Neural Cell Adhesion Molecule (N-CAM) Is Required for Cell Type Segregation and Normal Ultrastructure in Pancreatic Islets

Farzad Esni,* Inge-Bert Täljedal,‡ Anne-Karina Perl,§ Harold Cremer,‖ Gerhard Christofori,§ and Henrik Semb*

*Department of Microbiology and ‡Department of Histology and Cell Biology, Umeå University, S-901 87 Umeå, Sweden; §Research Institute of Molecular Pathology, A-1030 Vienna, Austria; and ‖Developmental Biology Institute of Marseille, University de Mediterranee, CNRS/INSERM, Campus de Luminy, 13288 Marseille cedex 09, France

Abstract. Classical cell dissociation/reaggregation experiments with embryonic tissue and cultured cells have established that cellular cohesiveness, mediated by cell adhesion molecules, is important in determining the organization of cells within tissue and organs. We have employed N-CAM-deficient mice to determine whether N-CAM plays a functional role in the proper segregation of cells during the development of islets of Langerhans. In N-CAM-deficient mice the normal localization of glucagon-producing α cells in the periphery of pancreatic islets is lost, resulting in a more randomized cell distribution. In contrast to the expected reduction of cell–cell adhesion in N-CAM-deficient mice, a significant increase in the clustering of cadherins, F-actin, and cell–cell junctions is observed suggesting enhanced cadherin-mediated adhesion in the absence of proper N-CAM function. These data together with the polarized distribution of islet cell nuclei and Na^+/K^+-ATPase indicate that islet cell polarity is also affected. Finally, degranulation of β cells suggests that N-CAM is required for normal turnover of insulin-containing secretory granules. Taken together, our results confirm in vivo the hypothesis that a cell adhesion molecule, in this case N-CAM, is required for cell type segregation during organogenesis. Possible mechanisms underlying this phenomenon may include changes in cadherin-mediated adhesion and cell polarity.

Key words: N-CAM • knockout • pancreas • cadherin • organogenesis

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CELL–CELL interactions mediated by cell–cell adhesion molecules (CAMs)[1] are thought to be crucial for tissue formation and organogenesis by regulating the spatial and temporal dissociation, migration, aggregation, and sorting of cells. Neural cell adhesion molecule (N-CAM), a member of the immunoglobulin super-gene family of CAMs mediating homophilic and heterophilic cell–cell interactions, has been implicated in some of these processes during development of the nervous system (Schachner, 1989; Rutishauser, 1991, 1993; Walsh and Doherty, 1991, 1997; Thiery, 1996). N-CAM is encoded by a single gene whose primary transcript exhibits a complex pattern of alternative splicing (Gennarini et al., 1986; Cunningham et al., 1987; Owens et al., 1987; Santoni et al., 1989; Walsh and Dickson, 1989). The various isoforms of N-CAM can be categorized into three main groups according to the size of their cytoplasmic tails and cell surface membrane association: N-CAM-120, -140, and -180 (Cunningham et al., 1983; Chuong and Edelman, 1984; Gennarini et al., 1984). During early phases of embryogenesis these proteins are expressed in cells from all three germ layers. However, as development proceeds its expression pattern becomes more restricted to neuronal tissues (Goridis and Brunet, 1992). Recently, N-CAM mutant mice, lacking either all N-CAM forms (Cremer et al., 1994) or only the 180-kD isoform (N-CAM-180; Tomasiewicz et al., 1993) were generated. Although these mice appear healthy and are fertile, a reduction of the olfactory bulb and deficits in spatial learning are observed in adult mice, phenotypes attributed to defects in cell migration. In adult animals N-CAM is also expressed in various nonneuronal cells, for example in pancreatic endocrine cells (Langley et al., 1989; Rouiller et al., 1990; Moller et al., 1992). However, N-CAM's functional role in these cell types, in particular during organogenesis, remains elusive.

Pancreatic development involves both cytodifferentiation and morphogenesis, in that order (Gittes and Rutter,

Address correspondence to Henrik Semb at Institute of Medical Biochemistry, Göteborg University, Box 440, S-405 30 Göteborg, Sweden, Tel.: 46 31 773 3779. Fax: 46 31 41 61 08. E-mail: henrik.semb@medkem.gu.se

1. *Abbreviations used in this paper:* CAMs, cell adhesion molecules; Cy3, indocarbocyanine; dpc, days postcoitum; eGFP, enhanced green fluorescence protein; N-CAM, neural cell adhesion molecule.

