, the population of receptors was concentrated in focal contacts and fibrillar streaks associated with microfilament bundles and, in these sites, the mobile fraction was small ($16 \pm 8\%$). When cells were in a stage intermediate between highly motile and stationary, the population of fibronectin receptors was distributed both in focal contacts with a small mobile fraction and in a diffuse pattern with a reduced mobile fraction ($33 \pm 9\%$) relative to the diffuse population in highly locomotory cells. The mobile fraction of the fibronectin receptor was found to be temperature dependent in locomoting but not in stationary cells. The mobile fraction could be modulated by affecting the interaction between the receptor and the substratum. The strength of this interaction could be increased by growing cells on a substratum coated with polyclonal antibodies to the receptor. This caused the mobile fraction to decrease. The interaction could be decreased by using a probe, monoclonal antibodies to the receptor known to perturb the adhesion of certain cell types which caused the mobile fraction to increase. From these results, we conclude that in locomoting embryonic cells, most fibronectin receptors can readily diffuse in the plane of the membrane. This degree of lateral mobility may be correlated to the labile adhesions to the substratum presumably required for high motility. In contrast, fibronectin receptors in stationary cells are immobilized in focal contacts and fibrillar streaks which are in close association with both extracellular and cytoskeletal structures; these stable complexes appear to provide firm anchorage to the substratum.

D URING early embryonic development, certain groups of cells can transiently express locomotory properties that allow them to migrate long distances from their sites of origin and populate other areas of the embryo (for reviews, see references 16, 41, 56, 57). There is a large body of evidence that suggests that cell motility results from the conjunction of environmental influences and intrinsic properties of cells. For example, the extracellular matrix glycoprotein fibronectin is known to promote cell locomotion in vitro (3, 46, 47, 61) and in vivo; its presence has often been correlated with migration of embryonic cells (for reviews, see references 16, 57). In addition, both in vivo and in vitro, direct interaction of fibronectin molecules with the embryonic cell surface is required for cell movement (5, 6, 17, 49).

Intrinsic characteristics of motile cells can be appreciated by contrasting modes of anchorage to the extracellular matrix of motile and stationary cells. The latter interact with the substratum primarily at restricted sites of the membrane, called focal contacts and close contacts; much of the rest of

the ventral membrane is not in contact with the substratum (10, 13, 17, 32). In contrast locomotory cells interact more uniformly with the substratum at broad close contacts, and develop only a limited number of focal contact sites (13, 17, 39). In stationary cells, actin filaments are bundled with other proteins into contractile stress fibers whereas, in motile cells, actin filaments are not extensively bundled and are mainly distributed as a network in the cellular cortex (17, 26, 58). In addition, unlike stationary cells, locomotory cells lack the ability to retain fibronectin on their surfaces (14, 17, 24, 45, 52). Finally, motile cells are unable to induce distortions in silicon–rubber sheets, suggesting that they are not attached firmly to the substratum or that they do not generate sufficient forces (58). These data suggest that locomotory and stationary cells are specialized for labile adhesion and firm attachment, respectively.

Due to its key location at the interface of the intracellular and extracellular compartments, the fibronectin receptor is thought to play a critical role in both the control of cell–substratum interactions and in the organization of the cytoskeleton (for reviews, see references 7, 30, 48, 60). In avian species, the fibronectin receptor is comprised of three subunits of ∼120, 140, and 160 kD that are held together in noncovalently linked complexes of unknown stoichiometry (23, 38). Organization as an oligomer is apparently essential for its binding function (8). Immunofluorescent analyses have located the receptor complex near focal cell–substratum contact sites where actin bundles terminate and in fibrillar streaks that align with fibronectin fibrils externally and with microfilament bundles internally (11, 15, 17). Direct association of the fibronectin receptor with talin, one of the cytoskeletal proteins concentrated at focal contacts, has been demonstrated using purified forms of these proteins (28). Thus, the fibronectin receptor appears to provide a link between the extracellular matrix and the cytoskeleton. In motile cells, the fibronectin receptor complex has been observed as a nearly homogeneous pattern over the entire surface of cells, in striking contrast to stationary cells where it appears predominantly in focal contacts and fibrillar streaks (17). These distinct distribution patterns of the fibronectin receptor in motile and stationary cells are indicative of differences in the modes of interaction of the receptor and both fibronectin molecules and cytoskeletal elements.

An approach to study this phenomenon is to analyze the lateral mobility of the fibronectin receptor within the plane of the membrane using the fluorescence recovery after photobleaching (FRAP)[1] technique (for review, see reference 37). In the present report we show that a large fraction of the population of the fibronectin receptors in locomoting cells is mobile, whereas in stationary cells the receptors are concentrated in focal contacts and fibrillar streaks where they are immobile. In addition, the mobile fraction of the receptor can be modulated by both temperature and the degree of interaction between the receptor and its ligands.

Materials and Methods

Embryos

Japanese quail (*Coturnix Japonica*) and Peterson/Arbor Acres chick (*Gallus*

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1. *Abbreviation used in this paper:* FRAP, fluorescence recovery after photobleaching.

domesticus) embryos were used throughout the study. Eggs were incubated at 37° ± 1°C in a humidified air chamber and staged according to the duration of incubation and the number of somite pairs.

Cell Cultures

Neural crest and somitic cell cultures were generated as described previously (17, 47). Briefly, the caudal regions of embryos incubated for 60 h were excised with a scalpel, and the fragments were incubated for 30–60 min at room temperature with 2.5 U/ml Dispase (Boehringer Mannheim Diagnostics, Inc., Houston, TX) in DME. Somites and neural tubes were teased apart with tungsten needles and incubated for recovery from enzyme treatment for 30 min in DME supplemented with 1% FCS. Cardiac fibroblasts were cultured as follows, according to Couchman and Rees (13). Hearts from 8–10-d embryos were minced into pieces with a razor blade and washed in DME. Tissue pieces were then briefly incubated at 37°C in a trypsin–EDTA solution (Gibco Laboratories, Grand Island, NY) in order to obtain small cell clusters. After enzyme inactivation by serum in excess, clusters were harvested by centrifugation. Dermal fibroblasts were obtained by removal of the skin from the dorsum of 8–14-d chick embryos. The skin was incubated in trypsin–EDTA and repeatedly pipetted until a suspension of cells was achieved. Trypsin was inactivated with DME containing 10% FCS. The suspension was allowed to sit for 5 min for feathers to settle out, and the cells in suspension were washed with DME plus FCS before plating. Fibroblasts for this study were used at passages 3–5. Tissues were explanted onto glass coverslips coated for 1 h with 10–100 μg/ml human plasma fibronectin in PBS followed by a 1-h saturation with BSA in PBS (3 mg/ml). Dermal fibroblasts were deposited onto uncoated coverslips or coverslips treated for 1 h with 50 μg/ml human plasma fibronectin in PBS followed by a brief PBS wash. All cells were cultured in DME supplemented with 10% FCS at 37°C in a humidified 7% CO_2/93% air incubator.

Antibodies

Methods for the production and characterization of a rat monoclonal IgG, termed ES66-8, directed against the beta subunit of the avian fibronectin receptor have been reported elsewhere (43). JG22E mouse monoclonal IgG against the beta subunit of the avian fibronectin–receptor complex has been described and characterized previously (11, 22). These antibodies were conjugated with lissamine rhodamine B-sulfonyl chloride (Molecular Probes Inc., Eugene, OR) essentially as described previously (51). The dye-to-protein molar ratio was 3.9 for the ES66-8 antibody and 2.7 for the JG22E antibody. Conjugated antibodies were subsequently checked for specificity and for the presence of free dye by immunofluorescence labeling of mouse fibroblasts as described below. The production and purification of rabbit polyclonal antibodies to the beta subunit of the avian fibronectin receptor were described elsewhere (11).

