Cross Talk between Adhesion Molecules: Control of N-cadherin Activity by Intracellular Signals Elicited by β1 and β3 Integrins in Migrating Neural Crest Cells

Frédérique Monier-Gavelle and Jean-Loup Duband

Institut Jacques Monod, Centre National de la Recherche Scientifique (CNRS) et Université Paris 7-Denis Diderot, and Laboratoire de Biologie Moléculaire et Cellulaire du Développement, CNRS et Université Pierre et Marie Curie, 75252 Paris, France

Abstract. During embryonic development, cell migration and cell differentiation are associated with dynamic modulations both in time and space of the repertoire and function of adhesion receptors, but the nature of the mechanisms responsible for their coordinated occurrence remains to be elucidated. Thus, migrating neural crest cells adhere to fibronectin in an integrin-dependent manner while maintaining reduced N-cadherin–mediated intercellular contacts. In the present study we provide evidence that, in these cells, the control of N-cadherin may rely directly on the activity of integrins involved in the process of cell motion. Prevention of neural crest cell migration using RGD peptides or antibodies to fibronectin and to β1 and β3 integrins caused rapid N-cadherin–mediated cell clustering. Restoration of stable intercellular contacts resulted essentially from the recruitment of an intracellular pool of N-cadherin molecules that accumulated into adherens junctions in tight association with the cytoskeleton and not from the redistribution of a preexisting pool of surface N-cadherin molecules. In addition, agents that cause elevation of intracellular Ca^{2+} after entry across the plasma membrane were potent inhibitors of cell aggregation and reduced the N-cadherin–mediated junctions in the cells. Finally, elevated serine/threonine phosphorylation of catenins associated with N-cadherin accompanied the restoration of intercellular contacts. These results indicate that, in migrating neural crest cells, β1 and β3 integrins are at the origin of a cascade of signaling events that involve transmembrane Ca^{2+} fluxes, followed by activation of phosphatases and kinases, and that ultimately control the surface distribution and activity of N-cadherin. Such a direct coupling between adhesion receptors by means of intracellular signals may be significant for the coordinated interplay between cell–cell and cell–substratum adhesion that occurs during embryonic development, in wound healing, and during tumor invasion and metastasis.

CADHERINS are integral membrane receptors that mediate Ca^{2+}-dependent cell–cell adhesion among most, if not all, tissues (for reviews see 31, 96). At the cellular level, cadherins are primarily concentrated in the adherens junctions where they are connected with the actin cytoskeleton. In these junctions, cadherin molecules interact through their cytoplasmic domains with cytoskeleton-associated proteins, namely α-, β-, and γ-catenins, α-actinin, and $p120^{cas}$, and this association is essential for their cell-binding function (for reviews see 33, 62).

Although they form complex multimolecular structures, adherens junctions are highly dynamic and can be repeatedly and rapidly assembled and disassembled. In particular, during embryonic development and tumor progression, groups of cells are constantly remodeled so that neighboring cells that are initially in tight contact become separated, disperse into adjacent tissues, and ultimately reassociate with other cell types in different locations of the organism. A large number of data has been accumulated recently about the possible mechanisms involved in the down-regulation of cell–cell associations in tumor cells allowing their dissemination throughout the body (see for review 95). In a variety of infiltrating human cancers, E-cadherin was found to be either absent or at least in significantly reduced amounts (e.g., see 30, 103). Consistent with this finding, noninvasive transformed cells in vitro become invasive upon abolishing E-cadherin function or expression by antibodies or by introduction of E-cadherin antisense RNA (4, 106). Conversely, transfection of highly invasive epithelial tumor cell lines by E-cadherin cDNA totally abrogates their invasiveness potential (106). A number of tumors, however, can disaggregate and metastasize despite an abundant expression of cadherin molecules on the cells' surface. In these cases, several mechanisms have been proposed that would account for the suppression of cadherin activity (48, 64, 89, 107). In particular, elevated

Please address all correspondence to Jean-Loup Duband, Equipe Adhésion et Migration Cellulaires, Laboratoire de Biologie Moléculaire et Cellulaire du Développement, CNRS URA 1135 et Université Pierre et Marie Curie, 9 quai Saint-Bernard, 75252 Paris Cedex 05, France. Fax: (33) 1-44-27-34-97. e-mail: duband@ccr.jussieu.fr

levels of tyrosine phosphorylation of β-catenin, p120^{cas}, and, to a lesser extent, of cadherins are believed to cause unstable cell–cell adhesion in invasive cancer cell lines or in cultured cells transformed with a *v-src* gene (5, 35, 49, 65, 92, 108, 109). However, this assumption has been challenged recently in a study revealing that phosphorylation of β-catenin is dispensable for diminishing cadherin-mediated cell–cell associations in src-transformed cells (93).

In contrast with tumor cells, relatively little is known about the mechanisms involved in the regulation of cadherin-based cellular interactions during embryonic development. Because of their spectacular migration throughout the embryo, accompanied by sequential modulations in their intercellular cohesion, neural crest cells provide a powerful paradigm for exploring these mechanisms (see for reviews 12, 26, 28, 56, 75). Thus, various in vivo studies clearly established an inverse correlation between the expression of N-cadherin and the migratory behavior of neural crest cells, suggesting a precisely regulated, negative control of the expression and function of N-cadherin molecules during migration (2, 13, 23, 37, 73). Under in vitro conditions, consistent with the in vivo situation, neural crest cells do not establish extensive and stable intercellular contacts during migration. However, they express intact N-cadherin molecules on their surface but, contrasting with nonmotile cells, the bulk of these molecules are excluded from the regions of cell–cell contacts (71). In addition, inhibition of serine-threonine kinases, tyrosine kinases, and phosphotyrosine phosphatases by specific inhibitors restored tight cell–cell cohesion among cells accompanied by N-cadherin accumulation to the regions of intercellular contacts, suggesting that N-cadherin–mediated interactions in migrating neural crest cells are under the control of a complex cascade of intracellular signals involving kinases and phosphatases and presumably elicited by surface receptors (71).

Receptors for growth factors have been proposed to control E-cadherin activity in various epithelial cell lines, such as MDCK, carcinoma, and mammary cells. In particular, the EGF receptor has been identified among the proteins associated with E-cadherin and catenins, and binding to its ligand provokes immediate tyrosine phosphorylation of β-catenin followed by rapid deterioration of adherens junctions (45). Conversely, expression of *Wnt-1*, a gene encoding a putative growth factor, results in enhancement of β-catenin and E-cadherin expression accompanied by increased stability of the catenin–cadherin complex and greater cell adhesion (42). In migrating neural crest cells, the nature of the cell surface receptors eliciting the cascade of intracellular signals that would regulate N-cadherin–based junctions is not known yet. Interestingly, at the end of migration, as cells aggregate into peripheral ganglia, N-cadherin is reexpressed on their surface coincidently with the disappearance of fibronectin (2, 13, 23, 37, 73, 99), suggesting that, locally, the lack of an appropriate substratum for migration could provoke N-cadherin–mediated aggregation of neural crest cells. As integrins have been shown to constitute the major receptors for extracellular matrix molecules involved in neural crest cell migration (11, 20, 21, 53), this raises the intriguing possibility that they might be part of the regulatory mechanism of N-cadherin activity in neural crest cells. In the present study we demonstrate in vitro that blocking neural crest cell migration by agents that interfere with fibronectin-to-integrin interactions causes rapid N-cadherin–mediated cell–cell aggregation. This aggregation process is mediated by a cytoplasmic pool of N-cadherin molecules recruited to the membrane and incorporated into adherens junctions in association with cytoskeletal elements. We also found that the control of cadherin function in neural crest cells is via transmembrane Ca^{2+} fluxes possibly mediated by voltage-independent Ca^{2+} channels activated by integrins involved in cell migration. Finally, we provide evidence that phosphorylation of β- and γ-catenins and of a 180-kD protein is increased most likely on serine and threonine residues in aggregated neural crest cells. Our data are therefore in favor of the existence of cross talk mechanisms between cell–cell and cell–substratum adhesion systems responsible for their coordinated activity in migrating cells.

Materials and Methods

Adhesive Proteins and Antibodies

Bovine and human plasma fibronectin and Arg-Gly-Asp-Ser (RGDS) peptides were purchased from Sigma Chemical Co. (St. Louis, MO). Gly-Arg-Asp-Gly-Ser (GRDGS) peptides were provided by Dr. K.M. Yamada (National Institutes of Health, Bethesda, MD). Cyclic Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala (GPenGRGDSPCA) peptides were purchased from GIBCO BRL (Gaithersburg, MD). The mAb 333 to human fibronectin (3, 27), the polyclonal antibody (2992) directed against the chicken β1 integrin subunit (17, 21), and the mAbs ES66-8 and ES46-8 also to the chicken β1 subunit (22, 24, 72) were kindly provided by Dr. K.M. Yamada. The CSAT hybridoma (anti–chicken β1 integrin subunit; 44) was kindly donated by Dr. C. Buck (The Wistar Institute, Philadelphia, PA). The mAb LM609 to the human αvβ3 integrin cross-reacting with its avian counterpart (14, 20) was a gift of Dr. D.A. Cheresh (The Scripps Research Institute, La Jolla, CA). The mAb HP2/1 to the human α4 integrin chain (82) was kindly provided by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). The mAb B5G10 to the human α4 chain (38) was kindly provided by Dr. M.E. Hemler (The Dana Farber Cancer Institute, Boston, MA) or purchased from UBI (Euromedex, France). Both antibodies were found to recognize the α4 integrin subunit in a variety of species including quail (59). The mAb P1B5 to the human α3 integrin subunit (111) was purchased from Becton Dickinson & Co. (Mountain View, CA). Using both immunoprecipitation and immunocytochemistry, we could demonstrate that this antibody recognizes the avian α3 integrin subunit. mAbs to chicken N-cadherin (mAb CC-11 and ID-7.2.3; 23, 110) were a kind gift of Dr B. Geiger (The Weizmann Institute, Rehovot, Israel). The rat mAb NCD-2 to chicken N-cadherin (36) was kindly donated by Dr. M. Takeichi (Kyoto University, Kyoto, Japan). A rabbit antiserum directed against a peptide corresponding to the 24 COOH-terminal amino acids of chicken N-cadherin and the mAb PT-66 antiphosphotyrosine were purchased from Sigma Chemical Co.

Metabolic Agents

Drugs that affect macromolecule synthesis and signal transduction pathways are listed on Table I. They were purchased from Sigma Chemical Co., Biomol Research Laboratories (Tebu, France), or Calbiochem-Novabiochem Corp. (La Jolla, CA). Agents of limited aqueous solubility were prepared as stock solutions in a minimum volume of solvent, e.g., DMSO or methanol, to reduce solvent concentration in assays below 0.1% (vol/vol). Effects of solvents were evaluated in separate controls and were found not to affect cell viability, spreading, and motility. Each agent was tested for toxicity. Cell viability was assessed by cell morphology under an inverted microscope and by videomicroscopy. As recommended by the manufacturers, metabolic agents were generally used at concentrations ranging from 1× to 100× the inhibition constants or the concentrations of 50% inhibition measured in vitro (values obtained generally from Calbiochem-Novabiochem Corp. or Biomol Research Laboratories).

Table I. List of Metabolic Agents Used in the Present Study

Metabolic agent	Effect	Source	Solvent/stock concentration	Concentration range studied	References
Cycloheximide	Inhibits protein synthesis	Sigma	DMSO/10 mg/ml	0.01–10 μg/ml	/
Tunicamycin	Inhibits N-glycosylation	Sigma	Methanol/1 mg/ml	0.01–1 μg/ml	/
Brefeldin A	Inhibits secretion	Sigma	Methanol/5 mg/ml	0.01–5 μg/ml	50
Monensin	Inhibits secretion	Sigma	Methanol/10 mM	0.01–1 μM	81
Ionomycin	Ca^{2+} ionophore	Sigma	DMSO/10 mM	0.01–5 μM	100
A23187	Ca^{2+} ionophore	Sigma	DMSO/1 mM	1–100 nM	81
Thapsigargin	Inhibits Ca^{2+} ATPase	Sigma	DMSO/10 mM	0.1–50 nM	98
Nifedipine	Ca^{2+} channel blocker	Sigma	DMSO/100 mM	1–100 μM	67
NiCl	Ca^{2+} channel blocker	Sigma	H_2O/500 mM	0.05–5 mM	47
Staurosporine	Inhibits ser-thr kinases	Sigma	DMSO/2 mM	0.1–100 nM	97
H7	Inhibits ser-thr kinases	Sigma	DMSO/100 mM	1–100 μM	40
Bisindolylmaleimide	Inhibits PKC	Calbiochem	DMSO/25 mM	0.5–50 μM	101
Sphingosine	Inhibits PKC	Sigma	DMSO/30 mM	0.3–30 μM	34
PMA	Stimulates PKC	Sigma	DMSO/10 mg/ml	0.01–10 μg/ml	77
Calmidazolium	Inhibits calmodulin	Sigma	DMSO/100 mM	1–10 μM	32
Trifluoperazine	Inhibits calmodulin	Sigma	DMSO/100 mM	1–25 μM	32
Erbstatin analog	Inhibits tyr kinases	Biomol	DMSO/100 mM	0.01–100 μM	104
Herbimycin A	Inhibits tyr kinases	Biomol	DMSO/2 mg/ml	0.02–2 μg/ml	102
Tyrphostin 25	Inhibits tyr kinases	Sigma	DMSO/500 mM	0.05–1,000 μM	61

Embryos and Cell Cultures

Japanese quail embryos were used throughout the study. Eggs were incubated at 38 ± 1°C and staged according to the number of somite pairs. Neural crest cell cultures were generated essentially as described (25). Neural tubes were deposited in bacteriological petri dishes coated with fibronectin at 2–5 μg/ml in PBS followed by saturation with heat-denatured BSA at 10 mg/ml in PBS. Cells were cultured in DME supplemented with 3% bovine serum previously depleted in fibronectin. Time-lapse videomicroscopy analyses were performed in Terasaki plates as described elsewhere (27).

Immunofluorescent Staining

For immunofluorescent labeling of cell cultures, neural tubes were explanted onto fibronectin-coated glass coverslips. Cultures were fixed either in cold methanol for 5 min followed by cold acetone for 1 min to retain the total cellular pool of N-cadherin, or in 3.7% formaldehyde–0.2% Triton X-100–5% sucrose in PBS for 3 min followed by a 1-h incubation in 3.7% formaldehyde–5% sucrose in PBS to visualize the detergent-insoluble pool. After several rinses in PBS, cultures were subjected to immunofluorescent staining using biotinylated secondary antibodies and fluorescein-conjugated streptavidin (Amersham Intl., Little Chalfont, UK). Preparations were observed with an epifluorescence microscope (Leica, Wetzlar, Germany) and photographed using TMAX-400 Kodak film (Eastman Kodak Co., Rochester, NY).