1992). Initially, pancreatic progenitor cells form a dorsal and ventral evagination of the foregut endoderm. During the outgrowth of these buds a branched ductular tree containing pancreatic progenitors is formed. It is thought that the organization of endocrine cells in islets of Langerhans is established through a series of morphogenetic events involving cell sorting, cell migration, and cell reaggregation, processes for which cell adhesion is thought to be important (Pictet and Rutter, 1972; Slack, 1995). Initially, the endocrine cells are present in the primitive pancreatic duct epithelium. These cells eventually sort out of the duct epithelium and begin to aggregate into islets of Langerhans with a distinct cell architecture; non-β cells (α, D, and PP cells) in the periphery and β cells in the center. The fact that islet cell organization is perturbed in humans with diabetes and in animal models for the disease, suggest that the ability to organize endocrine cells properly could be crucial for islet function (Gepts and Lecompte, 1981; Gomez Dumm et al., 1990; Tokuyama et al., 1995). Moreover, it was demonstrated that the molecular mechanisms that regulate insulin secretion depend on β cell contacts, further emphasizing the need for intact cell–cell interactions within islets of Langerhans (Lernmark, 1974; Halban et al., 1982; Bosco et al., 1989; Salomon and Meda, 1986; Philippe et al., 1992).

Using a transgenic approach, we recently demonstrated that members of the cadherin family of CAMs are required for the aggregation of endocrine cells into pancreatic islets (Dahl et al., 1996). However, with these experiments we could not explore whether cadherins are also necessary for sorting of cell types into the typical islet cell architecture. Based on a series of experiments that involved the reaggregation of dissociated rat pancreatic islet cells in vitro, it was recently proposed that N-CAM may regulate cell type segregation of pancreatic islet cells (Cirulli et al., 1994). We have employed N-CAM knockout mice (Cremer et al., 1994) to test this hypothesis in vivo.

Our data reveal that N-CAM is essential for islet cell type segregation, thus providing in vivo evidence for the requirement of a CAM in cell sorting during organogenesis. Alterations in the subcellular distribution of cell polarity markers and cell–cell junctional components and structures suggest that cell polarity is affected and that cadherin-mediated adhesion may be enhanced in N-CAM mutant mice. Furthermore, our data indicate that N-CAM-mediated cell–cell interactions are important for the turnover and activity of intracellular organelles within islets.

Materials and Methods

Animals

Control (+/+), N-CAM heterozygous (+/−), and N-CAM homozygous (−/−) mutant mice in a C57Bl/6J background were used (Cremer et al., 1994). Control experiments demonstrated that no N-CAM protein is expressed in the pancreas of N-CAM −/− mice (see Fig. 2).

Islet Isolation

Mice were killed and pancreata were perfused with HBSS (GIBCO BRL) with 3 U/ml collagenase (Worthington Biochemical Corporation), and 10 μg/ml DNase (Sigma Chemical Co.), incubated at 37°C for 30 min, and washed once with HBSS (GIBCO BRL) with 3% goat serum and three times in RPMI (GIBCO BRL) supplemented with 10% FBS (GIBCO

BRL). Islets were handpicked under microscope and incubated overnight in RPMI (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL).

Cell Transfections

L cells and L cells transfected with cDNAs for murine E-cadherin (LE; Nose et al., 1988) and N-cadherin (LN; Miyatani et al., 1989) were generously supplied by Dr. M. Takeichi (Kyoto University, Kyoto, Japan). These cell lines were transiently transfected with expression-vectors containing cDNAs for enhanced green fluorescence protein (eGFP; Clon-Tech), murine N-cadherin (pRc/CMV; Invitrogen), and murine N-CAM-120, N-CAM-140, or N-CAM-180 (pRc/CMV; Invitrogen), by using Lipofectamine Reagent according to the manufacturer's instructions (GIBCO BRL). Except for eGFP, which was regulated by the MSV-LTR promoter (pMexNeo), all cDNAs were under the influence of the CMV promoter. Cells were either fixed in 4% paraformaldehyde for 10 min or in methanol at −20°C for 5 min, washed, and processed for immunofluorescence staining as described under immunohistochemistry, with the exception that the incubation-time for primary antibodies were 3 h (see below). Samples were analyzed both by standard fluorescence and confocal laser scanning microscopy.

Histological Analysis

Pancreata were removed and fixed in 4% paraformaldehyde overnight, washed in PBS overnight, dehydrated in graded alcohols, and embedded in paraffin. 6-μm sections were stained with hematoxylin-eosin and photographed using a Zeiss Axioplan light microscope.