Immunofluorescence Labeling of Living Cells

For immunofluorescent staining, neural tubes, somites, and cardiac cell clusters were cultured on fibronectin-coated coverslips essentially as described in Duband et al. (17). Neural crest, somite, and heart cultures were briefly rinsed in DME buffered with Hepes (50 mM) at pH 7.4 (DME-Hepes). They were then incubated for 10 min at room temperature in 70 μg/ml ES66-8 monoclonal antibody in DME-Hepes. After washes in DME-Hepes, cultures were fixed in ethanol at −20°C for 20 min and rinsed extensively in DME-Hepes. This was followed by a 10-min incubation with biotinylated sheep antibodies to rat IgG, then with fluorescein-conjugated streptavidin (Amersham International, Amersham, United Kingdom). After washes in DME-Hepes, cultures were mounted in glycerol. Dermal fibroblasts were incubated in 25 μg/ml ES66-8 in DME or 60 μg/ml JG22E in DME for 10 min at 37°C. Cells were then washed in PBS and fixed in 3.7% formaldehyde in PBS for 5 min. After extensive washing, a fluorescein-ated second antibody was applied for 30 min and cells were then washed and mounted in Mowiol (Calbiochem, La Jolla, CA). Cultures were observed with Leitz Orthoplan or Zeiss IM-35 epifluorescence microscopes.

Time-Lapse Video Microcinematography

To examine the locomotory behavior of cells in culture, time-lapse video cinematography was performed with a Leitz inverted phase-contrast microscope equipped with a video camera (×3,400; 0.5 Lux; Sanyo Electronic Co., Ltd., Osaka, Japan) connected to a television monitor (model WV

5400; National Matsushita Co., Osaka, Japan), a time generator, and a time-lapse recorder (model NV8030; National Matsushita Co.).

Cell-Substratum Spreading Assay

To test the possible effect of the ES66-8 monoclonal antibodies on embryonic cells, cells were cultured on fibronectin-coated glass coverslips in the presence of varying amounts of antibodies in DME as described previously (17). Cultures were then observed using a Leitz inverted phase-contrast microscope up to 24 h after adding the antibody.

Fluorescence Recovery After Photobleaching

Cells were cultured on 12-mm-square glass coverslips and labeled for 10 min with rhodamine-conjugated ES66-8 monoclonal antibody at 50–100 µg/ml in DME at room temperature for the spot FRAP measurements. For dermal fibroblasts, rhodamine-conjugated ES66-8 antibody was used at 25 µg/ml and rhodamine-conjugated JG22E antibody was used at 60 µg/ml. These were the lowest concentrations that produced clearly visible direct staining in our system. After two washes in DME and one wash in PBS, coverslips were mounted in PBS on a stainless steel chamber described elsewhere (36). FRAP measurements were routinely made at room temperature with a Leitz microscope that has been previously described in detail (34, 36), equipped with a ×40 phase contrast 1.3 NA oil immersion objective which produces a 2-µm-diam spot (e^{-2} width) focused on the cell surface. A 350-µm pinhole was positioned in an intermediate image plane which limited the collection depth of the photomultiplier to ±1.2 µm about the in-focus plane. The depth is defined as the distance from the in-focus plane at which the collection efficiency drops to 50% of maximum.

When only isotropic lateral diffusion occurs, the diffusion coefficient, D, is calculated from the half-time for recovery ($\tau_{1/2}$ = time required to obtain 50% of final recovery) by the equation $D = W_s^2 \gamma/4\tau_{1/2}$, where W_s is the e^{-2} radius of the Gaussian profile laser beam used for both photobleaching and measuring fluorescence, and γ is a parameter that depends on the extent of photobleaching (4). The mobile fraction of the measured population of probe molecules is obtained from the degree to which the final fluorescence level approaches the prebleach fluorescence value. For motile cells, the bleach power was in the range of 150–250 mW (measured as laser output, not microscope throughput) and the bleach time was between 350 and 1,000 ms. For stationary cells, the bleach power was between 25 and 50 mW and the bleach time between 100 and 250 ms. After the bleach, fluorescence recovery was measured during 0.5–1.0-s interrogation pulses of 0.001 times the intensity of the bleach beam which were spaced every 5 s for the first 30 s of recovery (or until the shape of the recovery phase was ascertained) and then every 15 s until the end of the measurement (when F_∞, the final fluorescence, could be approximated). The intensity of fluorescence was minimal on all controls which included measurements on the substrate between cells and staining with irrelevant rhodamine-conjugated antibodies. FRAP measurements at temperatures other than 25°C were conducted with a thermoelectric temperature stage (Sensortek, Sadlebrook, NJ). The actual temperature of the cells during a FRAP experiment was determined with a thermocouple microprobe (Sensortek) mounted on a stainless steel chamber as described previously (36).

Results

When cultured on fibronectin substrates, neural crest, somite, and heart cells displayed different morphologies and behavior depending on the duration of culture (13, 17, 47). During the first 2 d of culture, these cells were highly locomotory. Whereas neural crest cells showed a typical stellate morphology, somitic and cardiac cells were mainly bipolar with a wide leading edge and a narrow trailing edge. By 3 d in culture, nearly all cells became larger and polygonal with a concomitant loss of motile behavior. In the case of neural crest cells however, part of the cell population remained highly motile without any notable change in cell shape. These cells appeared to be melanocyte precursors (not shown, but see reference 42). In contrast to the other cell types, dermal fibroblasts cultured on glass after several passages showed no

noticeable locomotory activity as judged by constancy of location in time-lapse video recording.

Cytological and Biological Characterization of the ES66-8 Monoclonal Antibody

A preliminary series of experiments verified that the ES66-8 monoclonal antibody fulfilled several requirements in order for it to be useful in a study correlating receptor distribution and lateral mobility with cell locomotion. First, the distribution of the fibronectin receptor–beta subunit visualized by direct and indirect immunofluorescence using ES66-8 antibody was compared with the pattern described previously using a rabbit polyclonal antibody (17). As shown in Fig. 1 a, motile neural crest cells displayed uniform, diffuse, bright labeling over the entire cell surface. Stationary neural crest cells showed a strikingly different fibronectin receptor distribution. Fluorescence was almost exclusively concentrated in the areas of cell–substratum contact sites and on fibrillar streaks (Fig. 1 b). These patterns were also seen using direct immunofluorescence labeling with rhodamine-conjugated ES66-8 antibody (not shown). These results were very similar to those obtained with polyclonal antibodies (17), except that no visible patches were detected with the ES66-8 monoclonal antibody, suggesting that it did not induce extensive receptor clustering. We observed that, after 2 d in culture, most neural crest and somite cells showed a marked decrease in their locomotory activity and were stained both diffusely over their cell surface and in numerous focal contact sites (Fig. 1 c). This behavior was detected before cells became completely stationary and was interpreted as an intermediate state between highly motile and stationary states. Melanocyte precursors (Fig. 1 d) as well as motile somitic and cardiac fibroblasts displayed a staining pattern very similar to that of crest cells cultured for 24 h (not shown).

Second, in order to verify that the ES66-8 monoclonal antibody did not interfere with the morphology of cells, both motile and stationary cells were incubated for 24 h in the presence of the antibody at concentrations up to 250 µg/ml. ES66-8 did not detectably perturb the spread morphology of cells (not shown) as compared with other monoclonal antibodies, such as JG22E or CSAT, which caused cells to round up and detach from fibronectin substrates (6, 22, 44).