Cell Extraction with Triton X-100

For preparation of Triton X-100–insoluble fractions of cells, neural crest cells were obtained from an equal number of neural tube explants for each experimental condition. Cells were rinsed in PBS and extracted at 4°C for 15 min in ice-cold extraction buffer consisting of 2.5% Triton X-100, 0.1 M Tris, pH 7.4, 0.15 M NaCl, 2 mM $CaCl_2$, 2 mM PMSF, 1 mM leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A. The cells were then scraped from the dish in extraction buffer and the cell residue was pelleted at 11,000 *g* for 10 min. The pellet corresponding to the Triton-insoluble fraction was resuspended at 90°C for 5 min in SDS sample buffer. Protein concentration was determined using the bicinchoninic acid protein assay kit (BCA; Pierce Chemical Co., Rockford, IL). The samples were subsequently subjected to SDS-PAGE followed by immunoblotting analysis.

Immunoblotting

For immunoblotting of whole cell extracts, neural crest cells were obtained from an equal number of neural tube explants for each experimental condition. Cells were detached from the substratum using a solution of 0.01% trypsin (type XI; Sigma Chemical Co.) in 0.1 M Tris, pH 7.2, 0.15 M NaCl, 10 mM Hepes, and 2 mM $CaCl_2$ at 37°C for 5–7 min. Once cells were detached, trypsin was inactivated by addition of 0.01% soybean trypsin inhibitor (type II; Sigma Chemical Co.). Cells were then collected by centrifugation, counted, and lysed at 90°C with SDS sample buffer under reducing conditions. The extracts were clarified by centrifugation and subjected to SDS-PAGE after determination of protein concentration using the Pierce BCA protein assay kit. PAGE was performed in Laemmli buffer system on slab 7.5%-polyacrylamide minigels. The protein bands were electroblotted for 1.5 h onto nitrocellulose in 50 mM Tris-glycine, 20% methanol buffer. The nitrocellulose membranes were then incubated with BSA at 5 mg/ml in PBS for 1 h at ambient temperature, followed by incubation with antibody solution for 12 h at 4°C. The sheets were rinsed in PBS supplemented with 0.2% Tween-20 and incubated first with biotinylated secondary antibodies for 1 h, and then with ^{125}I-streptavidin (Amersham Intl.) at 0.1 μCi/ml for 1 h at room temperature. After rinsing, the nitrocellulose was dried and subjected to autoradiography. Immunolabelings were quantitated with a PhosphorImager using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation

For immunoprecipitation, neural crest cells obtained from an equal number of neural tube explants for each experimental condition were metabolically labeled at 37°C with [^{35}S]methionine (Amersham Intl.) at 250 μCi/ml or with [^{32}P]orthophosphate (Amersham Intl.) at 1 mCi/ml for 5–8 h. The viability of cells during labeling was regularly confirmed with an inverted microscope. After radioactive labeling, cells were washed four times in PBS supplemented with 1 mM $CaCl_2$ and $MgCl_2$ and were extracted for 20 min on ice with extraction buffer (0.1 M Tris, pH 7.2, 0.15 M NaCl, 2 mM $CaCl_2$, 1% NP-40, 1% Triton X-100, 2 mM PMSF, 1 mM leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). Sodium orthovanadate (Na_3V0_4) was added to the extraction buffer at the final concentration of 0.5 mM in the case of [^{32}P]orthophosphate labeling. All subsequent steps were performed at 4°C. Lysates were clarified by centrifugation at 11,000 *g* for 15 min. The extracts containing equal amounts of acid-precipitable radioactivity were preabsorbed by incubation with nonimmune rabbit serum for 30 min under constant mixing followed by a 1-h incubation with a 50% suspension of protein A–Sepharose (Sigma Chemical Co.). After centrifugation to remove the beads, extracts were incubated with constant mixing, first in the presence of rabbit antibodies to the cytoplasmic domain of N-cadherin for 2 h, and then with an excess of a 50% suspension of protein A–Sepharose for 1 h. The beads were subsequently washed five times with 1% Triton X-100 in 0.1 M Tris, pH 7.2, 0.15 M NaCl, 2 mM $CaCl_2$, and protease inhibitors and, when necessary, 0.5 mM Na_3V0_4, and extracted at 95°C for 5 min in 2% SDS in 0.1 M Tris, pH 7.2, 0.15 M NaCl. Samples were analyzed by SDS-PAGE on 7.5%-acrylamide

gels under reducing conditions and subjected to fluorography or autoradiography. Immune precipitates from ^{32}P-labeled cells were quantitated with a PhosphorImager.

Results

Induction of Neural Crest Cell Aggregation upon Inhibition of Fibronectin Adhesion In Vitro

We determined at first whether neural crest cell aggregation could occur in vitro upon inhibition of cell locomotion on fibronectin, using a battery of agents known to interfere with the binding of integrins to fibronectin, i.e., RGDS peptides and inhibitory antibodies to fibronectin or to integrins (8, 10, 21, 27, 83). GRDGS peptides and noninhibitory antibodies were used as controls. Neural tube explants were deposited on fibronectin substrata, and neural crest cells were allowed to migrate out of the explant for ~15 h. Under these conditions, the neural crest population organized into an outgrowth of ~1,500–2,000 cells around

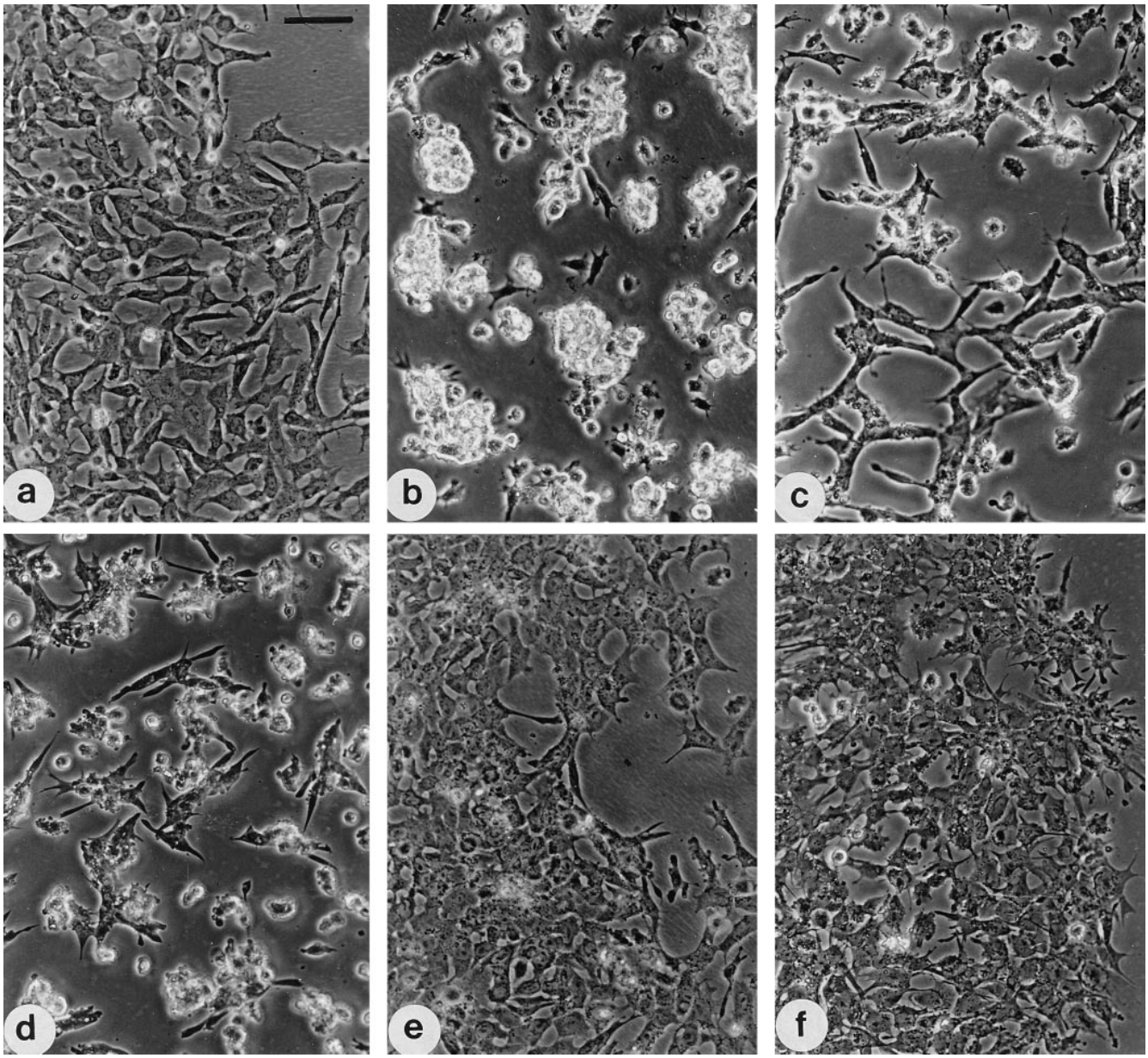

Figure 1. Inhibition of neural crest cell adhesion to fibronectin by RGDS peptides or antibodies to fibronectin and integrins provokes cell clustering. Neural crest cells were cultured for 15 h on fibronectin substrata at 3 μg/ml. Cells were subsequently confronted for 5 h with control GRDGS peptides (*a*), RGDS peptides (*b*) both at 1 mg/ml, mAb 333 to fibronectin at 50 μg/ml (*c*), polyclonal antibodies 2992 anti–β1 integrins at 1 mg/ml (*d*), mAb HP2/1 to the α4 integrin subunit at 40 μg/ml (*e*), and mAb LM609 ascite to the αVβ3 integrin at dilution 1:100 (*f*). In the presence of RGDS peptides and antibodies to fibronectin and β1 integrins, most neural crest cells are retracted and regrouped into compact clusters. In the presence of antibodies to the α4 and αVβ3 integrins, cells remain flattened but form a monolayer of tightly adherent cells. Bar, 50 μm.

the neural tube. Inhibitory probes were then added to the culture medium, and their effects on the cell culture were evaluated for the subsequent 5–8 h.

In the presence of GRDGS peptides at 1 mg/ml (Fig. 1 *a*) or of noninhibitory antibodies to fibronectin or to integrins, neural crest cells retained a well-spread morphology with several processes per cell during the time course of the experiment. Although the cell population was dense, the contours of individual cells could be easily visualized, allowing us to distinguish them from their neighbors. Videomicroscopy studies revealed that cells exchanged neighbors frequently and formed only transient intercellular contacts.

When neural crest cells were confronted with RGDS peptides at 0.5–2 mg/ml, cells retracted immediately and became round within an hour after addition of the peptide. Coincidently, adjacent cells regrouped and formed clusters that gradually increased in size as they collided with other clusters or isolated cells. After 5 h in the presence of the peptide (Fig. 1 *b*), most cells formed aggregates of about several cells up to 10 cells. The situation did not evolve significantly during the subsequent hours. By videomicroscopy, it could be observed that, once cells joined clusters and adhered to them, they rarely detached from them. At lower concentrations of the peptide (e.g., 0.1 mg/ml), cell retraction was less extensive and cells became round only occasionally. However, a significant proportion of the cells established tight intercellular contacts and formed small clusters of spread cells (not shown).

In the presence of the inhibitory mAb 333 to fibronectin at 50 μg/ml, neural crest cells retracted, but a number of them did not round up; instead, they showed an elongated, bipolar morphology. However, these cells were connected with their neighbors and formed a continuous network of cells (Fig. 1 *c*). Cells that rounded up also formed compact aggregates, but of sizes generally smaller than in the presence of RGDS peptides.

Polyclonal antibodies 2992 to the β1 integrin subunit at 0.5–1 mg/ml produced the same degree of retraction and aggregation of neural crest cells as mAb 333 (Fig. 1 *d*). Similarly, mAb CSAT also directed against the β1 subunit caused extensive cell aggregation at 10–50 μg/ml (not shown). Conversely, the noninhibitory mAbs ES46-8 and ES66-8 to the β1 chain did not cause neural crest cell aggregation. As migrating neural crest cells express multiple β1 integrins, including α3β1, α4β1, α5β1, and αvβ1, as potential fibronectin receptors (Delannet, M., S. Testaz, and J.-L. Duband, manuscript in preparation), specific antibodies to the various α chains have been assayed for causing neural crest cell aggregation. Among them, the function-blocking mAb HP2/1 to the α4 chain caused at 20–50 μg/ml only limited retraction of cell processes but provoked progressively, after several hours, an important regroupment of neighboring cells that organized locally as a dense monolayer resembling a cluster of epithelial cells (Fig. 1 *e*). The nonblocking mAb B5G10 to α4, in contrast, did not induce neural crest cell compaction. Finally, mAb P1B5 to the α3 chain did not affect the cohesion of neural crest cells at all the concentrations tested (not shown). In addition to β1 integrins, neural crest cells have also been found to use β3 integrins for migration on extracellular matrices (20; Delannet, M., S. Testaz, and J.-L. Duband, manuscript in preparation). The effect of the inhibitory mAb LM609 to the αvβ3 integrin complex on neural crest cell cohesion was then evaluated. Like mAb HP2/1, this antibody provoked a substantial cell clustering but no apparent rounding up of the cells (Fig. 1 *f*). However, in contrast with mAb HP2/1, mAb LM609 effect was rapid as it could be detected only after an hour of incubation. Finally, cyclic Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala (GPen-GRG DSPCA) peptides known to affect selectively αv integrins (79) induced aggregation of neural crest cells at 0.1–1 mg/ml (not shown).

Table II. Quantitation of the Effect of Competitors of Fibronectin–Integrin Interactions on the Cohesion of Neural Crest Cells

Inhibitor of cell–substratum adhesion	Concentration	Number of particles
	μg/ml	
None	/	230 ± 20
RGDS	100	165 ± 19
	500	78 ± 5
	1,000	50 ± 2
	2,000	53 ± 7
GRDGS	1,000	215 ± 10
GPenGRGDSPCA	100	137 ± 24
	250	99 ± 14
	500	58 ± 14
	1,000	64 ± 18
mAb 333 anti-fibronectin	5	129 ± 60
	50	56 ± 14
Ab 2992 anti-β1 integrin	100	113 ± 12
	1,000	42 ± 5
mAb CSAT anti-β1 integrin	10	162 ± 25
	20	54 ± 9
	40	44 ± 10
mAb ES66-8 anti-β1 integrin	100	215 ± 10
mAb HP2/1 anti-α4 integrin	10	138 ± 22
	20	80 ± 24
	40	68 ± 15
mAb B5G10 anti-α4 integrin	50	220 ± 25
mAb LM609 anti-αVβ3 integrin	1/250*	203 ± 16
	1/100*	88 ± 38
	1/25*	67 ± 11

Results are expressed as the number of particles that were counted in an arbitrarily defined region of each neural crest outgrowth and covering ~0.2 mm^2, after 5 h of incubation with the competitor. Values represent the mean ± SD of a minimum of eight measurements in at least two independent experiments.
*Dilution of ascitic fluid.