Immunoblotting

Islets were solubilized by boiling in sample buffer (63 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, and 10 μg/ml of bromphenol blue) for 5 min, separated by SDS–polyacrylamide gels, and electrophoretically transferred onto nitrocellulose filters (Bio-Rad) in 192 mM glycine, 20% methanol, and 25 mM Tris-HCl. Blocking (overnight) and all antibody incubations were in HBST-Ca^{2+} (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and 0.1% Tween 20). The first and secondary antibodies were applied for 3 h and 60 min, respectively. To visualize the antigen–antibody complexes, filters were incubated with a conjugated secondary antibody, which was visualized by chemiluminiscence using the ECL-detection kit (Amersham) according to the manufacturer's specifications.

Immunohistochemistry

Tissues (pancreata) were collected and fixed in HBS (10 mM Hepes, pH 7.4, 150 mM NaCl) supplemented with 4% paraformaldehyde for 2 hours at room temperature. For cryostat protection, tissues were incubated in serial sucrose solutions (12, 15, and 18%) in HBS supplemented with 1 mM $CaCl_2$ for 2–3 h each at 4°C. Tissues were embedded in Tissue Tek compound and frozen in liquid nitrogen. 8-μm-thick sections on polylysine (Sigma)-coated glass slides were washed in HBS, heated in a microwave oven (only for N-cadherin antibody), postfixed in −20°C methanol for 20 min, and blocked in TBS-Ca^{2+} (10 mM Tris, pH 7.6, 150 mM NaCl, and 1 mM $CaCl_2$) supplemented with 5% skim milk for 30 min at room temperature. The first antibody was added in TBS-Ca^{2+} supplemented with 5% skim milk overnight at 4°C. Secondary antibodies, FITC- or Cy3-streptavidin were added for 60 min each. When HRP staining was used (Vectastain ABC kit) endogenous peroxidase was blocked with 3% H_2O_2 in methanol during postfixation (see above).

Insulin and Glucagon Measurements

Pancreatic insulin and glucagon were measured in total pancreatic extracts from five fed animals (4–5 mo of age) of each genotype using a commercially available radioimmunoassay for rat insulin and glucagon (Linco Research, Inc.). Total pancreatic protein concentration was determined using Bio-Rad protein assay (Bio-Rad). Values of pancreatic insulin and glucagon were within the normal range according to the manufacturer (Linco Research, Inc.). Statistical analysis was performed using the Chi-square test.

Immunoreagents

The following antibodies were used at the indicated dilutions for immunoblotting and immunohistochemistry experiments; rat mAb against E-cad-

herin (ECCD-2; Shirayoshi et al., 1986; 1:40); rat mAb against N-cadherin (MNCD-2; Matsunami and Takeichi, 1995; 1:200); affinity-purified rabbit anti-Na$^+$/K$^+$-ATPase (Nelson and Hammerton, 1989; 1:100); rabbit anti-N-CAM (Rasmussen et al., 1982; 1:1,000); rat mAb against ZO-1 (Chemicon; 1:100); rabbit anti–rat amylase (Przybyla et al., 1979; 1:1,000); rabbit anti-carboxypeptidase (Biogenesis; 1:1,000); rabbit anti-PDX1 (Ohlsson et al., 1993; 1:400); guinea pig anti-insulin (Linco Research, Inc.; 1:1,000); rabbit anti-glucagon (Linco Research, Inc.; 1:500); FITC-conjugated anti-guinea pig and anti-rabbit (Molecular Probes; 1:500); indocarbocyanine (Cy3)-conjugated anti-rabbit (Molecular Probes; 1:300); biotin-conjugated anti–rat and anti–rabbit (Molecular Probes; 1:500). Cy3-conjugated streptavidin were purchased from Molecular Probes and used at 1:1,000 dilution. Rhodamine-phalloidin was purchased from Molecular Probes and used according to the manufacturer's instructions. The Vectastain ABC kit was from Vector Laboratories, Inc.

Cell Distribution Measurements within Islets

To estimate the distribution and the number of α cells in islets, whole pancreata were sectioned and sections separated by 200 μm were stained with anti-glucagon polyclonal antibodies. Data were obtained by analyzing more than 80 individual islets per mouse in four animals (4–8 mo old) of each genotype. Normal islets were defined as islets in which all α cells were found within the three most peripheral cell layers. Islets with a radius of five or less cell layers were not included. Analysis of variance (ANOVA) and the Scheffe multiple comparison test were used to compare the values in Table I. Statistics were performed by using SPSS 7.5 for Windows (SPSS Inc.).