Lateral Diffusion of the Fibronectin Receptor in Various Cells

The lateral diffusion of the complex of ES66-8 and the beta subunit of the fibronectin receptor was measured using rhodamine-conjugated ES66-8 monoclonal antibody on locomotory and stationary cells. For locomoting cells, bleaches were performed preferentially over the perinuclear region but also on lobopodia. Cellular shape was recorded before and after the FRAP measurement. For stationary neural crest, somite, and heart cells, bleaches were performed either on regions covering focal contacts and fibrillar streaks or outside of these regions. On dermal fibroblasts, FRAP measurements were made exclusively on regions, each of which contained part of a focal contact or fibrillar streak. Whenever possible, the laser beam was focused on the ventral surface; this was easily achieved on less motile cells but more difficult on motile neural crest cells. Thus, on the motile cells the measurements should be considered to reflect
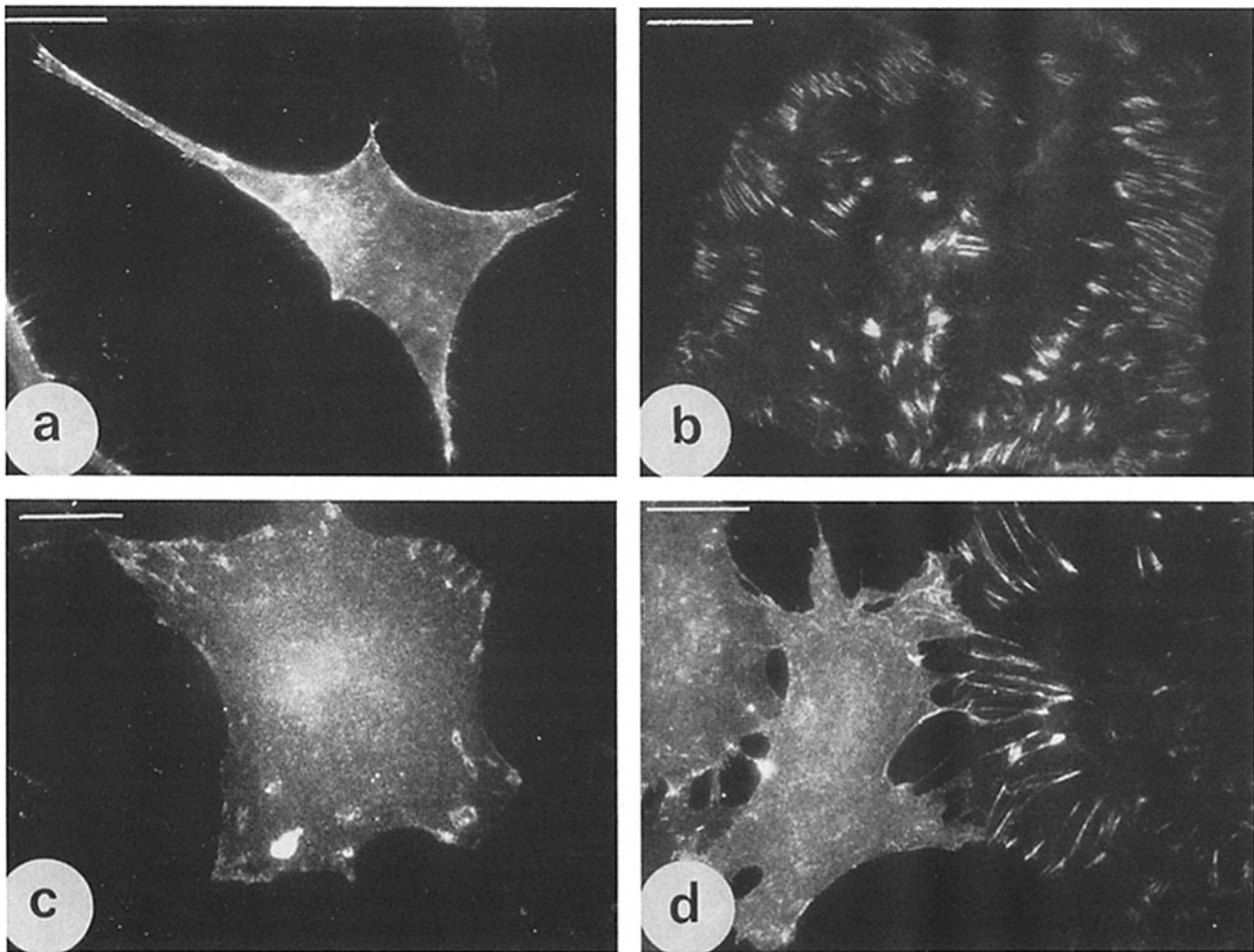
Figure 1. Immunofluorescent distribution of the fibronectin receptor on unfixed cultured neural crest cells as revealed with the ES66-8 monoclonal antibody. (*a*) Motile cells, (*b*) stationary cells, and (*c*) cells in a state intermediate between motile and stationary, and (*d*) melanocyte precursors. Motile cells show a uniform, diffuse labeling over their entire surfaces, whereas stationary cells display labeling almost exclusively in cell–substratum contact sites and along fibrillar streaks. Cells in the process of becoming immobilized (*c*) exhibit receptors both uniformly on their surface and in focal contact sites. Note that very few fibrillar streaks can be visualized on these cells. On melanocyte precursors obtained after several days in culture, the receptor shows a diffuse pattern very similar to that of motile neural crest cells. Bars: (*a*) 5 μm; (*b*) 10 μm; (*c*) 7 μm.

receptor mobility on both surfaces as opposed to stationary cells on which measurements were made primarily on the ventral surface.

Fig. 2 presents typical fluorescence recovery curves on motile and stationary cells. Three major types of recordings were obtained. On both moderately motile cells and stationary cells outside of the focal contact and fibrillar streak regions, the FRAP curves showed substantial recovery (Fig. 2 *a*). On highly motile cells the shape of which changed rapidly during measurement, the FRAP curves generally exhibited breaks corresponding to the sudden displacement of the cell and F_∞, the final fluorescence, was often slightly higher than F_i, the prebleach fluorescence (Fig. 2 *b*). Such curves were not analyzed for D and mobile fraction. On focal contacts and fibrillar streaks of stationary cells, the FRAP curves showed much less recovery (Fig. 2 *c*).

FRAP measurements on locomoting cells yielded high mean values for the mobile fraction ranging from 66 to 84%;

lateral diffusion coefficients ranged from 2×10^{-10} cm²/s to 3.3×10^{-10} cm²/s (Table I). Values for the mobile fraction in neural crest cells were distributed over a range from 30 to 100% recovery (Fig. 3 *a*). Most diffusion coefficient values were in the range of $1.0–3.0 \times 10^{-10}$ cm²/s (Fig. 3 *a'*). FRAP measurements were performed on different areas of motile cells, including the perinuclear region and the lobopodia to check for systematic regional differences. The value of the mobile fraction in this random analysis was apparently independent of the location of the bleached area on the cell surface.