To quantify the degree of aggregation induced by the peptides and the antibodies, the number of particles was counted in a representative region of the neural crest outgrowth. The surface of the region considered was ~0.2 mm^2, corresponding approximately to one-fifth to one-tenth of the total surface occupied by the neural crest population. A particle was defined as an entity that could be distinguished from its neighbors, i.e., an isolated cell, either round or spread, which showed little or no intercellular contacts with other cells, an aggregate of round cells, or a monolayer of tightly apposed cells. Table II shows the number of particles obtained with peptides and antibodies to fibronectin and to integrins at various concentrations after 5 h of incubation. This time was chosen essentially because the number of particles did not evolve signifi-

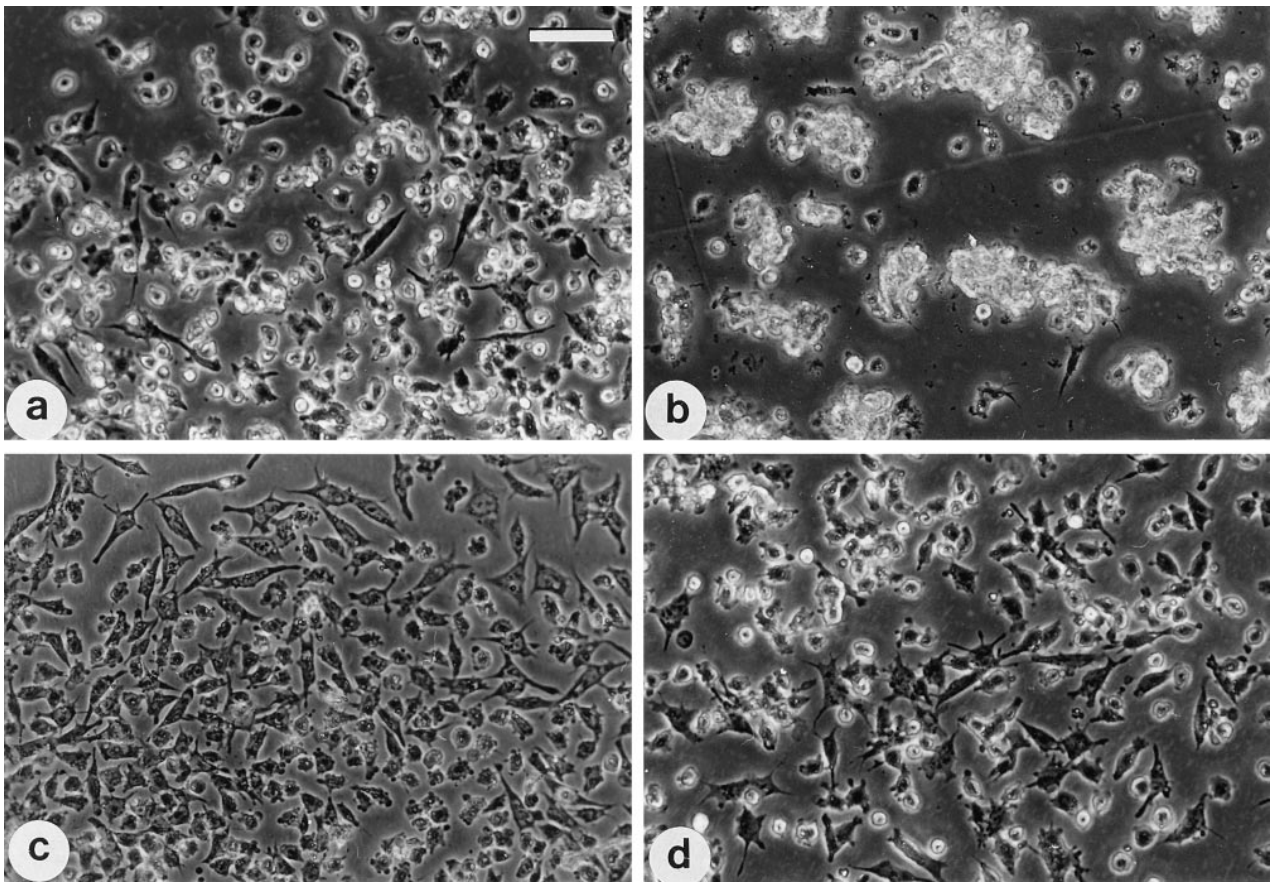

Figure 2. Neural crest cell aggregation induced by RGDS peptides and antibodies to integrins is mediated by N-cadherin. Effect of antibodies to N-cadherin on neural crest cell aggregation induced by RGDS peptides at 1 mg/ml (*a* and *b*), by mAb HP2/1 to α4 integrin subunit at 40 μg/ml (*c*), and by mAb LM609 ascite to the αVβ3 integrin at dilution 1:100 (*d*). Cells were confronted during 5 h with peptides or anti-integrin antibodies in the presence of antibodies to N-cadherin either inhibitory (A-CAM CC-11; *a*, *c*, and *d*) or noninhibitory (A-CAM ID-7.2.3; *b*), both at 5 μg/ml. Note that cells treated with both anti–N-cadherin antibodies and antibodies to α4 or αVβ3 integrins are less spread than when antibodies to N-cadherin are omitted (compare with Fig. 1, *e* and *f*). Bar, 50 μm.

cantly during the subsequent hours. About 200–250 particles were found in the absence of any competitor or in the presence of GRDGS peptides or noninhibitory antibodies (e.g., mAb ES66-8 to β1 and mAb B5G10 to α4) even at high doses. In contrast, both the RGD-containing peptides (RGDS and GPenGRGDSPCA) and inhibitory antibodies to integrins (mAb CSAT and Ab 2992 to β1, mAb HP2/1 to α4, and mAb LM609 to αvβ3) or to fibronectin (mAb 333) caused neural crest cell aggregation in a dose-dependent manner; the number of particles dropped to ~50 in the presence of the inhibitors at the highest concentrations tested.

Cell Aggregation Induced by Inhibitors of Substratum Adhesion Is Mediated by N-cadherin

We then examined whether neural crest cell aggregation upon inhibition of cell adhesion to fibronectin was mediated by N-cadherin, using both functional and immunodetection assays. Neural crest cells were confronted with RGDS peptides or antibodies to integrins in the presence of antibodies to N-cadherin either inhibitory or not. Fig. 2 shows the morphology and cohesion of cells and Table III gives the values of the numbers of particles. The inhibitory mAbs to N-cadherin tested (mAbs NCD-2 or A-CAM CC-11) severely blocked aggregation induced by the RGDS peptides in a dose-dependent manner. The number of particles systematically reached values >150 at the highest concentration of the mAbs tested. Most cells remained round and isolated, and the few clusters that could be detected were composed of no more than five cells that were loosely attached together (Fig. 2 *a*). In contrast, the noninhibitory mAb A-CAM ID-7.2.3 did not perturb cell aggregation at all (Fig. 2 *b*). The inhibitory mAbs to N-cadherin were also extremely potent in blocking cell aggregation induced by antibodies to fibronectin or to the β1 integrin subunit (not shown), by mAb HP2/1 to the α4 subunit (Fig. 2 *c*), by mAb LM609 to the αvβ3 integrin (Fig. 2 *d*), or lastly by the cyclic GPenGRGDSPCA peptide (not shown).

The surface expression of N-cadherin was analyzed by immunofluorescence. As shown previously (71), when formaldehyde fixation combined with Triton X-100 was used to reveal the insoluble pool of N-cadherin, immunoreactivity was essentially concentrated at the tip of the cell processes in regions of intercellular contacts in the absence of any adhesion inhibitor (Fig. 3 *a*). When cells were fixed in

Table III. Effect of Antibodies to N-cadherin on Neural Crest Cell Aggregation Induced by RGDS-containing Peptides or by Antibodies to Integrins

Inhibitor of cell–substratum adhesion	Antibody to N-cadherin	Antibody concentration	Number of particles
		μg/ml	
None	None	/	227 ± 12
None	A-CAM CC-11	5	250 ± 11
RGDS 1 mg/ml	None	/	55 ± 8
	NCD-2	5	151 ± 16
	A-CAM CC-11	5	219 ± 49
		1	130 ± 15
		0.25	95 ± 13
	A-CAM ID-7.2.3	5	71 ± 15
GPenGRGDSPCA 0.5 mg/ml	None	/	51 ± 10
	A-CAM CC-11	5	244 ± 27
Ab 2992 1 mg/ml	None	/	35 ± 5
	A-CAM CC-11	5	203 ± 24
mAb HP2/1 40 μg/ml	None	/	86 ± 21
	A-CAM CC-11	5	201 ± 18
mAb LM609 1/100	None	/	62 ± 16
	A-CAM CC-11	5	250 ± 30

Results are expressed as the number of particles that were counted in an arbitrarily defined region of each neural crest outgrowth after 5 h of treatment. Values represent the mean ± SD of a minimum of 10 measurements in at least two independent experiments.

cold methanol to visualize the total cellular pool of N-cadherin, a diffuse staining over the entire cell surface became also apparent (not shown, but see 71). Aggregates of cells in the presence of RGDS peptides or anti–β1 integrin antibodies showed a conspicuous staining over the entire cell surface and in regions of cell–cell contacts, both in cells fixed with methanol and with formaldehyde and Triton X-100 (Fig. 3, *b* and *c*). In cells treated with mAb HP2/1 (Fig. 3 *d*) or mAb LM609 (not shown), immunoreactivity was distributed in cell peripheries in an almost continuous belt in regions of cell–cell contacts, regardless of the type of fixation used.

The relative amounts of N-cadherin synthesized and expressed in aggregated and nonaggregated cells were evaluated by immunoblots and immunoprecipitations of [^{35}S]methionine-labeled extracts of cells using polyclonal antibodies to the cytoplasmic domain of the N-cadherin molecule. In immunoblots of total cell extracts, the antibodies recognized mainly a band of ~130 kD, corresponding to N-cadherin (36), as well as a faint band of a higher molecular mass, corresponding to its biosynthetic precursor (78). As shown in Fig. 4 *a*, lanes *1–3*, aggregated and nonaggregated neural crest cells expressed similar amounts of N-cadherin, and no difference in the apparent molecular mass of the protein was observed. Quantitation of the intensity of the bands using a PhosphorImager demonstrated that the total amount of N-cadherin was nearly identical in nontreated cells and in cells treated with RGDS peptides or antibodies to integrins. In immune precipitates of metabolically labeled cell extracts, both N-cadherin and its precursor were in similar amounts in treated and nontreated extracts (Fig. 4 *b*). Likewise, the other components that were coprecipitated with N-cadherin, including α-, β-, and γ-catenins, showed also almost identical electrophoretic patterns in all extracts. Finally, we have evaluated by immunoblotting the relative amount of N-cadherin in the Triton X-100–insoluble fraction of cells that presumably reflects the proportion of cadherin molecules associated with the cytoskeleton (1, 41, 66, 74). Consistent with the immunofluorescence studies, the relative amount of N-cadherin in the Triton X-100–insoluble fraction of cells was significantly higher in treated cells (Fig. 4 *a*, lanes *4–6*). PhosphorImager quantitation analyses of six independent experiments showed a mean increase of 36% and 54% in cells confronted with mAb CSAT and RGDS peptides, respectively. The proportion of N-cadherin molecules in the Triton X-100–insoluble pool relative to the total cellular amount of N-cadherin was estimated to be ~30–35% in untreated neural crest cells and reached 50% in cells treated with peptides or antibodies. Thus, induction of cell clustering upon inhibition of fibronectin adhesion corresponded primarily to an increase in the proportion of cadherin–catenin complexes associated with the cytoskeleton.

Cell Aggregation Requires Recruitment of an Intracellular Pool of N-cadherin Molecules

We next determined whether the increase in cell cohesion in cells treated with inhibitors of integrin function resulted from the redistribution of the preexisting N-cadherin molecules on the cell surface or from the recruitment of a cytoplasmic pool of molecules into the developing junctions. To address this question, we first analyzed the effects of agents known to affect macromolecule synthesis and secretion on the aggregation of neural crest cells. Cycloheximide, an inhibitor of protein synthesis, caused a significant decrease of cell aggregation in the presence of RGDS peptides, but only after a long preincubation period of at least 5 h (Figs. 5 *a* and 6 *a*). After shorter preincubations (1–3 h), it did not affect cell clustering at all. In contrast, brefeldin A, a potent inhibitor of protein transfer from the Golgi apparatus to the membrane, abrogated cell aggregation almost totally at concentrations above 1 μg/ml, and no preincubation with the drug was required to obtain strong inhibition (Fig. 5 *b*). Cells were round and isolated and only occasionally regrouped into clusters of less than five cells (Fig. 6 *a*). Likewise, monensin, a sodium ionophore also known to perturb glycoprotein secretion, produced the same effect as brefeldin A (Fig. 5 *c*). In agreement with previous studies (90), tunicamycin, an inhibitor of N-glycosylation, did not affect cell aggregation even at high doses (not shown). Expression of N-cadherin in cells treated with RGDS peptides in the presence of these drugs was analyzed by immunoblotting (Fig. 7 *a*). Cycloheximide caused the complete disappearance of the biosynthetic precursor of N-cadherin. In contrast, brefeldin A and monensin provoked an important increase in the amount of the precursor, probably resulting from its accumulation in the ER. When cells were confronted with RGDS peptides in the presence of brefeldin A (or monensin) combined with cycloheximide, the amount of the precursor band was considerably reduced. Interestingly, treatment with brefeldin A reduced the proportion of the N-cadherin amount in the Triton X-100–insoluble pool to levels close to those found in control cells in the absence of RGDS peptides (Fig. 7 *b*).