Transmission Electronmicroscopy

Specimens were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C, rinsed in phosphate buffer, postfixed in 1% OsO$_4$ in 0.1 M phosphate buffer for 2 h, dehydrated in graded alcohol solutions, and embedded in Poly/Bed (Polyscience Inc.). Ultrathin sections were cut on a LKB ultratome. The sections were placed on gold grids, stained with uranyl acetate and lead citrate, and examined in a Jeol 100 CX electron microscope.

Results

N-CAM Expression during Pancreatic Ontogeny

N-CAM's expression pattern during pancreatic development is reminiscent of its general expression pattern in the embryo, i.e., initially it is expressed more or less ubiquitously, whereas at later stages of development and in adult tissue its expression becomes more restricted. During the early stages of pancreatic ontogeny, N-CAM is expressed

Table I. Islet Cell Type Segregation Is Affected in N-CAM Mutant Mice

Genotype	Normal	Mixed	Missorted α-cells	α-Cells/islet section[¶]
	%*	%‡	%§	
N-CAM +/+	78 ± 4	22 ± 4	14 ± 0.6	24 ± 1
N-CAM +/−	29 ± 1	71 ± 1	20 ± 0.3	25 ± 2
	(P < 0.0001)	(P < 0.0001)	(P < 0.01)	(P = 0.5)
N-CAM −/−	33 ± 1	67 ± 1	21 ± 0.4	27 ± 3
	(P < 0.0001)	(P < 0.0001)	(P < 0.005)	(P = 0.2)

Values are mean ± SEM.
*Percentage of islets in which all α-cells were found within the three most peripheral cell layers.
‡Percentage of islets containing one or more α-cells positioned centrally to the three most peripheral cell layers.
§Percentage of α-cells positioned centrally to the three most peripheral cell layers within "mixed" islets.
¶Number of α-cells per islet section.

both in the pancreatic mesenchyme and endoderm (Fig. 1, a and b). Gradually, N-CAM becomes confined to aggregating endocrine cells (Fig. 1, c–f), which in addition to peripheral nerve endings and ganglia are the only pancreatic cells that express N-CAM in adult mice (Fig. 1 g). In contrast to pancreatic islets in the rat, which express higher levels of N-CAM in non-β cells than in β cells (Rouiller et al., 1990; Moller et al., 1992), the mouse appears to express similar levels in all endocrine cells, at least when judged by immunohistochemistry (Fig. 1 g). The predominant N-CAM polypeptide expressed in adult islets is the glycosylphosphatidylinositol-linked 120-kD isoform (Fig 1 h). In conclusion, the pattern of N-CAM expression during pancreatic organogenesis suggests an involvement in islet morphogenesis.

N-CAM Is Required for Islet Cell Type Segregation

N-CAM does not appear to be required for the differentiation of pancreatic cells or for major pancreatic morphogenetic events, since a normal pancreas, consisting of all pancreatic cell types and islets of normal size and number scattered within the exocrine tissue, formed in N-CAM-deficient mice (Fig. 2, a–d). However, close examination of the mutant animals revealed alterations in the organization and morphology of islet cells. Notably, these changes are apparent in both heterozygous (N-CAM +/−) and homozygous (N-CAM −/−) animals. Control experiments demonstrated that N-CAM protein is completely absent in N-CAM −/− islets and that N-CAM +/− islets express ~50% of the protein levels detected in wild-type mice (Fig. 2, e–h). The subcellular distribution of N-CAM in N-CAM +/− islets was unaltered as compared with wild-type islets, i.e., N-CAM is predominantly expressed in cell–cell contacts (Fig. 2 f).