When FRAP measurements were performed on focal contacts and fibrillar streaks on stationary cells using ES66-8 antibody, mobile fractions of the receptor were found to be quite low, mean values ranging from 13 to 18% depending on cell type (Table I); however, diffusion coefficients were not greatly different from those measured in motile cells (Table I). As shown in Fig. 3, *c* and *c'*, fibronectin receptors
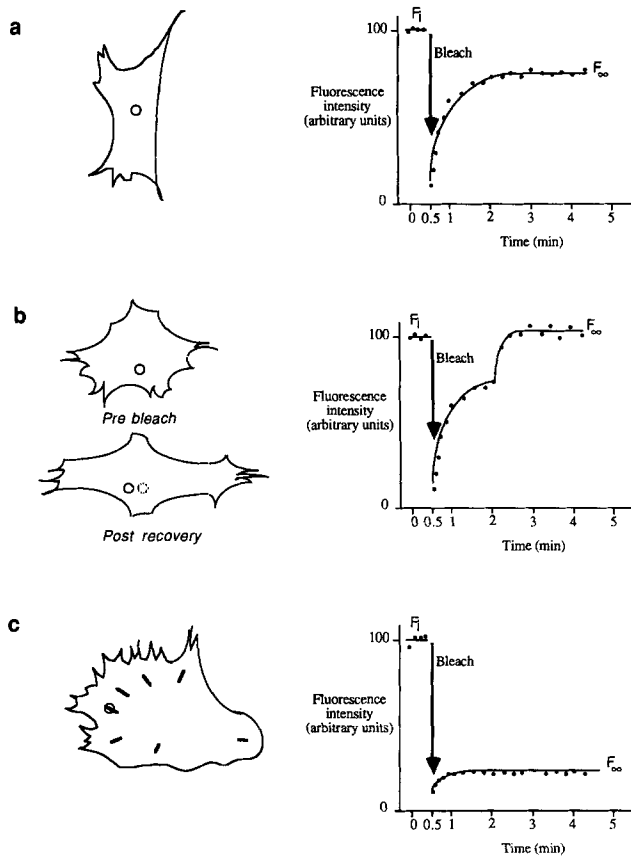
Figure 2. Fluorescence recovery after photobleaching curves for antibody-labeled fibronectin receptors on moderately motile (*a*), highly motile (*b*), and stationary cells. (*c*). Left panels show the morphology of the cells during FRAP measurement and the location of the bleached region is indicated by a circle. On moderately motile cells, curves display substantial recovery, where the intensity of fluorescence after recovery (F_∞) approaches the prebleach fluorescence level (F_i). In some cases for highly motile cells, sudden cell displacement may occur during FRAP measurement, resulting in changes in the location of the measuring beam on the cell. Breaks occur in the curves and recovery levels can be slightly higher than before bleach. On focal contacts in stationary cells, curves show only minimal recovery.

Table I. Lateral Diffusion of the Fibronectin Receptor β Subunit on Various Embryonic Cells as a Function of the Locomotory State and of the Bleached Area*

Locomotory state	Bleached area		Mobile fraction ($\pm$ SD)	Diffusion coefficient ($\pm$ SD)	*n*
	Diffuse staining	Focal contacts			
			%	$cm^2/s \times 10^{10}$	
Neural[‡] crest cell					
Locomotory	+		66 $\pm$ 19	2.0 ($\pm$ 1.0)	127
Intermediate	+		33 $\pm$ 9	2.3 ($\pm$ 1.2)	20
Intermediate		+	16 $\pm$ 7	2.1 ($\pm$ 1.0)	11
Stationary	+		57 $\pm$ 19	2.2 ($\pm$ 0.8)	17
Stationary		+	16 $\pm$ 8	2.0 ($\pm$ 1.0)	27
Somitic[‡] fibroblast					
Locomotory	+		66 $\pm$ 14	3.1 ($\pm$ 1.9)	19
Intermediate	+		30 $\pm$ 9	2.1 ($\pm$ 0.5)	10
Stationary	+		68 $\pm$ 24	3.4 ($\pm$ 2.8)	11
Stationary		+	18 $\pm$ 7	2.8 ($\pm$ 1.6)	25
Heart fibroblast					
Locomotory	+		73 $\pm$ 18	2.1 ($\pm$ 0.7)	23
Stationary		+	14 $\pm$ 8	2.0 ($\pm$ 0.5)	17
Dermal fibroblast					
Stationary		+	13 $\pm$ 9	3.4 ($\pm$ 1.3)	87
Melanoblast					
Locomotory	+		84 $\pm$ 15	3.3 ($\pm$ 1.4)	21

Results are expressed as mean value $\pm$ SD. FRAP measurements were performed at 25°C using rhodamine-conjugated ES66-8 monoclonal antibody.
* The bleached area corresponds either to regions where the receptor is diffusely distributed or to regions of focal contacts and fibrillar streaks.
‡ The intermediate state corresponds to a low cellular mobility associated with both a uniform distribution of the receptor and focal contacts.

located in fibrillar streaks and focal contacts exhibited a relatively narrow range of mobile fraction and diffusion coefficient values. In contrast, when measurements were performed on areas outside of fibrillar streaks and focal contacts, values for mobile fraction were more widely distributed, but the mean values (57 $\pm$ 19% for stationary neural crest cells and 68 $\pm$ 24% for stationary somitic fibroblasts) were similar to that obtained for locomoting cells (Table I). It should be stressed, however, that the level of fluorescence in these areas was much lower than in focal contacts (see Fig. 1 *b*) and that, as estimated by monitoring of the relative fluorescence intensity, the density of fibronectin receptors located out of fibrillar streaks and focal contacts was ∿10-fold lower than in focal contacts and fibrillar streaks. This difference is probably >10-fold in stationary dermal fibroblasts and so measurements outside focal contacts and fibrillar streaks on this cell type were not feasible.

The lateral mobility of the fibronectin receptor was also measured on neural crest and somite cells at a stage intermediate between highly motile and stationary. FRAP measurements were performed either on focal contacts or on areas devoid of focal contacts. As opposed to stationary cells, the relative intensity of fluorescence in focal contacts on these intermediate motility cells was estimated to be only twice that of the intensity detected outside of these regions. Although FRAP measurements in focal contact areas yielded similar values for mobile fractions and diffusion coefficients as for focal contacts in stationary cells (Table I), the mobile fraction of the receptor outside of these areas was significantly reduced as compared with both motile cells and stationary cells. The mean mobile fraction in these areas of diffuse distribution on intermediate motility cells was between that for the mobile fraction of highly motile cells and that for focal contacts and streak regions of stationary cells ($MF = 33 \pm 9\%$ for neural crest cells and 30 $\pm$ 9% for somite cells; Table I and Fig. 3, *b* and *b'*).

The values of FRAP measurements as a function of the locomotory state of the cells are summarized in Fig. 4.

Modulation of the Lateral Mobility of the Fibronectin Receptor Influence of Temperature