We also evaluated whether neural crest cell clustering could be achieved with RGDS peptides at low tempera-

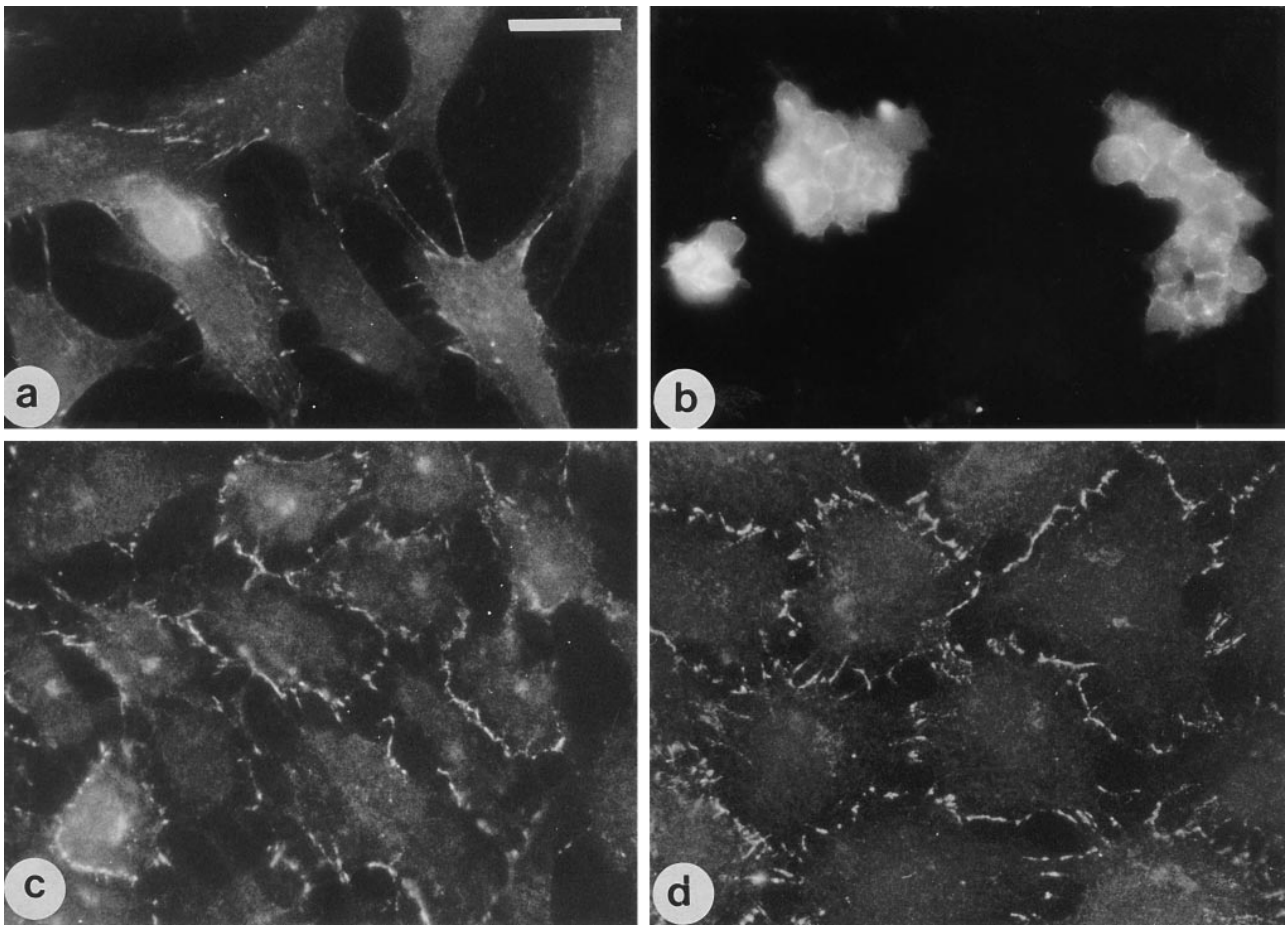

Figure 3. Immunofluorescence staining for N-cadherin on neural crest cells confronted with RGDS peptides or antibodies to integrins. Cells cultured on fibronectin were incubated for 5 h to control nonperturbing peptides (*a*), to RGDS peptides at 1 mg/ml (*b*), to mAb CSAT to β1 integrins at 20 μg/ml (*c*), or to mAb HP2/1 to α4 integrin subunit at 40 μg/ml (*d*), fixed in 3.7% formaldehyde in the presence of 0.2% Triton X-100, and processed for immunofluorescence staining. Under normal conditions, N-cadherin immunoreactivity is essentially concentrated at the tip of cell processes in contact with other cells (*arrows*). In cells aggregated by RGDS peptides, N-cadherin staining is conspicuous over most of the cell surface, particularly in regions of cell–cell contacts. In cells treated with mAb CSAT or HP2/1, staining is essentially accumulated in the cell periphery in areas of intercellular contacts. Bar, 20 μm.

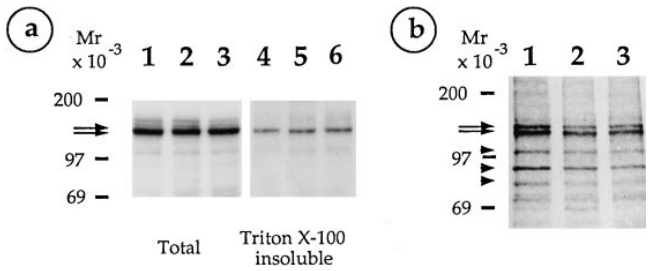

Figure 4. Immunoblot and immunoprecipitation analyses of N-cadherin expression and localization in neural crest cells using antibodies to the cytoplasmic domain of the N-cadherin molecule. (*a*) Immunoblot of total cell extracts (lanes *1–3*) and of Triton X-100–insoluble fractions (lanes *4–6*) of untreated cells (lanes *1* and *4*) and of cells confronted with the mAb CSAT anti-β1 integrin at 20 μg/ml (lanes *2* and *5*) and RGDS peptides at 1 mg/ml (lanes *3* and *6*). Equal amounts of material were loaded in lanes *1–3* and *4–6*, respectively. The relative intensities of the N-cadherin bands in the Triton X-100–insoluble fractions measured using a PhosphorImager were estimated to be 100, 133, and 145% for the untreated, mAb CSAT–treated, and RGDS peptide–treated cells, respectively. (*b*) Immunoprecipitation of extracts of metabolically labeled neural crest cells in the absence of inhibitors of substratum adhesion (lane *1*) or in the presence of the mAb CSAT anti-β1 integrin at 20 μg/ml (lane *2*) and RGDS peptides at 1 mg/ml (lane *3*). Equal amounts of material were loaded in each lane. Arrows point at N-cadherin and its biosynthetic precursor at 130 and 135 kD, respectively, and arrowheads at α-, β-, and γ-catenins at ~105, 95, and 85 kD. Positions of molecular mass markers are indicated on the left.

tures (i.e., below 20°C), as it is known that cytoplasmic vesicular traffic and protein secretions are considerably reduced at those temperatures. Cells incubated at 18–20°C in the absence of peptides remained attached to the substratum and, although they showed a slight retraction of cellular extensions, they did not regroup into clusters (Figs. 5 *d* and 6 *c*). Cells treated for 5 h with RGD peptides at the same temperatures did not regroup into multicellular aggregates, and values for the number of particles were comparable to controls in the absence of peptides (Figs. 5 *d*

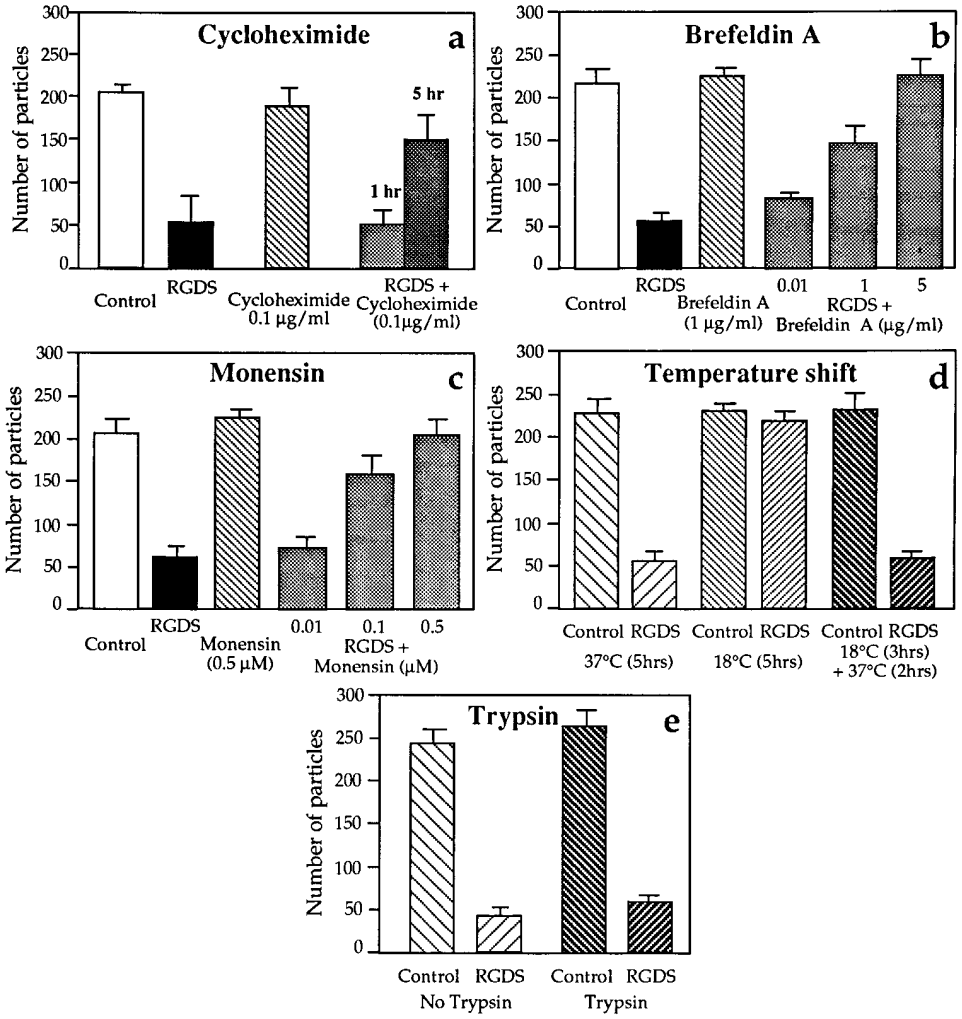

Figure 5. Neural crest cell aggregation is mediated by N-cadherin molecules recruited from an intracellular pool. (*a–c*) Effects of inhibitors of macromolecule synthesis (cycloheximide; *a*) and secretion (brefeldin and monensin; *b* and *c*) on neural crest cell aggregation induced by RGDS peptides at 1 mg/ml. Cells were preincubated with cycloheximide for 1–5 h or with brefeldin A or monensin for 30 min, before addition of the peptides for the subsequent 5 h. (*d*) Effect of low temperatures on neural crest cell aggregation. Cells were confronted with RGDS peptides at 1 mg/ml at 37° or 18°C for 5 h or were incubated first at 18°C in the presence or not of the peptides for 3 h followed by a 2-h incubation at 37°C. (*e*) Effect of removal of the surface pool of N-cadherin molecules on neural crest cell aggregation. Cells were treated or not for 5 min with a trypsin solution at 0.001% in Ca^{2+}-free medium before addition of the RGDS peptides at 1 μg/ml for the subsequent 5 h. Results are expressed as the number of particles that were counted in an arbitrarily defined region of each neural crest outgrowth and covering ~0.2 mm^2. Values represent the mean ± SD of a minimum of eight measurements in at least two independent experiments.

and 6 *d*). However, when cells were shifted to 37°C after a preincubation period of 3 h at 18°C in the presence of peptides, aggregation was restored to levels normally obtained when cells were continuously treated at 37°C (Figs. 5 *d* and 6 *f*). In contrast, in the absence of peptides, cells shifted from 18° to 37°C regained their flattened morphology and did not form clusters (Figs. 5 *d* and 6 *e*).

Finally, to further demonstrate that the preexisting pool of surface N-cadherin molecules is dispensable for neural crest cell aggregation caused by RGDS peptides, we analyzed the effect of these peptides on cells that have been briefly treated with trypsin to remove surface N-cadherin molecules. Cells were incubated for 5–10 min with trypsin at low concentrations (0.001%) in Ca^{2+}-free medium, followed by incubation with 0.01% soybean trypsin inhibitor, a treatment sufficient to remove the bulk of surface cadherin molecules without damaging other surface molecules (94). In addition, to prevent extensive inactivation of integrins and cell detachment from the substratum, Mg^{2+} at 2 mM was added to the trypsin solution. Under these conditions, cells remained spread onto the bottom of the dish and cell processes retracted only slightly (Fig. 8 *a*). The reduction in surface N-cadherin after trypsin treatment was assessed both by immunostaining of cells and by immunoblotting. By immunofluorescence, N-cadherin staining was almost undetectable on the cells' surface and was confined intracellularly to perinuclear vesicles (Fig. 8 *b*, compare with Fig. 3 *a*). PhosphorImager quantitation analyses of immunoblots of total cell extracts showed a 80% reduction in the overall amount of N-cadherin, the remaining 20% corresponding possibly to the intracellular pool of N-cadherin molecules inaccessible to the enzyme treatment (not shown). When confronted with RGDS peptides at 1 mg/ml, neural crest cells treated with trypsin formed aggregates of sizes very similar to those in untreated cells (Figs. 5 *e* and 8 *c*), and we did not observe any delay in cell clustering.

N-cadherin Function in Neural Crest Cells Is Dependent upon Transmembrane Ca^{2+} Fluxes and Activity of Kinases

The results described above show that inhibition of integrin activity by adhesion blockers causes rapid cell–cell

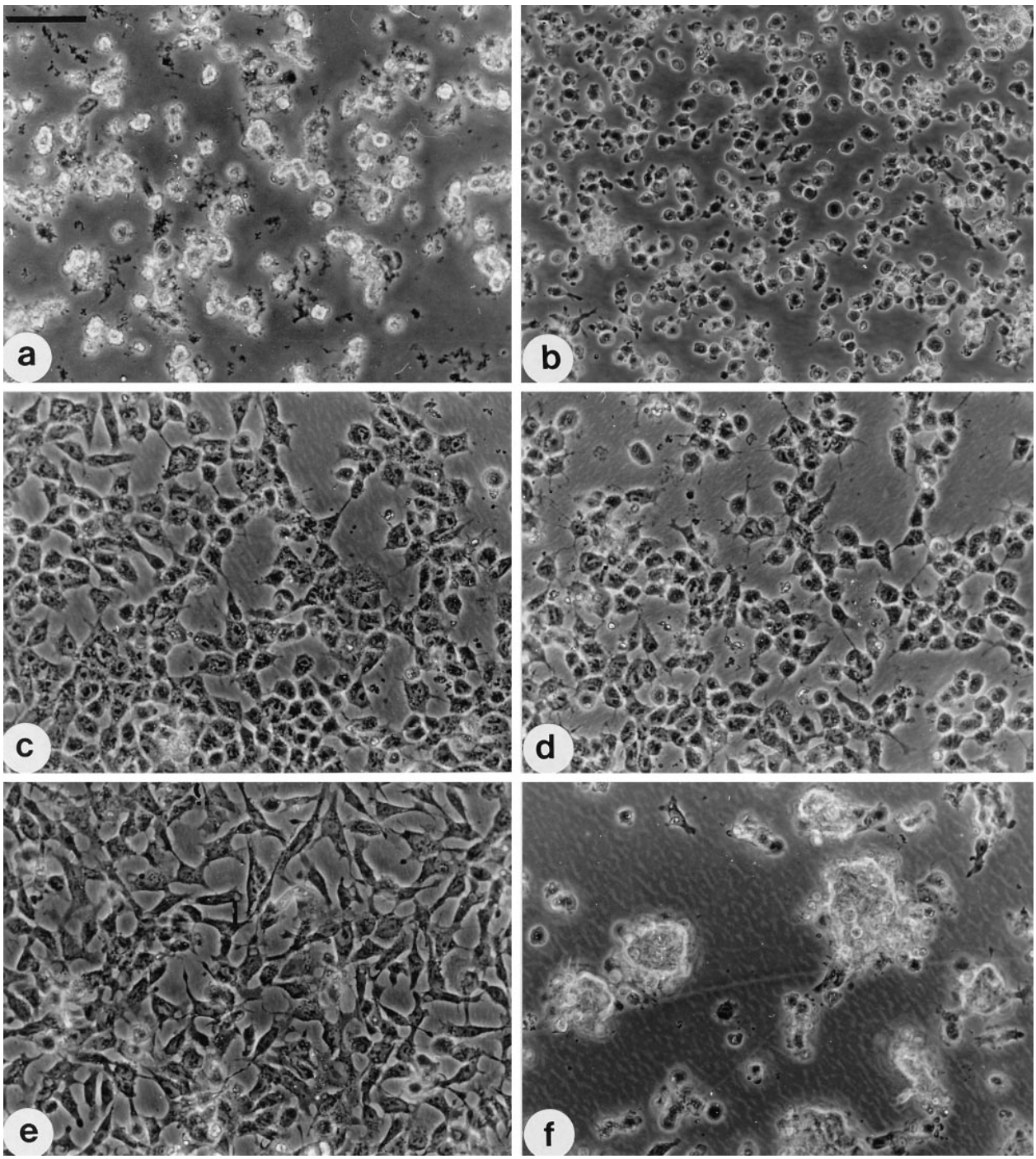

Figure 6. (*a* and *b*) Cell–cell interactions among cells confronted during 5 h with peptides in the presence of cycloheximide at 0.1 mg/ml (*a*) and brefeldin A at 1 μg/ml (*b*). In the case of cycloheximide, cells were preincubated with the drug for ~5 h before addition of the peptide. (*c–f*) Cell–cell interactions among cells treated (*c*) or not (*d*) with RGDS peptides at 18°C during 5 h and among cells treated first at 18°C during 3 h and then at 37°C during 2 h either in the absence (*e*) or presence (*f*) of RGDS peptides at 1 mg/ml. Bar, 50 μm.

aggregation mediated by N-cadherin molecules that are recruited from a trypsin-resistant pool that is likely translocated to the cell surface and presumably associated with the actin cytoskeleton. We have shown previously that, in motile neural crest cells, N-cadherin–based junctions can be restored upon inhibition of tyrosine kinases, phosphotyrosine phosphatases, and protein kinases C, suggesting that, during migration, N-cadherin activity is repressed by a cascade of intracellular signaling events (71). To determine which signals are elicited by integrins, we have analyzed the effects of agents known to interfere specifically with signaling pathways on the neural crest cell aggregation induced by RGDS peptides or antibodies to integrins. Agents were also tested in the absence of these adhesion blockers.

As described previously (71), inhibitors of tyrosine ki-

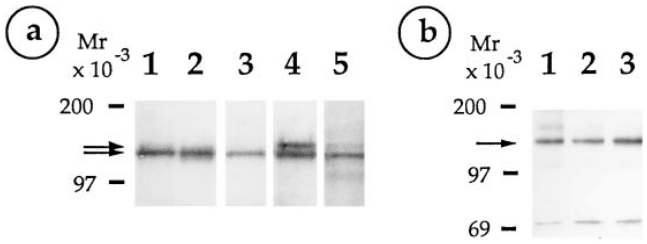

Figure 7. Immunoblotting analyses of N-cadherin expression and localization in cells treated with inhibitors of protein synthesis and secretion using antibodies to the cytoplasmic domain of the N-cadherin molecule. (*a*) Total cell extracts of untreated cells (lane *1*) and of cells treated with RGDS peptides at 1 mg/ml alone (lane *2*) or in combination with cycloheximide at 0.1 μg/ml (lane *3*), brefeldin A at 1 μg/ml (lane *4*), and brefeldin A plus cycloheximide (lane *5*). (*b*) Triton X-100–insoluble fractions of untreated cells (lane *1*) and of cells treated with RGDS peptides at 1 mg/ml in combination with brefeldin A at 1 μg/ml (lane *2*) or alone (lane *3*). The relative intensities of the N-cadherin bands in the Triton X-100–insoluble fractions measured using a PhosphorImager were estimated to be 100, 108, and 161% for lanes *1*, *2*, and *3*, respectively. Arrows point at N-cadherin and its biosynthetic precursor at 130 and 135 kD. Positions of molecular mass markers are indicated on the left. Equal amounts of material were loaded in each lane in *a* and *b*, respectively.

nases, such as herbimycin, tyrphostins, or erbstatin analog, and inhibitors of protein kinases C, such as bisindolylmaleimide and sphingosine, all produced rapid and strong clustering of neural crest cells in the absence of RGDS peptides or anti-integrin antibodies. In addition, these agents neither amplified nor diminished peptide- or antibody-induced cell aggregation (not shown). On the other hand, phorbol esters, which activate protein kinases C, and inhibitors of calmodulin-dependent kinases, such as calmidazolium and trifluoperazine, did not interfere with the effect of RGDS peptides on neural crest cells nor did they provoke cell aggregation when applied alone (not shown).

In contrast, the Ca^{2+} ionophores, ionomycin and the compound A23187, were both found to inhibit in a dose-dependent manner the RGDS peptide-induced aggregation of neural crest cells (Fig. 9, *a* and *b*). This inhibitory effect was apparently restricted to Ca^{2+} ionophores, as valinomycin, a potassium ionophore, was ineffective on neural crest cell aggregation at all the concentrations tested (not shown). At high concentrations, ionomycin and A23187 totally abrogated cell aggregation and, at low concentrations, both compounds still diminished cell aggregation significantly (Fig. 9, *a* and *b*). In the presence of these drugs, neural crest cells treated with RGDS peptides were round and isolated and, when present, clusters of cells were composed of a few individuals (Fig. 10 *a*). Ionomycin also blocked in a dose-dependent manner clustering of neural crest cells in the presence of cyclic GpenGRGDSPCA peptides and of antibodies to β1 integrins, to the α4 subunit or to αvβ3 (Fig. 9 *a*). Interestingly, in the absence of RGDS peptides or of antibodies to integrins, ionomycin and A23187 reduced significantly the degree of cell cohesion among the neural crest cell population (Figs. 9, *a* and *b*, and 10 *b*).

As Ca^{2+} ionophores severely reduced neural crest cell aggregation, we tested whether agents known for blocking Ca^{2+} channels would produce the opposite effect in the absence of peptides or antibodies to integrins. La^{3+}, a commonly used blocker of voltage-independent Ca^{2+} channels, could not be used because it precipitated phosphate from the culture medium and neural crest cells did not survive long in phosphate-depleted media. However, as shown in Fig. 9 *c*, Ni^{2+}, another blocker of voltage-independent Ca^{2+} channels, provoked a rapid cell aggregation among the neural crest population at concentrations between 0.5 and 5 mM. In the presence of 2.5 mM Ni^{2+}, for example, cells remained spread but with few cellular processes, and they formed extensive intercellular contacts as in epithelial sheets (Fig. 10 *c*). This clustering effect of Ni^{2+} on neural crest cells could be abolished by antibodies to N-cadherin (Fig. 9 *c*). In contrast, none of various inhibitors of voltage-dependent Ca^{2+} channels tested, like nifedipine (Figs. 9 *d* and 10 *d*) and verapamil (data not shown), induced aggregation of neural crest cells. These data therefore suggest that the control of N-cadherin activity in neural crest cells involves voltage-independent, receptor-activated Ca^{2+} channels. To further confirm this hypothesis, we evaluated the effect of thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase known to cause a transient increase of cytosolic free Ca^{2+} without involvement of extracellular Ca^{2+}, hydrolysis of phosphoinositides, and activation of protein kinases C (98). We found that thapsigargin did not perturb aggregation of neural crest cells at all the concentrations tested (Fig. 9 *e*).

Expression of N-cadherin on neural crest cells confronted with RGDS peptides in the presence of ionomycin (or A23187) was further analyzed by immunoblotting, immunoprecipitation, and immunofluorescence. Ionomycin did not affect the overall content of N-cadherin in cells treated with RGDS (Fig. 11 *a*). Consistent with this finding, by immunofluorescence, we observed no major change in the intensity of N-cadherin immunoreactivity on neural crest cells with RGDS peptides plus ionomycin (Fig. 10 *e*), compared with peptides alone (Fig. 3 *b*). Isolated round cells showed a strong staining on their surface. However, ionomycin significantly reduced the relative amount of N-cadherin in the Triton X-100–insoluble fraction of neural crest cells, even in cells that were not treated with RGDS peptides (Fig. 11 *b*). Quantitative analyses using a PhosphorImager revealed that the N-cadherin content in the Triton X-100–insoluble pool of cells with ionomycin is ~80% of that in control cells. This reduction was also observed in immunofluorescence studies showing an almost complete absence of intercellular contacts in cells treated with ionomycin alone (Fig. 10 *f*).

Elevation of the intracellular Ca^{2+} concentration is known to initiate a complex series of cytoplasmic signaling events, among which activation of the calmodulin-dependent serine-threonine kinases and the phosphatase calcineurin is most important. To investigate which downstream events might be activated after Ca^{2+} influxes in neural crest cells, we have tested whether the effect of ionomycin on RGDS peptide-induced neural crest cell aggregation could be challenged by addition of various kinase inhibitors. Neural crest cells were first confronted with RGDS peptides in the presence of ionomycin for ~3 h, i.e., until cells were clearly round and isolated, kinase in-

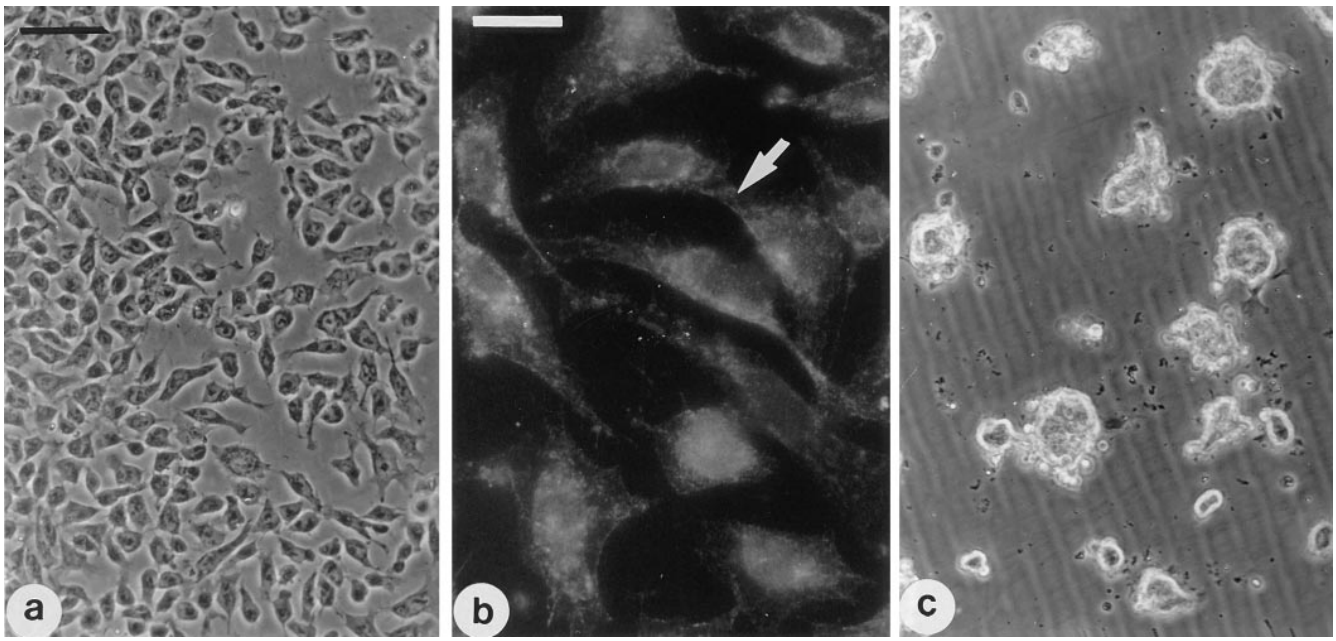

Figure 8. Effect of removal of the surface pool of N-cadherin molecules on neural crest cell aggregation. (*a* and *b*) Morphology of neural crest cells (*a*) and immunofluorescence staining for N-cadherin on neural crest cells (*b*), immediately after a 5-min treatment with a trypsin solution at 0.001% in Ca^{2+}-free medium. N-cadherin staining has almost entirely disappeared from the cell surface including from intercellular contacts (*arrow*) and is only detectable intracellularly. (*c*) Cell–cell interactions among neural crest cells confronted for 5 h with RGDS peptides at 1 mg/ml after trypsin treatment. Note that cells form aggregates as compact as untreated cells. Bars: (*a*) 50 μm; (*b*) 20 μm.

hibitors were then added, and the intercellular contacts were scored during the subsequent hours. As shown in Table IV, staurosporine and H7, which affect a broad spectrum of serine-threonine kinases, were able to significantly reverse the effect of ionomycin on neural crest cells. In contrast, ionomycin effect could not be abolished by addition of inhibitors of tyrosine kinases or of protein kinases C alone.

Cell Aggregation Is Correlated with Changes in Catenin Phosphorylation

Elevation of tyrosine phosphorylation of constituents of adherens junctions, including β-catenin and cadherins, has been proposed to cause alterations in their stability (5, 35, 65, 92, 108, 109). We then examined whether phosphorylation of N-cadherin and catenin molecules was modified in neural crest cells upon treatment with RGDS peptides. Cells incubated or not with peptides were metabolically labeled with ^{32}P, extracted with detergents, and subjected to immunoprecipitations using antibodies to the cytoplasmic domain of N-cadherin. As shown on Fig. 12 *a*, N-cadherin was phosphorylated in neural crest cells whether or not they were confronted with peptides. Quantitation of the radioactivity incorporated into the bands with a PhosphorImager revealed that the level of phosphorylation of N-cadherin was not significantly different in RGDS-treated and untreated cells. In contrast, a number of other bands that coprecipitated with N-cadherin, and more particularly β- and γ-catenins and a band of ~180 kD, were phosphorylated strongly in cells treated with RGD peptides and only poorly in untreated cells. We next analyzed the tyrosine-specific phosphorylation of N-cadherin and catenins. N-cadherin–catenin complexes were immunoprecipitated using antibodies to N-cadherin and analyzed for reactivity to anti-phosphotyrosine antibodies by immunoblotting (Fig. 12 *b*). No bands corresponding to N-cadherin, catenins, or the 180-kD protein reacted with the antibodies to phosphotyrosine in extracts of both RGDS-treated and untreated cells, suggesting that elevation of phosphorylation upon RGDS peptide treatment occurred primarily on serine and threonine residues.