The lateral mobility of the fibronectin receptor was measured at 15°, 25°, and 37°C on motile and stationary neural crest
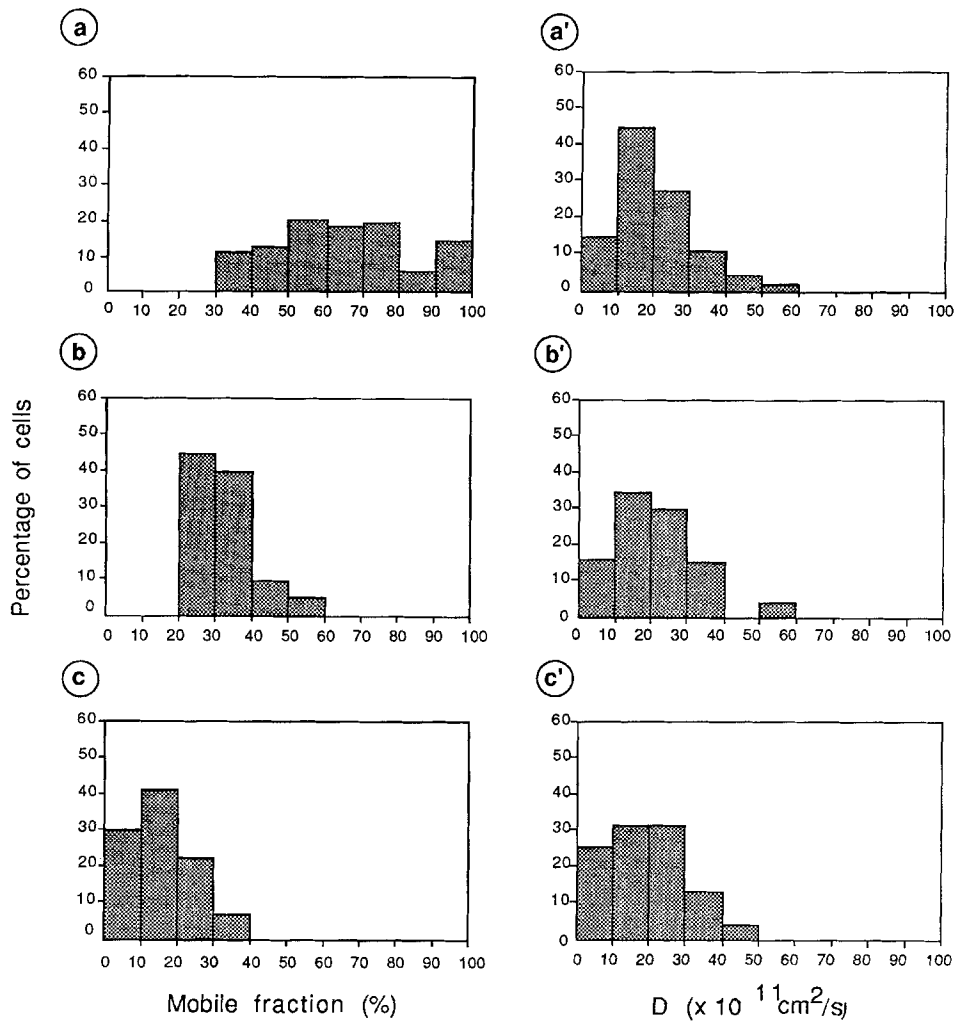
Figure 3. Distribution of the mobile fractions and diffusion coefficients for the fibronectin receptor in motile (*a,a'*), intermediate motility (*b,b'*), and stationary (*c,c'*) neural crest cells. The mobile fraction (*left histograms*) is highly dependent on the degree of cell motility whereas the diffusion coefficient (*right histograms*) shows little dependence on motility. Measurements were performed at 25°C using rhodamine-conjugated ES66-8 antibody.

cells and somitic fibroblasts. The results were similar for both cell types. At 37°C, fluorescence recovery curves more frequently exhibited breaks corresponding to a marked increase in cell locomotion (see Fig. 2 *b*), whereas, these breaks were rarely found at 15°C, presumably because of the reduced cell motility. The mean values for the mobile fraction of the receptor showed a pronounced positive temperature dependence for motile cells (Fig. 5, *upper left*). The diffusion coefficient for the receptor was only slightly affected by temperature (Fig. 5, *lower left*). In contrast, neither

the diffusion coefficient value nor the mobile fraction of the fibronectin receptor located in focal contacts and fibrillar streaks of stationary cells exhibited any detectable temperature dependence (Fig. 5, *right* panels).

Effect of Increasing Receptor Binding to the Substrate

To provide stronger binding between substrate and receptor, motile neural crest cells were cultured on a substratum of purified polyclonal antibodies to the fibronectin receptor,
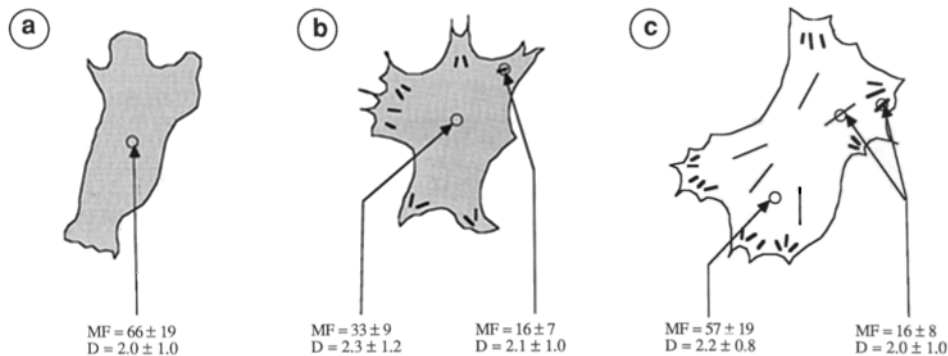

Figure 4. Diagram showing the distribution of the fibronectin receptor on motile (*a*), intermediate motility (*b*), and stationary cells (*c*), and the corresponding values for mobile fraction (*MF*) and diffusion coefficient (*D*). Areas where FRAP measurements were performed are indicated by circles. The grey areas correspond to diffuse, uniform distribution of the receptor, and focal contacts and fibrillar streaks are represented by short and long lines, respectively. *MF* is in percent and *D* in ($\times$ 10^{10} cm^2/s).
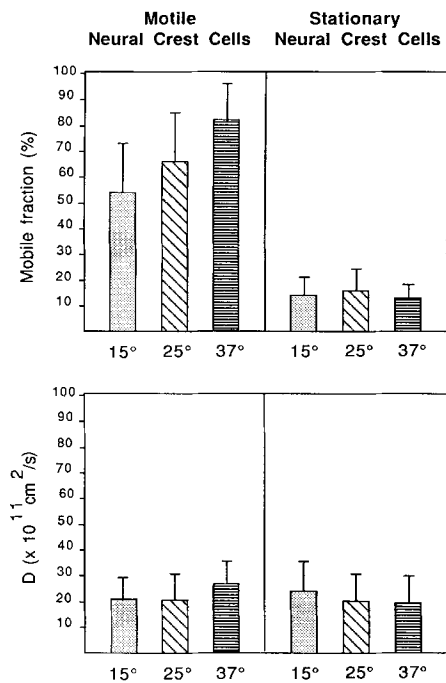
Figure 5. Values of the mobile fractions and diffusion coefficients for the fibronectin receptor in motile and stationary neural crest cells as a function of temperature.

Substratum	Temperature	Mobile fraction (± SD)	Diffusion coefficient (± SD)	n
	°C	%	cm²/s × 10¹⁰	
Ab to FN receptor*	15	20 ± 11	2.6 (± 1.0)	38
FN‡	15	54 ± 19	2.1 (± 0.8)	60
Ab to FN receptor	25	32 ± 13	2.0 (± 0.9)	33
FN	25	66 ± 19	2.0 (± 1.0)	127
Ab to FN receptor	37	34 ± 10	2.3 (± 1.6)	17
FN	37	82 ± 14	2.5 (± 0.8)	32

* Antibody (Ab) to the fibronectin (FN) receptor was coated onto glass coverslips at 50 μg/ml.
‡ Human plasma fibronectin was coated onto glass coverslips at 50 μg/ml.

which had been adsorbed onto coverslips using solution antibody concentrations from 10–100 μg/ml. We showed previously that, under these conditions, neural crest cells were able to leave the neural tube but migrated poorly with a locomotion speed of 15 μm/h (17). These cells formed a layer resembling epithelia, were unusually flattened, and adhered to each other. In contrast, cells migrating on fibronectin substrates migrated at over 60 μm/h. Using immunofluorescence labeling with the ES66-8 monoclonal antibody, fibronectin receptors appeared to be uniformly distributed on the entire surface of neural crest cells and no focal contact-like

structures could be detected (Fig. 6). Labeling was somewhat weaker than on neural crest cells grown on fibronectin indicating an expected competition between the two anti-receptor antibodies. The lateral mobility of fibronectin receptors has been measured on these cells at 15°, 25°, and 37°C and the results are shown on Table II. Values for mobile fraction were reduced markedly as compared with cells cultured on fibronectin substrates and showed a slight positive temperature dependence. In contrast, diffusion coefficients were not significantly different from those obtained using cells cultured on fibronectin.