Discussion

Descriptive studies of the expression patterns of cell-to-cell and cell-to-substratum adhesion molecules during the development of the neural crest have revealed precisely coordinated and dynamic modulations in the repertoire of adhesion systems both at initiation and cessation of cell migration (see for reviews 26, 28, 46, 58). Recent functional studies further demonstrated that, at the onset of migration, neural crest cells gradually acquire cell-to-substratum adhesion mediated by integrins and that, conversely, N-cadherin–based cell–cell contacts are reduced as a result of intracellular processes involving signaling events (19, 71, 76). However, the regulatory mechanisms that both spatially and temporally control the occurrence of these changes are poorly understood. In particular, the inverse correlation between the activity of N-cadherin and the migratory behavior of cells raises the important, as yet unanswered question as to whether the cell–cell and cell–substratum adhesion systems in neural crest cells are regu-

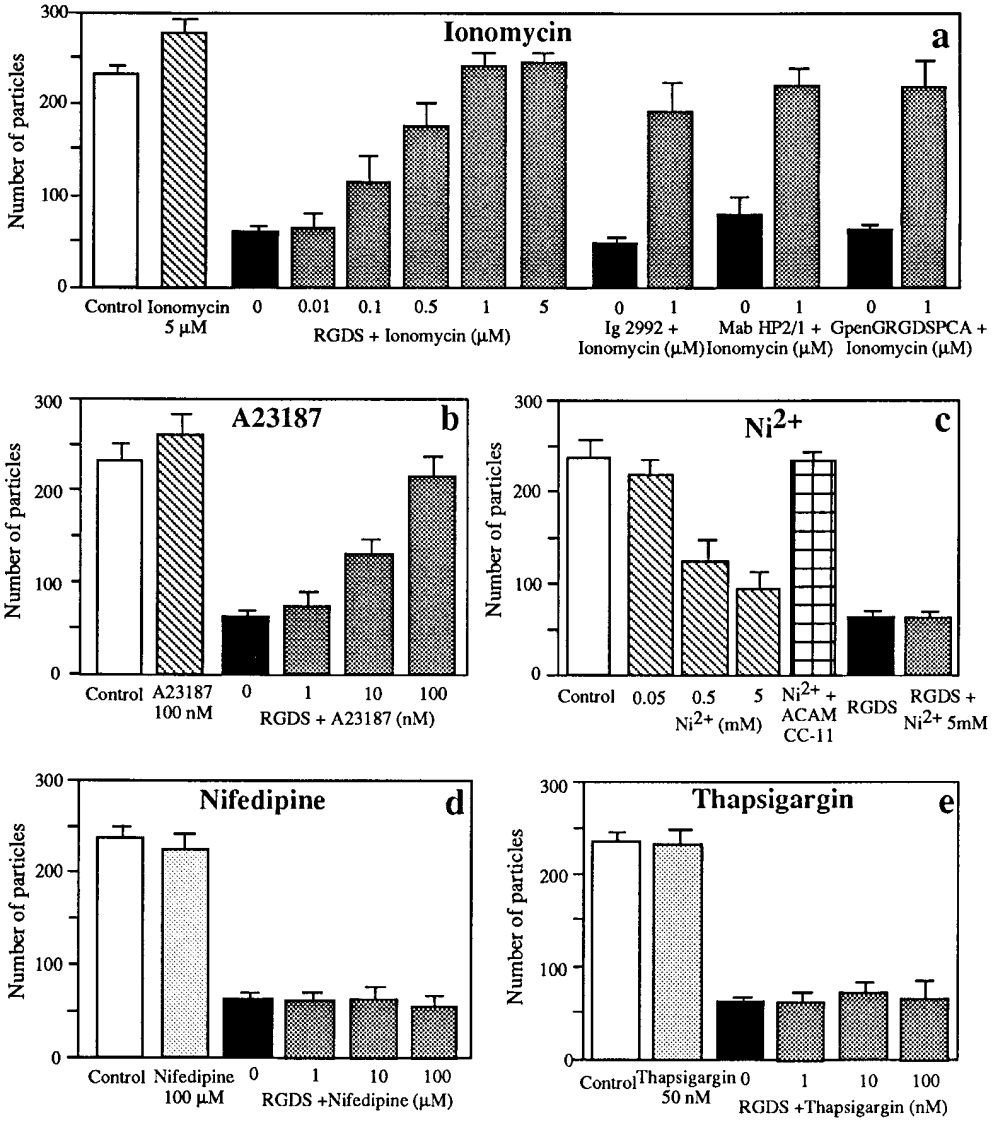

Figure 9. Quantitation of the effect of agents that affect transmembrane Ca^{2+} fluxes and intracellular Ca^{2+} concentration on the aggregation of neural crest cells. In *c*, the mAb A-CAM CC-11 was applied at the concentration of 5 μg/ml. Results are expressed as the number of particles that were counted in an arbitrarily defined region of each neural crest outgrowth and covering ~0.2 mm^2, after 5 h of incubation with the agent combined or not with RGDS peptides and antibodies to integrins. Values represent the mean ± SD of a minimum of eight measurements in at least two independent experiments.

lated separately by distinct mechanisms or, alternatively, if they belong to a cascade of tightly connected, interdependent events. In the present study, to address this question of the possible inter relationship between the cell–cell and cell–substratum adhesion systems, we investigated whether repression of N-cadherin function in neural crest cells might be directly related to the integrin-dependent migratory process or if this event requires additional signals independently of cell motion. Although they do not exclude the possible implication of external factors, our data are in favor of a direct, negative control of the surface distribution and activity of N-cadherin by intracellular signals elicited by integrins during cell migration, thus exemplifying possible cross talk mechanisms between cell–cell and cell–substratum adhesion systems in neural crest cells.

Formation of adherens junctions is a complex, multistep process that has been much studied in epithelial MDCK cells (1, 41, 66, 74). In these cells, E-cadherin and β-catenin are first assembled in the ER and progressively exported to the plasma membrane where they become soon associated with α-catenin. While the synthesis of N-cadherin and formation of cadherin–catenin complexes in migrating neural crest cells does not differ significantly from that of MDCK cells or of other nonmotile cells, there appear to be major differences in the stabilization of the developing contacts. In stationary MDCK cells, the E-cadherin–catenin complexes are almost immediately and entirely incorporated into adherens junctions in association with the cytoskeleton upon arrival at the plasma membrane, providing rapid initiation of tight cell–cell interactions. In neural crest cells, in contrast, the bulk of the N-cadherin–catenin complexes is kept excluded from adherens junctions, with only ~30% of the cadherin molecules being incorporated into the Triton X-100–insoluble fraction, thus resulting in caracteristically unstable intercellular contacts. In the presence of RGDS peptides or anti-integrin antibodies, while the overall surface amount of N-cadherin is not substantially modified, the proportion of N-cadherin molecules in the Triton-X-100–insoluble cellular fraction is significantly increased. In addition, inhibition of intracellular vesicular traffic abrogated cell aggregation. These data suggest that, upon addition of in-

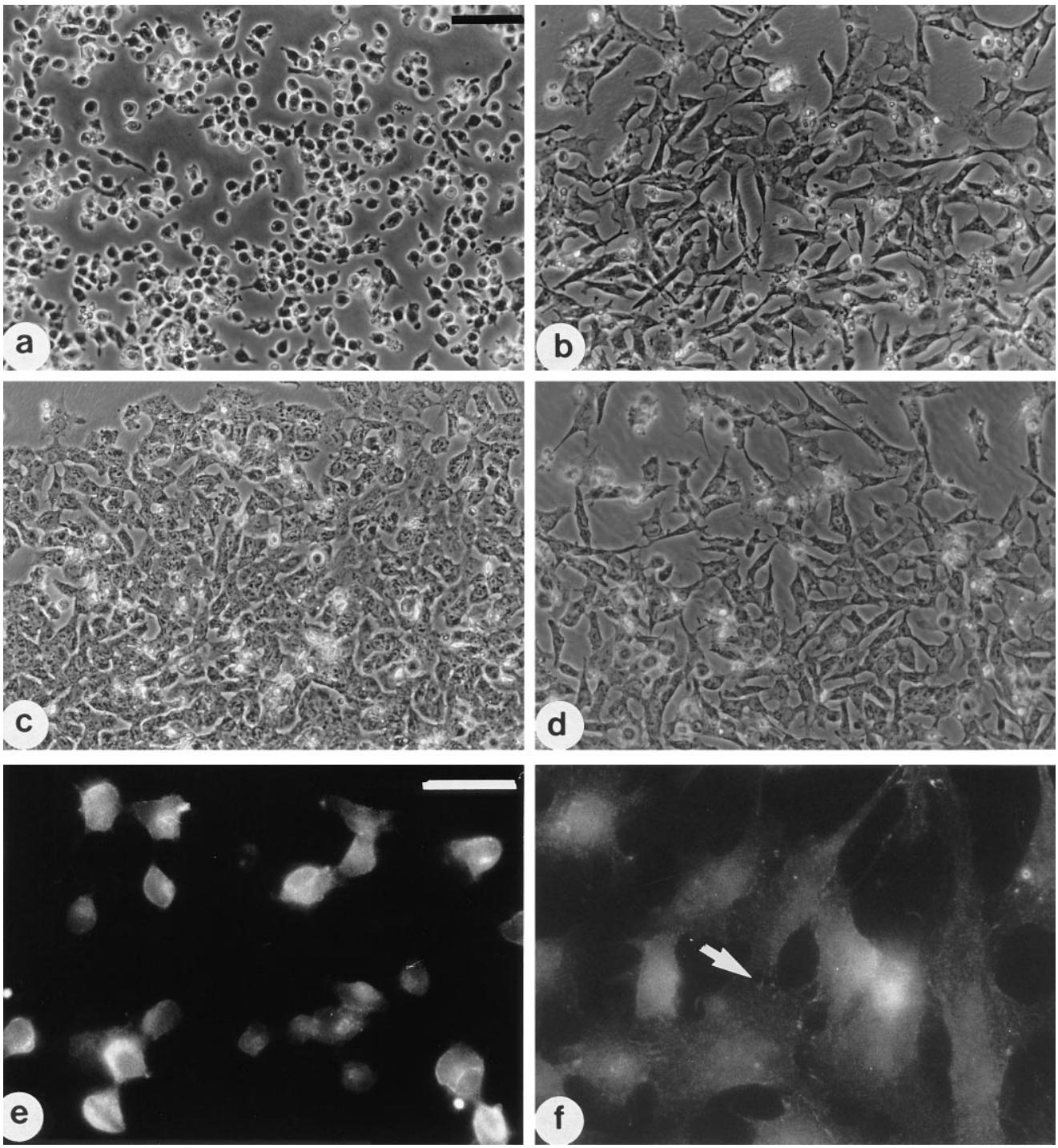

Figure 10. (*a* and *b*) Effect of the Ca^{2+} ionophore ionomycin on neural crest cell aggregation induced by RGDS peptides at 1 mg/ml. Cells were confronted during 5 h with ionomycin at 1 μM in the presence (*a*) or absence (*b*) of RGDS peptides at 1 mg/ml. (*c* and *d*) Effects of Ni^{2+}, a blocker of voltage-independent Ca^{2+} channels (*c*), and nifedipine, a blocker of voltage-dependent Ca^{2+} channels (*d*), on neural crest cell cohesion in the absence of RGDS peptides after 5 h of treatment. (*e* and *f*) Immunofluorescence staining for N-cadherin on neural crest cells treated with ionomycin at 1 μM in the presence (*e*) or absence (*f*) of RGDS peptides. Note that N-cadherin staining has almost entirely disappeared from the regions of cell–cell contacts in cells treated with ionomycin alone (*arrow*). Bars: (*a*) 50 μm; (*e*) 20 μm.

hibitors of substratum adhesion, newly synthesized N-cadherin–catenin complexes are preferably targeted into the transient cell–cell contacts, which in turn become stabilized. Most importantly, the preexisting pool of N-cadherin molecules that are initially diffuse over the cell surface is apparently not recruited to adherens junctions when RGDS peptides or anti-integrin antibodies are added, thus implying that these molecules may be possibly irreversibly inactivated. Therefore, our data raise the intriguing possibility that, in neural crest cells, integrins might regulate

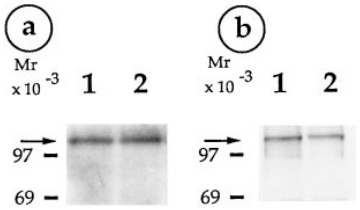

Figure 11. Immunoblotting analyses of N-cadherin expression and localization in cells treated with Ca^{2+} ionophores using antibodies to the cytoplasmic domain of the N-cadherin molecule. (*a*) Total cell extracts of untreated cells (lane *1*) and of cells treated with RGDS peptides at 1 mg/ml in combination with ionomycin at 1 μM (lane *2*). (*b*) Triton X-100–insoluble fractions of untreated cells (lane *1*) and of cells treated with ionomycin at 1 μM (lane *2*). The relative intensities of the N-cadherin bands in the Triton X-100–insoluble fractions measured using a PhosphorImager were estimated to be 100 and 85% for the untreated and the ionomycin-treated cells, respectively. Arrows point at N-cadherin at 130 kD. Positions of molecular mass markers are indicated on the left. Equal amounts of material were loaded in each lane in *a* and *b*, respectively.

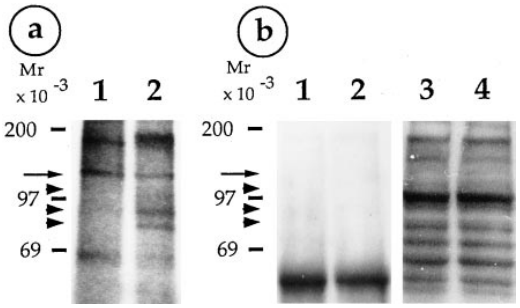

Figure 12. Analysis of N-cadherin phosphorylation in neural crest cells confronted with RGDS peptides. (*a*) Immunoprecipitation of total cell extracts of ^{32}P-labeled untreated neural crest cells (lane *1*) and cells treated with RGDS peptides at 1 mg/ml (lane *2*) using antibodies to the cytoplasmic domain of the N-cadherin molecule. (*b*) Tyrosine phosphorylation of N-cadherin in neural crest cells. Extracts of untreated (lane *1*) and RGDS-treated (lane *2*) neural crest cells first were immunoprecipitated with antibodies to the cytoplasmic domain of N-cadherin, and then were analyzed for tyrosine phosphorylation using immunoblotting with antibodies to phosphotyrosine. (Lanes *3* and *4*) Total extracts of untreated and RGDS-treated cells, respectively, subjected to immunoblotting using antibodies to phosphotyrosine. The arrow points at N-cadherin, and arrowheads point at α-, β-, and γ-catenins at ~105, 95, and 85 kD. Positions of molecular mass markers are indicated on the left. Equal amounts of material were loaded in each lane in *a* and *b*, respectively.

N-cadherin activity in an exquisite manner that would essentially consist of keeping cell–cell contacts transient by preventing accumulation of cadherin–catenin complexes into these contacts.

The integrin-dependent control of N-cadherin activity in migrating neural crest cells appeared to involve intracellular signaling events. More specifically, the effects of agents selected for elevating the intracellular Ca^{2+} concentration or for affecting Ca^{2+} channels suggest that transmembrane Ca^{2+} fluxes across the plasma membrane may be a major step in the signaling cascade involved in the repression of cadherin function. Transient increase in the intracellular Ca^{2+} concentration is one of the numerous signal transduction pathways that are triggered by integrin engagement (see for reviews 18, 52, 91, 112). In endothelial cells, for example, this rise in intracellular Ca^{2+} occurs upon adhesion to fibronectin and vitronectin but not to collagen, and it is mediated by αv integrins (84, 85). In addition, the integrin-associated protein, a 50-kD protein physically associated with the αvβ3 integrin, is apparently required for this event (15, 87). Our finding that blocking αvβ3 integrin with specific inhibitory antibodies causes rapid neural crest cell clustering therefore suggests that this particular integrin might be responsible for the transmembrane Ca^{2+} fluxes that would initiate the cascade of events that ultimately repress N-cadherin activity. Whether integrin-associated protein or another mechanism coupling integrins to calcium signaling is involved in this process remains, however, to be determined (91).

Table IV. Reversal of Ionomycin Effect by Addition of Kinase Inhibitors

Initial treatment	Kinase inhibitor	Number of particles
None	None	210 ± 20
RGDS 1 mg/ml	None	40 ± 8
RGDS + ionomycin 1 μM	None	240 ± 17
None	Staurosporine 100 nM	68 ± 6
RGDS + ionomycin	Staurosporine 100 nM	111 ± 20
None	H7 250 μM	77 ± 17
RGDS + ionomycin	H7 250 μM	98 ± 15
None	Sphingosine 25 μM	52 ± 7
RGDS + ionomycin	Sphingosine 25 μM	220 ± 41
None	Bisindolylmaleimide 25 μM	70 ± 5
RGDS + ionomycin	Bisindolylmaleimide 25 μM	196 ± 22
None	Herbimycin 2 μg/ml	24 ± 19
RGDS + ionomycin	Herbimycin 2 μg/ml	220 ± 40

Neural crest cells were first treated with RGDS peptides at 1 mg/ml combined with ionomycin at 1 μM until they became round and isolated (designated as initial treatment), inhibitors of kinases were then added, and the cohesion of the cell population was analyzed during the following 3–5 h. Results are expressed as the number of particles that were counted in an arbitrarily defined region of each neural crest outgrowth after 5 h of treatment. Values represent the mean ± SD of a minimum of 10 measurements in at least two independent experiments.

Interestingly, it has been shown that the αvβ3 integrin-mediated Ca^{2+} influxes observed in endothelial cells and in neutrophils are required for cell locomotion on vitronectin and fibronectin but are dispensable for cell spreading (57, 63, 85). This would then suggest that the control of N-cadherin activity by integrin-dependent signals in neural crest cells may be intimately and specifically related to cell motion. Consistent with this finding, aggregation of neural crest cells was achieved upon inhibition of α4β1 and αvβ3, which are precisely the major integrins implicated in the dynamics of cell locomotion on fibronectin and vitronectin in neural crest cells as well as in various other cell types (e.g., see 16, 20, 27, 57; Delannet, M., S. Testaz, and J.-L. Duband, manuscript in preparation). In addition, noninhibitory antibodies to integrins were systematically ineffective in inducing cell aggregation, thus ruling out the possibility that cell aggregation might result from the clustering effect of the antibodies, known to activate a number of the integrin-dependent intracellular sig-

naling events (68, 69). On the other hand, neural crest cells cultured on high affinity substrata composed of antibodies to the β1 integrin subunit or migrating in vitro from immature neural tube explants exhibit reduced locomotory competence, move as a cohesive cell sheet, and form extensive cell–cell contacts essentially as a result of the inability of cells to detach from the substratum (19, 24). Interestingly, in contrast with nonmotile cells that display broad and stable adherens junctions while being firmly anchored to the extracellular matrix (21, 71), transformed cells and tumor cells also maintain reduced cell–cell contacts while showing active integrin-dependent migratory properties (see for reviews 9, 95, 105), suggesting that control of cadherin activity by integrins might be a general feature common to all motile cells.

We tentatively searched for the intracellular events that are secondarily activated after Ca^{2+} entry through the membrane in neural crest cells. We found that inhibitors of serine-threonine kinases with limited selectivity such as H7 and staurosporine but not by inhibitors of protein kinases C, such as bisindolylmaleimide, could reverse to some extent the effect of the Ca^{2+} ionophore ionomycin on neural crest cell aggregation, meaning that the propagation of the Ca^{2+} signal involves kinases distinct from protein kinases C. Other known targets of Ca^{2+}, such as the Ca^{2+}-dependent serine-threonine phosphatase calcineurin, are likely to be also triggered in neural crest cells (see below), but this has not been investigated in the present study. Inhibitors of tyrosine kinases, such as herbimycin, could not, in contrast, challenge the effect of Ca^{2+} ionophores. However, tyrosine kinases and phosphotyrosine phosphatases have been shown previously to participate in the regulation of N-cadherin–based junctions in migrating neural crest cells (71). A plausible explanation is that the activity of these tyrosine kinases and phosphotyrosine phosphatases may not be directly connected with integrin signals, but instead would be driven by other surface receptors. The nature of these receptors remains to be determined, but receptors for growth factors with tyrosine kinase activity are likely candidates. Several recent studies have indeed demonstrated the linkage of cadherins to signaling pathways elicited by receptors for the EGF, the hepatocyte growth factor/scatter factor, and Wnt-1 (42, 45, 88). Therefore, N-cadherin activity in neural crest cells would possibly be under the dual control of integrins and growth factor receptors. Synergistic interactions between signals originating from matrix and growth factor receptors have been found previously to be critical for the occurrence of a variety of cellular events such as cell proliferation and cell migration (e.g., see 51, 70, 86).

In an attempt to determine how, at the molecular level, N-cadherin activity is restored in neural crest cells, we searched for possible changes in the phosphorylation level of N-cadherin molecules during aggregation, and we found that β- and γ-catenins, as well as a 180-kD protein, became heavily phosphorylated most likely on serine-threonine residues in aggregated cells. This situation is in striking contrast with the one found in transformed cells. In these cells, inactivation of cadherin molecules was found to be accompanied by elevated tyrosine phosphorylation of catenins (5, 35, 49, 65, 92, 108, 109), whereas, in neural crest cells, activation correlates with increased serine/threonine phosphorylation of the same set of molecules. Thus, β- and γ-catenins would possibly exist under three different forms differing in their level of phosphorylation: an "active" form phosphorylated essentially on serine and threonine residues, which is incorporated into stable associations with the actin cytoskeleton, and two "inactive" forms that are not found in stable contacts and that are either hypophosphorylated or hyperphosphorylated on tyrosine residues. Moreover, this would suggest that the intimate mechanisms of regulation of cadherin activity, and more broadly of cell adhesion, might be fundamentally different in normal, embryonic cells and in transformed cells, and that abnormal tyrosine phosphorylation of surface proteins causing their inactivation may be specific for virally infected cells. On the other hand, the nature of the 180-kD protein associated with N-cadherin has not been determined yet, but a protein exhibiting a similar molecular mass and identified as the EGF receptor has been described in immune precipitates of MDCK cells and human A431 carcinoma cells using antibodies to E-cadherin and to catenins (e.g., see 41, 45). In this event, our observation would raise the intriguing possibility that changes in the activity of integrins would affect the level of phosphorylation and eventually the activity of the EGF receptor in neural crest cells.

The absence of phosphorylation of catenins in migrating neural crest cells is likely to result from the activity of the Ca^{2+}-dependent serine-threonine phosphatase calcineurin activated by integrin-mediated Ca^{2+} influxes. Most interestingly, calcineurin has also been found to be implicated in the regulation of cell-to-substratum adhesion in CHO cells and in migrating neutrophils (for reviews see 39, 80, 91). In CHO cells, inhibition of calcineurin activity was found to abolish interaction between the α5β1 integrin and fibronectin in an in vitro assay (80). In motile neutrophils, in contrast, inhibition of Ca^{2+} entry or of calcineurin activity was found to reduce cell motility merely because it prevented cell detachment from the substratum and not cell attachment or spreading (39). Thus, in these cells, calcineurin inhibition provokes accumulation of the αvβ3 integrin to the ventral surface of the cell at the trailing edge, suggesting that its function would consist chiefly in down-regulating binding of αvβ3 integrin to the substratum (55). In this context, the αvβ3 integrin can be regarded in motile cells, such as neural crest cells and neutrophils, as a key regulatory element that would be at the origin of a cascade of signals involving Ca^{2+} fluxes and that would control both cell release from the substratum and prevention of intercellular contacts, two critical events necessary for active cell locomotion. In contrast with αvβ3, the precise role of the α4β1 integrin in the control of cadherin remains unclear. So far, Ca^{2+} influxes have never been described after engagement of α4β1. Nevertheless, ionomycin could abolish almost entirely the aggregation effect of the antibodies to α4β1. It should be stressed that aggregation produced by antibodies to α4β1 was significantly delayed as compared with that obtained with antibodies to αvβ3. Thus, α4β1 may not be directly involved in the regulation of cadherin activity but possibly may be important for other cellular processes necessary for cell locomotion, which in turn would affect αvβ3.

The existence of interplay between members of the dif-

ferent families of adhesion molecules has been suggested previously in other cellular systems. For example, in a strikingly similar fashion to neural crest cells, compaction of mesodermal cells in somites has been found to be promoted by soluble RGD peptides (54) and to be mediated by cadherins (Yamada, K.M., personal communication). Likewise, it has been shown recently that, in endothelial cells, β1 integrin engagement can signal to dephosphorylate PECAM-1, a member of the Ig domain superfamily, and that this signaling pathway is important for PECAM-1–mediated control of cell migration (60). Conversely, the loss of integrins that normally occurs in the epidermis during terminal differentiation of basal keratinocytes can be prevented by antibodies to E- and P-cadherin (43). Stable transfection of *Xenopus* XTC cells with E- or XB-cadherin was shown to cause drastic deterioration of substratum adhesion to fibronectin and laminin and to induce reduction of the surface amount and expression of both fibronectin and α3β1 integrin (29). Lastly, in phagocytic cells, ligation of αvβ3 with vitronectin was found specifically to inhibit the phagocytosis of fibronectin beads, a process mediated by the α5β1 integrin (6). In addition, the effect of αvβ3 on α5β1 requires a signal transduction pathway involving a serine-threonine kinase as well as integrin-associated protein (6, 7). Cross talk between adhesion molecules may therefore be a general mechanism that would operate throughout development as well as in the adult to ensure both rapid and flexible changes and efficient coordination between adhesion systems during cell motility, cell differentiation, wound repair, and host defense.

We are extremely grateful to K. Yamada for useful discussions and advice, for providing antibodies, and for critical reading of the manuscript. We also thank B. Geiger for the anti–A-CAM antibodies, F. Sanchez-Madrid for the antibodies to the α4 integrin subunit, D. Cheresh for the antibodies to the αVβ3 integrin, and M. Block for helpful discussions.

This work was supported by the Centre National de la Recherche Scientifique (Programme ATIPE), the Ministère de la Recherche et de la Technologie (91.T.0011), the Association pour la Recherche contre le Cancer (ARC-6517), the Institut National de la Santé et de la Recherche Médicale (CRE 910705), the Ligue contre le Cancer, the Association Française contre les Myopathies, and the Fondation pour la Recherche Médicale. F. Monier-Gavelle is a recipient of predoctoral fellowships from the Ministère de l'Enseignement Supérieur et de la Recherche.

Received for publication 13 June 1996 and in revised form 7 January 1997.

References

1. Adams, C.L., W.J. Nelson, and S.J. Smith. 1996. Quantitative analysis of cadherin-catenin-actin reorganization during development of cell–cell adhesion. *J. Cell Biol.* 135:1899–1911.
2. Akitaya, T., and M. Bronner-Fraser. 1992. Expression of cell adhesion molecules during initiation of and cessation of neural crest cell migration. *Dev. Dyn.* 194:12–20.
3. Akiyama, S.K., E. Hasegawa, T. Hasegawa, and K.M. Yamada. 1985. The interaction of fibronectin fragments with fibroblastic cells. *J. Biol. Chem.* 260:13256–13260.
4. Behrens, J., M.M. Mareel, F.M. Van Roy, and W. Birchmeier. 1989. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell–cell adhesion. *J. Cell Biol.* 108:2435–2447.
5. Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M.M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* 120:757–766.
6. Blystone, S.D., I.L. Graham, F.P. Lindberg, and E.J. Brown. 1994. Integrin αvβ3 differentially regulates adhesive and phagocytic functions of the fibronectin receptor α5β1. *J. Cell Biol.* 127:1129–1137.
7. Blystone, S.D., F.P. Lindberg, S.E. LaFlamme, and E.J. Brown. 1995. Integrin β3 cytoplasmic tail is necessary and sufficient for regulation of α5β1 phagocytosis by αvβ3 and integrin-associated protein. *J. Cell Biol.* 130:745–754.
8. Boucaut, J.-C., T. Darribère, T.J. Poole, H. Aoyama, K.M. Yamada, and J.P. Thiery. 1984. Biological active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J. Cell Biol.* 99:1822–1830.
9. Bracke, M.E., F.M. Van Roy, and M.M. Mareel. 1996. The E-cadherin/catenin complex in invasion and metastasis. *In* Attempts to Understand Metastasis Formation. I. Metastasis-related Molecules. U. Günthert and W. Birchmeier, editors. Springer, Berlin. 123–161.
10. Bronner-Fraser, M. 1985. Alteration in neural crest migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* 101:610–617.
11. Bronner-Fraser, M. 1986. An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Dev. Biol.* 117:528–536.
12. Bronner-Fraser, M. 1993. Neural crest cell migration in the developing embryo. *Trends Cell Biol.* 3:392–397.
13. Bronner-Fraser, M., J.J. Wolf, and B.A. Murray. 1992. Effects of antibodies against N-cadherin and N-CAM on the cranial neural crest and neural tube. *Dev. Biol.* 153:291–301.
14. Brooks, P.C., R.A.F. Clark, and D.A. Cheresh. 1994. Requirement of vascular integrin αvβ3 for angiogenesis. *Science (Wash. DC).* 264:569–571.
15. Brown, E., L. Hooper, T. Ho, and H. Gresham. 1990. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J. Cell Biol.* 111:2785–2794.
16. Chan, B.M.C., P.D. Kassner, J.A. Schiro, H.R. Byers, T.S. Kupper, and M.E. Hemler. 1992. Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. *Cell.* 68:1051–1060.
17. Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K.M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. *J. Cell Biol.* 100:1103–1114.
18. Clark, E.A., and J.S. Brugge. 1995. Integrins and signal transduction pathways: the road taken. *Science (Wash. DC).* 268:233–268.
19. Delannet, M., and J.-L. Duband. 1992. Transforming growth factor-β control of cell-substratum adhesion during avian neural crest cell emigration in vitro. *Development (Camb.).* 116:275–287.
20. Delannet, M., F. Martin, B. Bossy, D.A. Cheresh, L.F. Reichardt, and J.-L. Duband. 1994. Specific roles of the αVβ1, αVβ3 and αVβ5 integrins in avian neural crest cell adhesion and migration on vitronectin. *Development (Camb.).* 120:2687–2702.
21. Duband, J.-L., S. Rocher, W.-T. Chen, K.M. Yamada, and J.P. Thiery. 1986. Cell adhesion and migration in the early vertebrate embryo: location and possible role of the putative fibronectin–receptor complex. *J. Cell Biol.* 102:160–178.
22. Duband, J.-L., G.H. Nuckolls, A. Ishihara, T. Hasegawa, K.M. Yamada, J.P. Thiery, and K. Jacobson. 1988. The fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. *J. Cell Biol.* 107: 1385–1396.
23. Duband, J.-L., T. Volberg, I. Sabanay, J.P. Thiery, and B. Geiger. 1988. Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development (Camb.).* 103:325–344.
24. Duband, J.-L., S. Dufour, S.S. Yamada, K.M. Yamada, and J.P. Thiery. 1991. Neural crest cell locomotion induced by antibodies to β1 integrins: a tool for studying the roles of substratum molecular avidity and density in migration. *J. Cell Sci.* 98:517–532.
25. Duband, J.-L., M. Delannet, and F. Monier-Gavelle. 1995. Neural crest cells: strategies to generate lineage diversification in vitro. *In* Neural Cell Culture: A Practical Approach. J. Cohen and G.P. Wilkin, editors. Oxford University Press, Oxford, UK. 133–152.
26. Duband, J.-L., F. Monier, M. Delannet, and D.F. Newgreen. 1995. Epithelium-mesenchyme transition during neural crest development. *Acta Anat.* 154:63–78.
27. Dufour, S., J.-L. Duband, M.J. Humphries, M. Obara, K.M. Yamada, and J.P. Thiery. 1988. Attachment, spreading, and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2661–2671.
28. Erickson, C.A., and R. Perris. 1993. The role of cell–cell and cell-matrix interactions in the morphogenesis of the neural crest. *Dev. Biol.* 159:60–74.
29. Finnemann, S., M. Külh, G. Otto, and D. Wedlich. 1995. Cadherin transfection of Xenopus XTC cells downregulates expression of substrate-adhesion molecules. *Mol. Cell. Biol.* 15:5082–5091.
30. Frixen, U.H., J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Löchner, and W. Birchmeier. 1991. E-cadherin–mediated cell–cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113: 173–185.
31. Geiger, B., and O. Ayalon. 1992. Cadherins. *Annu. Rev. Cell Biol.* 8:307–332.
32. Gietzen, K., I. Sadorf, and H. Bader. 1982. A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca^{2+}-transport ATPase and brain phosphodiesterase by activators and inhibitors. *Biochem. J.* 207:541–548.