Effects of Using an Adhesion-perturbing Antibody to Label Receptor

We used a different probe for FRAP measurements on stationary chick dermal fibroblasts that interferes with the binding of the receptor to its ligand. The monoclonal antibody JG22E (11, 22) will cause decreased adhesion to fibronectin substrata in certain cell types. The adhesion-perturbing mech-
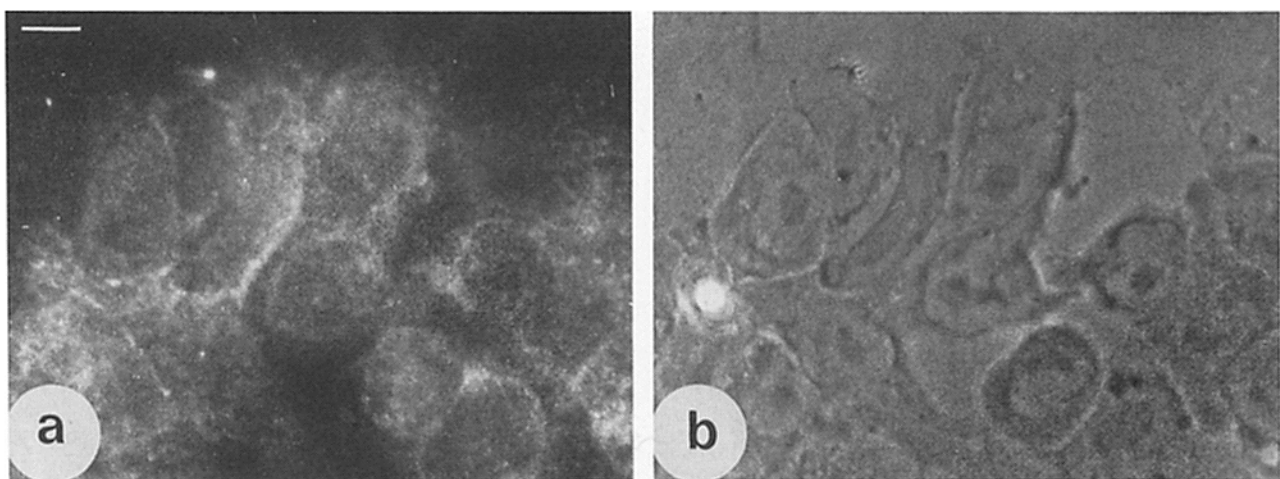

Figure 6. Immunofluorescent distribution of the fibronectin receptor in neural crest cells cultured on a substratum of polyclonal antibodies to fibronectin receptor. (a) Immunofluorescence labeling with ES66-8 monoclonal antibody, (b) phase-contrast image. Note that the cells are tightly juxtaposed and most exhibit a flattened shape with a broad lamellipodium. The receptor exhibits a uniform, diffuse distribution. Bar, 10 μm.
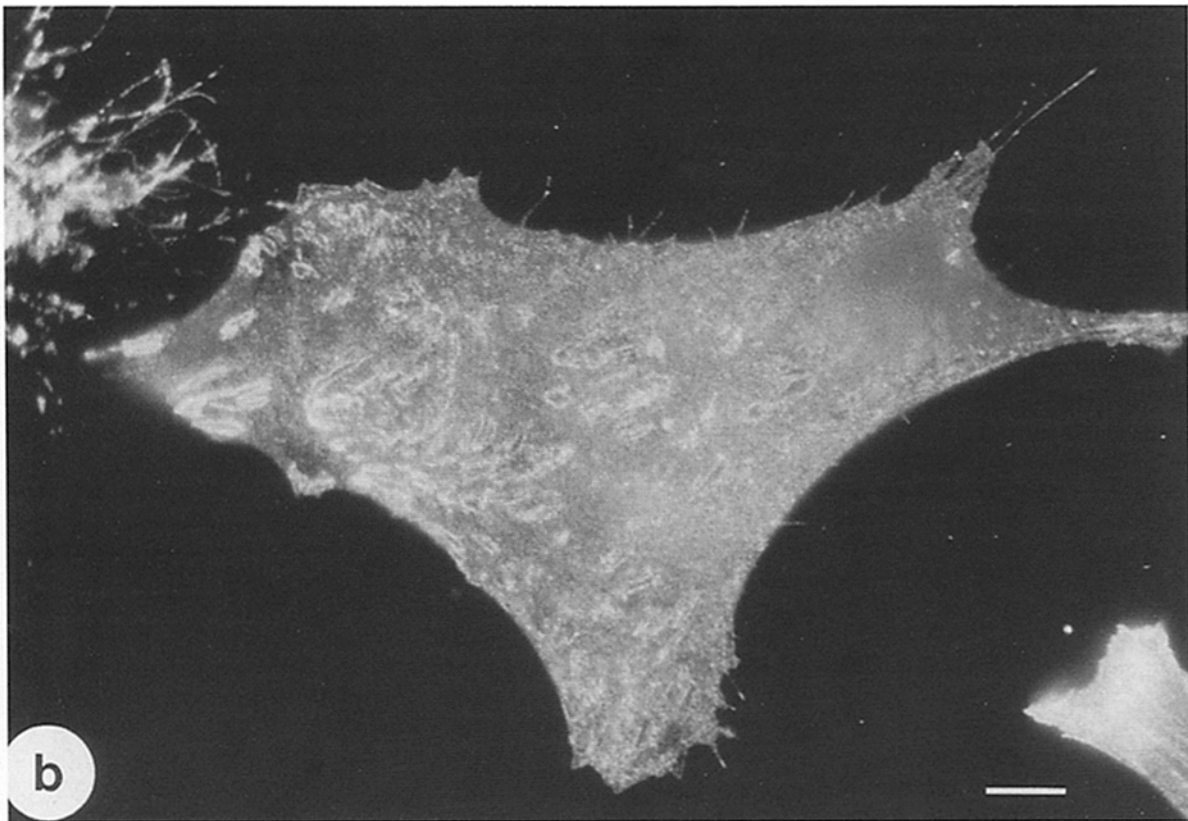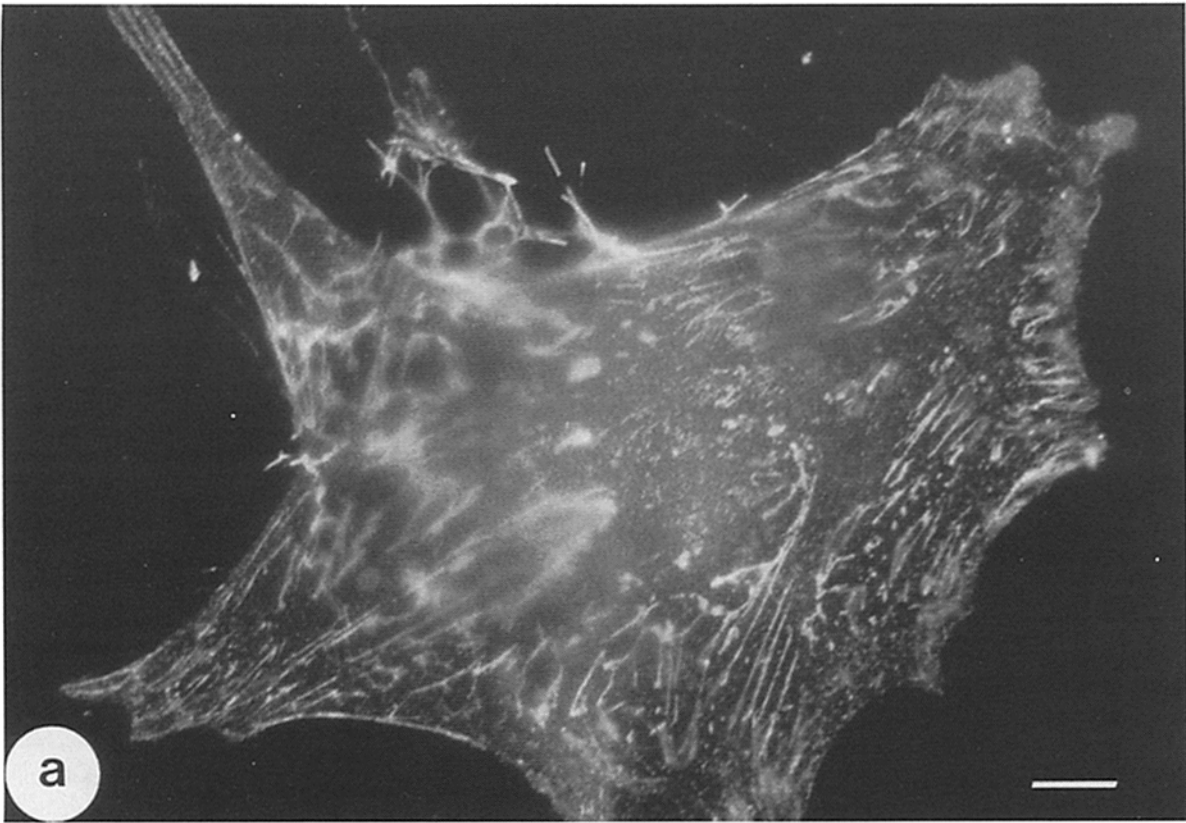
Table III. Effect of Antibody Labeling on Lateral
Mobility of Fibronectin Receptor*