33. Gumbiner, B.M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 84:345–357.
34. Hakomori, S. 1990. Bifunctional role of glycosphingolipid: modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.* 265:18713–18716.
35. Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. $p60^{v\text{-}src}$ causes tyrosine phoshorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:307–314.
36. Hatta, K., and M. Takeichi. 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature (Lond.).* 320:447–449.
37. Hatta, K., S. Takagi, H. Fujisawa, and M. Takeichi. 1987. Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev. Biol.* 120: 215–227.
38. Hemler, M.E., C. Huang, Y. Takada, L. Schwartz, J.L. Strominger, and M.L. Clabby. 1987. Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* 262:11478–11485.
39. Hendey, B., C.B. Klee, and F.R. Maxfield. 1992. Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin. *Science (Wash. DC).* 258:296–299.
40. Hidaka, H., and R. Kobayashi. 1992. Pharmacology of protein kinase inhibitors. *Annu. Rev. Pharmacol. Toxicol.* 32:377–397.
41. Hinck, L., I. Näthke, J. Papkoff, and W.J. Nelson. 1994. Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J. Cell Biol.* 125:1327–1340.
42. Hinck, L., W.J. Nelson, and J. Papkoff. 1994. Wnt-1 modulates cell–cell adhesion in mammalian cells by stabilizing β-catenin binding to the cell adhesion protein cadherin. *J. Cell Biol.* 124:729–741.
43. Hodivala, K.J., and F.M. Watt. 1994. Evidence that cadherins play a role in the downregulation of integrin expression that occurs during keratinocyte terminal differentiation. *J. Cell Biol.* 124:589–600.
44. Horwitz, A., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134–2144.
45. Hoschuetzky, H., H. Aberle, and R. Kemler. 1994. β-Catenin mediates the interaction of the cadherin–catenin complex with epidermal growth factor receptor. *J. Cell Biol.* 127:1375–1380.
46. Hynes, R.O., and A.D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell.* 68: 303–322.
47. Jones, K.T., and G.R. Sharpe. 1994. Ni^{2+} blocks the Ca^{2+} influx in human keratinocytes following a rise in extracellular Ca^{2+}. *Exp. Cell Res.* 212: 409–413.
48. Kadowaki, T., H. Shiozaki, M. Inoue, S. Tamura, H. Pka, Y. Doki, K. Iihara, S. Matsui, T. Iwazawa, A. Nagafuchi et al. 1994. E-cadherin and α-catenin expression in human esophageal cancer. *Cancer Res.* 54:291–296.
49. Kinch, M.S., G.J. Clark., C.J. Der, and K. Burridge. 1995. Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. *J. Cell Biol.* 130:461–471.
50. Klausner, R.D., J.G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071–1080.
51. Klemke, R.L., M. Yebra, E.M. Bayna, and D.A. Cheresh. 1994. Receptor tyrosine kinase signaling required for integrin αvβ5 directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.
52. Lafrenie, R.M., and K.M. Yamada. 1996. Integrin-dependent signal transduction. *J. Cell. Biochem.* 61:543–553.
53. Lallier, T., R. Deutzmann, R. Perris, and M. Bronner-Fraser. 1994. Neural crest cell interactions with laminin: structural requirements and localization of the binding site for α1β1 integrin. *Develop. Biol.* 162:451-464.
54. Lash, J.W., K.K. Linask, and K.M. Yamada. 1987. Synthetic peptides that mimic the adhesive recognition signal of fibronectin: differential effects on cell–cell and cell-substratum adhesion in embryonic chick cells. *Dev. Biol.* 123:411–420.
55. Lawson, M.A., and F.R. Maxfield. 1995. Ca^{2+}- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature (Lond.).* 377:75–79.
56. Le Douarin, N.M. 1982. The Neural Crest. Cambridge University Press, Cambridge, UK.
57. Leavesley, D.I., M.A. Schwartz, M. Rosenfeld, and D.A. Cheresh. 1993. Integrin β1- and β3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J. Cell Biol.* 121:163–170.
58. Levi, G., J.-L. Duband, and J.P. Thiery. 1990. Modes of cell migration in the vertebrate embryo. *Int. Rev. Cytol.* 123:201–252.
59. Lobb, R.R., and M.E. Hemler. 1994. The pathophysiologic role of α4 integrins in vivo. *J. Clin. Invest.* 94:1722–1728.
60. Lu, T.T., L.G. Yan, and J.A. Madri. 1996. Integrin engagement mediates tyrosine phosphorylation on platelet-endothelial cell adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* 93:11808–11813.
61. Lyall, R.M., A. Zilberstein, A. Gazit, C. Gilon, A. Levitzki, and J. Schlessinger. 1989. Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. *J. Biol. Chem.* 264:14503–14509.
62. Marinkovich, M.P., G.P. Lunstrum, D.R. Keene, and R.E. Burgeson. 1992. The dermal-epidermal junction of human skin contains a novel laminin variant. *J. Cell Biol.* 119:695–703.
63. Marks, P.W., B. Hendey, and F.R. Maxfield. 1991. Attachment to fibronectin or vitronectin makes human neutrophil migration sensitive to alterations in cytosolic free calcium concentration. *J. Cell Biol.* 112:149–158.
64. Matsuura, K., J. Kawanishi, S. Fujii, M. Imamura, S. Hirano, M. Takeichi, and Y. Niitsu. 1992. Altered expression of E-cadherin in gastric cancer tissues and carcinomatous fluid. *Br. J. Cancer.* 66:1122–1130.
65. Matsuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell–cell adhesion is perturbed by v-*src* tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703–714.
66. McNeill, H., T.A. Ryan, S.J. Smith, and W.J. Nelson. 1993. Spatial and temporal dissection of immediate and early events following cadherin-mediated epithelial cell adhesion. *J. Cell Biol.* 120:1217–1226.
67. Miller, R.J. 1987. Multiple calcium channels and neuronal function. *Science (Wash. DC).* 235:46–52.
68. Miyamoto, S., S. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.
69. Miyamoto, S., H. Teramoto, O.A. Coso, J.S. Gutkind, P.D. Burbelo, S.K. Akiyama, and K.M. Yamada. 1995. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J. Cell Biol.* 131:791–805.
70. Miyamoto, S., H. Teramoto, J.S. Gutkind, and K.M. Yamada. 1996. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J. Cell Biol.* 135:1633–1642.
71. Monier-Gavelle, F., and J.-L. Duband. 1995. Control of N-cadherin-mediated intercellular adhesion in migrating neural crest cells in vitro. *J. Cell Sci.* 108:3839–3853.
72. Mueller, S.C., T. Hasegawa, S.S. Yamada, K.M. Yamada, and W.-T. Chen. 1988. Transmembrane orientation of the fibronectin receptor complex (integrin) demonstrated directly by a combination of immunocytochemical approaches. *J. Histochem. Cytochem.* 36:297–306.
73. Nakagawa, S., and M. Takeichi. 1995. Neural crest cell–cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development (Camb.).* 121:1321–1332.
74. Näthke, I.S., L. Hinck, and W.J. Nelson. 1995. The cadherin/catenin complex: connections to multiple cellular processes involved in cell adhesion, proliferation and morphogenesis. *Semin. Dev. Biol.* 6:89–95.
75. Newgreen, D.F., and C.A. Erickson. 1986. The migration of neural crest cells. *Int. Rev. Cytol.* 103:89–145.
76. Newgreen, D.F., and J. Minichiello. 1995. Control of epitheliomesenchymal transformation. I. Events in the onset of neural crest cell migration are separable and inducible by protein kinase inhibitors. *Dev. Biol.* 170: 91–101.
77. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and in tumor promotion. *Nature (Lond.).* 308:693–698.
78. Ozawa, M., and R. Kemler. 1990. Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. *J. Cell Biol.* 111:1645–1650.
79. Pierschbacher, M.D., and E. Ruoslahti. 1987. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J. Biol. Chem.* 262:17294–17298.
80. Pomiès, P., P. Frachet, and M.R. Block. 1995. Control of α5β1 integrin/fibronectin interaction in vitro by the serine/threonine protein phosphatase calcineurin. *Biochemistry.* 34:5104–5112.
81. Pressman, B.C. 1976. Biological applications of ionophores. *Annu. Rev. Biochem.* 45:501–529.
82. Pulido, R., M.J. Elices, M.R. Campanero, L. Osborn, S. Schiffer, A. Garcia-Pardo, R. Lobb, M.E. Hemler, and F. Sanchez-Madrid. 1991. Functional evidence for three distinct and independently inhibitable adhesion activities mediated by the human integrin VLA-4. Correlation with distinct α4 epitopes. *J. Biol. Chem.* 266:10241–10245.
83. Rovasio, R.A., A. Delouvée, K.M. Yamada, R. Timpl, and J.P. Thiery. 1983. Neural crest cell migration: requirements for exogenous fibronectin and high cell density. *J. Cell Biol.* 96:462–473.
84. Schwartz, M.A. 1993. Spreading of human endothelial cells on fibronectin or vitronectin triggers elevation of intracellular free calcium. *J. Cell Biol.* 120:1003–1010.
85. Schwartz, M.A., and K. Denninghoff. 1994. αv integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they play a minor role in adhesion. *J. Biol. Chem.* 269:11133–11137.
86. Schwartz, M.A., and D.E. Ingber. 1994. Integrating with integrins. *Mol. Biol. Cell.* 5:389–393.
87. Schwartz, M.A., E.J. Brown, and B. Fazeli. 1993. A 50-kDa integrin-associated protein is required for integrin-regulated calcium entry in endothelial cells. *J. Biol. Chem.* 268:19931–19934.
88. Shibamoto, S., M. Hayakawa, H. Takeuchi, T. Hori, N. Oku, K. Miyazawa, N. Kitamura, M. Takeichi, and F. Ito. 1994. Tyrosine phosphorylation of β-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adh. Commun.* 1:295–305.
89. Shimoyama, Y., A. Nagafuchi, S. Fujita, M. Gotoh, M. Takeichi, S. Tsukita, and S. Hirohashi. 1992. Cadherin dysfunction in a human cancer

cell line: possible involvement of loss of α-catenin expression in reduced cell–cell adhesiveness. *Cancer Res.* 52:5770–5774.
90. Shore, E.M., and W.J. Nelson. 1991. Biosynthesis of the cell adhesion molecule uvomorulin (E-cadherin) in Madin-Darby canine kidney epithelial cells. *J. Biol. Chem.* 266:19672–19680.
91. Sjaastad, M.D., and W.J. Nelson. 1997. Integrin-mediated calcium signaling and regulation of cell adhesion by intracellular calcium. *Bioessays.* 19:47–55.
92. Sommers, C.L., E.P. Gelmann, R. Kemler, P. Cowin, and S.W. Byers. 1994. Alterations in β-catenin phosphorylation and plakoglobin expression in human breast cancer cells. *Cancer Res.* 54:3544–3552.
93. Takeda, H., A. Nagafuchi, S. Yonemura, S. Tsukita, J. Behrens, W. Birchmeier, and S. Tsukita. 1995. V-src kinase shifts the cadherin-based cell adhesion from the strong to the weak state and β-catenin is not required for the shift. *J. Cell Biol.* 131:1839–1847.
94. Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* 75:464–474.
95. Takeichi, M. 1993. Cadherins in cancer: implications for invasion and metastasis. *Curr. Opin. Cell Biol.* 5:806–811.
96. Takeichi, M. 1995. Morphogenetic roles of cadherins. *Curr. Opin. Cell Biol.* 7:619–627.
97. Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{++}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135:397–402.
98. Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.
99. Thiery, J.P., J.-L. Duband, and A. Delouvée. 1982. Pathways and mechanism of avian trunk neural crest cell migration and localization. *Dev. Biol.* 93:324–343.
100. Toeplitz, B.K., A.I. Cohen, P.T. Funke, W.L. Parker, and J.Z. Gougoutas. 1979. Structure of ionomycin, a novel diacidic polyether antibiotic having high affinity for calcium ions. *J. Am. Chem. Soc.* 101:3344–3446.
101. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771–15781.
102. Uehara, Y., and H. Fukazawa. 1991. Use and selectivity of herbimycin A as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* 201:370–379.
103. Umbas, R., A. Schalken, T.W. Aalders, B.S. Carter, H.F. Karthaus, H.E. Schaafsman, F.M.J. Debruyne, and W.B. Isaacs. 1992. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. *Cancer Res.* 52:5104–5109.
104. Umezawa, K., T. Hori, H. Tajima, M. Imoto, K. Isshiki, and T. Takeuchi. 1990. Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS Lett.* 260:198–200.
105. Varner, J.A., and D.A. Cheresh. 1996. Integrins and cancer. *Curr. Opin. Cell Biol.* 8:724–730.
106. Vleminckx, K., L. Vakaet, M. Mareel, W. Fiers, and F. Van Roy. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell.* 66:107–119.
107. Vleminckx, K.L., J.J. Deman, E.A. Bruyneel, G.M.R. Vandenbossche, A.A. Keirsebilck, M.M. Mareel, and F.M. Van Roy. 1994. Enlarged cell-associated proteoglycans abolish E-cadherin functionality in invasive tumor cells. *Cancer Res.* 54:873–877.
108. Volberg, T., B. Geiger, R. Dror, and Y. Zick. 1991. Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regul.* 2: 105–120.
109. Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1733–1742.
110. Volk, T., T. Volberg, I. Sabanay, and B. Geiger. 1990. Cleavage of A-CAM by endogenous proteinases in cultured lens cells and in developing chick embryos. *Dev. Biol.* 139:314–326.
111. Wayner, E.A., W.G. Carter, R.S. Piotrowicz, and T.J. Kunicki. 1988. The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J. Cell Biol.* 107: 1881–1891.
112. Yamada, K.M., and S. Miyamoto. 1995. Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* 7:681–689.