Probe	Mobile fraction (± SD)	Diffusion coefficient (± SD)
	%	$cm^2/s \times 10^{10}$
ES66-8	13 ± 9 (n = 89)	3.4 ± 1.4 (n = 45)
JG22E	58 ± 23 (n = 29)	2.7 ± 1.0 (n = 27)

All experiments were performed on chick embryonic dermal fibroblasts at passage 3–5 in tissue culture. Cells were grown for 18–48 h on uncoated or fibronectin-coated coverslips. FRAP measurements were at 25°C. ES66-8 was used at 25 μg/ml, and JG22E at 60 μg/ml. On certain recovery curves, F_{∞} was clearly measurable, even though the shape of the curve was not as clear. This provided a higher n number for mobile fraction than for diffusion coefficient.
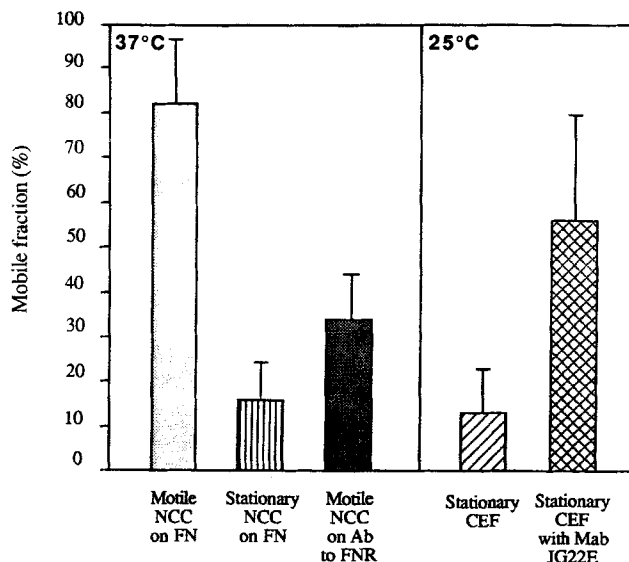

Figure 8. Effects of modulating interactions between the fibronectin receptor and the substratum on lateral mobility. Changing the affinity between the receptor and its ligand results in dramatic changes in the mobile fraction of the receptor. Increasing the binding of receptors to substrate by culturing neural crest cells on polyclonal antibodies to the receptor adsorbed to the substratum results in a marked reduction of the receptor mobile fraction (left). Conversely, decreasing the binding strength between the receptor and its ligand using JG22E antibodies induces a substantial increase in receptor mobile fraction in chick embryo fibroblasts (right). Temperature of experiments noted in upper left of each panel.

anism most likely results from antibody binding to a site on the receptor which is accessible only when the receptor is not bound to fibronectin.

To avoid the complications one would encounter in performing FRAP measurements on cells in the process of rounding, dermal fibroblasts were used in their third to fifth passages and were grown on coverslips for a minimum of 18 h. Under these conditions, these cells are resistant to the rounding effects of this antibody. These cells may express other extracellular matrix receptors not affected by this antibody, or only a small percentage of the receptors on a cell need be bound to fibronectin for normal morphology to be maintained.

Parallel studies were done with the ES66-8 monoclonal antibody. The distribution patterns of the receptor on living cells with the two antibody probes appeared strikingly different. Well-spread dermal fibroblasts stained for direct or indirect immunofluorescence with the ES66-8 antibody showed extensive focal contacts and fibrillar streaks with little labeling outside of these structures (Fig. 7 a). In contrast, direct or indirect immunofluorescence staining with the JG22E antibody gave a distinctively diffuse pattern with few focal adhesions visible by direct staining (Fig. 7 b). Even though JG22E did not induce changes in cell shape, the change in receptor distribution indicated that the antibody is affecting receptor binding to its ligand(s).

FRAP measurements using ES66-8 were performed on focal contacts and fibrillar streaks where the mobile fraction was low; measurements using JG22E were done on the areas of diffuse staining and yielded a much larger mobile fraction (58%; see Table III). In contrast, the difference in diffusion coefficients for the two antibody receptor complexes was not significant.

The modulation of the lateral mobility of the fibronectin receptor by temperature and factors affecting the binding between the receptor and the substratum are summarized in Fig. 8.

Discussion

The present analysis on the lateral mobility of the beta subunit of the fibronectin receptor was undertaken to examine the dynamics of the molecular interactions at cell–substratum contact sites which can provide either transient adhesion to the substratum in locomotory cells or stable anchorage in stationary cells. When the fibronectin receptor is diffusely distributed in motile cells, it exhibits high lateral mobility in terms of a large mobile fraction reaching a mean of 82% at 37°C. In contrast, receptors that are clustered in fibrillar streaks and focal contacts in various stationary cells are immobile on the time scale of the FRAP measurement. The mobile fraction of the fibronectin receptor is temperature dependent in motile cells and can be modulated by varying the strength of the association of the receptor with the substrate (Figs. 5 and 8). The receptor exhibits a relatively invariant lateral diffusion coefficient which is typical of many plasma membrane proteins ($1 \times 10^{-10} < D < 5 \times 10^{-10}$ cm²/s) (for reviews, see 33, 35).

We can consider the dynamics of molecular interactions between the fibronectin receptor and its corresponding ligands in motile and stationary embryonic cells in the context of a previous model (19, 35, 36). This model invokes rela-

Figure 7. Immunofluorescent distribution of the fibronectin receptor on cultured dermal fibroblasts using ES66-8 (a) and JG22E (b) monoclonal antibodies. While ES66-8 staining reveals extensive focal contacts and fibrillar streaks, JG22E staining gives a primarily diffuse pattern with a few focal contacts. This redistribution of the receptor on the cell surface probably results from the detaching effect of the JG22E antibody. Bar, 10 μm.

tively weak, rapid exchange interactions with peripheral membrane structures to retard the diffusion of integral membrane proteins. Diffusion is reduced in proportion to the time the protein spends visiting these peripheral binding sites. In the fibronectin receptor system, candidates for such interactions include fibronectin and other extracellular matrix proteins on the external side of the cell and talin as part of the cytoskeleton within the cell. Indeed, each of these molecules can bind the fibronectin receptor with a relatively low affinity ($K_d \sim 10^{-6}$ M; 1, 2, 28, 29).

The immobile fraction in this model is postulated to arise from slow exchange interactions with static peripheral structures where the "dwell time" for the association is long compared to the recovery half-time. In the present experimental system immobilization by stronger interactions can be produced artificially by coating substrates with high-affinity polyclonal anti–receptor antibodies, resulting in a substantial decrease in receptor mobile fraction in motile neural crest cells (Table II). Candidates for immobilizing interactions in the biological context include the static fibronectin-containing extracellular matrix (50) and the membrane-associated cortical cytoskeleton. In cells cultured on fibronectin-coated substrates, if we assume that fibronectin binds to the receptor with a $K_d \sim 10^{-6}$ M (29), simultaneous binding with similar affinity to a cytoskeletal protein could produce an overall dissociation constant of the order of 10^{-12} M, presumably sufficient for total immobilization of the receptor on the FRAP time scale. Our study shows that the fibronectin receptor is most probably totally immobilized within focal contact and fibrillar streak regions in stationary cells; this is based on the plausible contention that the low (13–18%) mobile fraction is due to recovery in the bleached diffuse pool of receptor immediately adjacent to the contact or streak. (Bleaching of such regions is inevitable considering that the streaks or contacts have at least one dimension smaller than the circular spot size [$\sim$2 μm diameter].) This has important implications: receptors in the contact or streak regions do not exchange with the surrounding pool of mobile receptors at a sufficient rate to cause recovery on the FRAP measurement time scale ($\sim$10 min). Also, exchange of receptors within a contact region must be minimal. Furthermore, over a limited temperature range (15°–37°C), no appreciable dissociation of receptor from these structures occurs as evidenced by the constant, low mobile fraction (Fig. 5, *upper right*). The very low, temperature-dependent lateral mobility of the fibronectin receptor in focal contacts and fibrillar streaks therefore suggests a stable association of this component with both the extracellular matrix and cytoskeletal elements. Early studies have established the partially coincident staining patterns of fibronectin fibrils and actin-containing stress fibers (3, 31, 53, 61), and these were extended by observations showing coincidence of staining patterns for fibronectin, fibronectin receptor, and microfilament-associated molecules, including actin, alpha actinin, vinculin, and talin (10, 15, 17, 21, 54; see reference 9 for a review).

Previous studies have shown that fibronectin molecules (50), membrane proteins (20), alpha actinin (55), and actin (40) are totally or partially immobilized when they are associated with focal contacts and fibrillar streaks. This study shows that when interactions between the receptor and fibronectin are abolished using JG22E antibodies that perturb adhesion of certain cells, the fraction of receptors in focal contacts and fibrillar streaks is decreased, and the overall lateral mobility of the population of receptors on a cell's surface is markedly increased. Similarly, oncogenic transformation induces the redistribution of the receptor on the cell surface (12, 27), the reduction of binding between the receptor and fibronectin and talin (7a), and an increase in the lateral mobility of alpha-actinin (55). Conversely, the addition of cellular fibronectin to the transformed cells can induce the receptors to redistribute into focal contacts and fibrillar streaks (12). These observations imply that under normal circumstances, focal contacts are stable structures in which the various components are intimately associated. In particular, binding between the fibronectin receptor and its corresponding ligands appears important in stabilizing the structural organization of focal contacts.

As far as the small diffuse pool of receptors in stationary cells is concerned, it has not been possible to measure the ratio of the amount of receptor in this pool to that in the fibrillar streaks and focal contacts because of the difficulty in gauging the relative surface area of the focal contacts and diffusely stained regions. However, it has been possible to estimate the relative density of receptors, which appeared to be $\sim$10-fold higher in focal contacts than outside of these sites. While receptors present in focal contacts are clearly involved in cell–substratum anchorage, the role of the diffusely distributed receptors in this process is not established. The high lateral mobility of these receptors would suggest that they are only transiently associated to both fibronectin and cytoskeleton and that, consequently, they are not involved in force transmitting cell–substratum attachment.

In contrast to stationary cells, the fibronectin receptor is diffusely and uniformly distributed on the surface of motile cells where it exhibits a high mobile fraction. It should be noted that FRAP measurements performed on different areas of motile cells did not reveal local variations in the lateral mobility of the receptor. One cannot exclude, however, some changes in the density of the receptor at discrete sites of the cell surface, e.g., at the tip of pulling lobopodia, where the mobility is transiently reduced. This high lateral mobility of the receptor can be interpreted as a large majority of receptors being bound to fibronectin molecules or cytoskeleton components, but these interactions are transient; thus the receptors rapidly dissociate from their ligands to establish new associations with other peripheral molecules. It should also be noted that locomotory cells have a small but measurable population of immobile receptors. Receptors in this immobilized state may serve to provide transient anchorage to the substrate permitting the cell to develop the traction required for motility. This receptor population is more labile than the receptors involved in focal contacts as it can be significantly modulated by temperature.

In intermediate-motility cells, a substantial fraction of the population of fibronectin receptors accumulates into focal contacts while a larger fraction continues to exhibit a diffuse pattern over the entire cell surface. FRAP measurements show that the latter population of receptors has an intermediate mobile fraction. This decrease in mobile fraction would suggest that the receptor is microclustered and that precursor structures to focal contacts and fibrillar streaks have formed but cannot be detected because they are beneath optical resolution. Such structures could act to retard cell translocation by participating in the formation of more stable sites of sub-

stratum anchorage. It remains to be determined if receptors in focal contacts and fibrillar streaks originate from the redistribution of the preexisting diffuse pool or from newly synthesized receptors directed to these sites.

It was found that the lateral mobility of the receptor can be modulated by various means, such as by affecting the interaction between the receptor and the substratum. Use of the adhesion-perturbing antibody JG22E, apparently releases receptor from the adhesive structures and into the diffuse pool which is normally present, but of very low concentration in ES66-8 stained cells. In contrast, increasing the binding strength between the receptor and the substratum induces a marked reduction of its lateral mobility. Binding of receptor to substrate bound polyclonal antibody may induce "global modulation" which reduces the mobile fraction of dorsal surface receptor (18, 25).

Summary and Overview

In motile neural crest cells on fibronectin substrates, the fibronectin receptor displays a large, temperature-dependent mobile fraction ($\sim$80% at 37°C) and a lateral diffusion coefficient ($\sim$2 × 10^{-10} cm^2/s) typical of several membrane proteins. When neural crest cells become stationary there is a correlated decrease in the receptor mobile fraction to a low, temperature-independent level as it becomes localized in focal contacts and fibrillar streaks, where it is most probably totally immobile. Similarly, the mobile fraction of the fibronectin receptor is very low in a variety of stationary fibroblasts. It seems likely, but is not proven, that a large portion of the receptor in these structures is recruited from a preexisting diffusely distributed plasma membrane pool.

The moderate lateral diffusion coefficient of the receptor indicates weak restraints on mobility, and is qualitatively explained by its transient monovalent binding to extracellular matrix proteins such as fibronectin or membrane-associated cytoskeletal components such as talin. At sites of sufficient concentration of both intracellular and extracellular ligands, simultaneous binding to both peripheral structures may explain the increases in receptor immobile fraction observed as cells become stationary. This multivalent, immobilized receptor state would provide stable anchorage to the substrate. It will be interesting in the future to examine how receptor is transported and localized to sites appropriate for locomotion, since the numbers and types of these sites presumably help regulate the locomotory state of the cell.

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