In the mouse, islets begin to form around 17.5–18 days postcoitum (dpc; Herrera et al., 1991). Already at this stage the islets begin to adopt their final cell organization, i.e., β cells in the center and non-β cells in the periphery. However, the segregation of these cell types is not yet complete and in many aggregates the cells are more or less intermixed. In fact, by using the distribution of glucagon-producing α cells as the criterion of cell type segregation after birth, it was noted that it is not until 4–5 wk of age that the majority of mouse islets adopt their final cell configuration (data not shown). This cell architecture is maintained at least up to 11 mo of age, which is the oldest age that we have analyzed. To investigate whether N-CAM has any influence on islet cell type segregation we compared the ratio of normal and mixed islets between control and N-CAM-deficient mice. We defined normal islets as islets with all α cells distributed within the three most peripheral cell layers and mixed islets as islets, which contain one or more α cells positioned centrally to the three most peripheral cell layers. Although mixed islets were found in control mice (22%), the majority of the islets was defined as normal (78%; Table I, Fig. 3). However, in N-CAM-deficient mice the ratio of normal and mixed islets completely changed. In these mice the number of normal islets were diminished, whereas the majority of the islets was defined as mixed (71% +/−, 67% −/− vs. 22% +/+; Table I, Fig. 3). This effect becomes apparent only when islet cell type segregation is usually complete, i.e., around 4–5 wk of
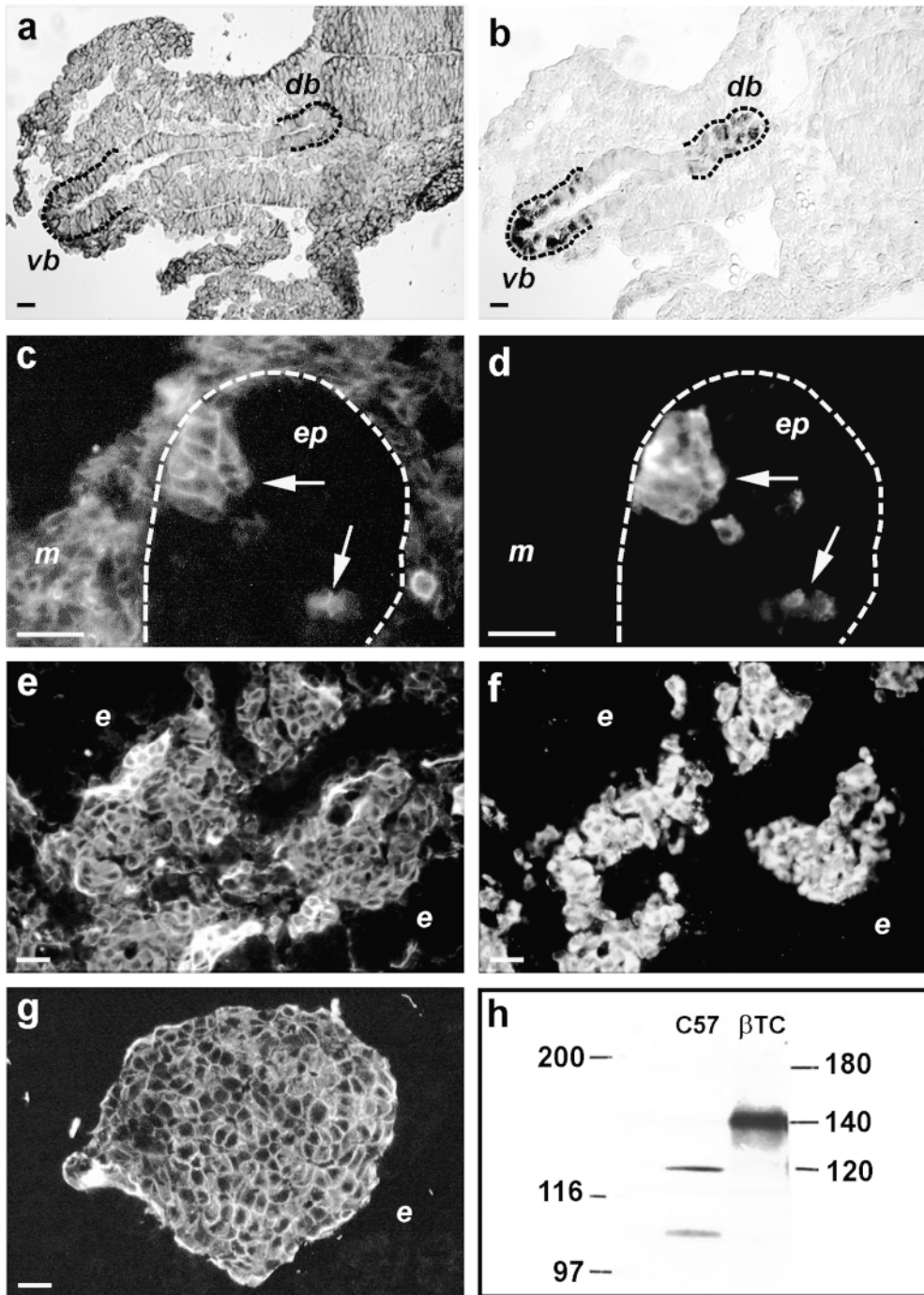
Figure 1. N-CAM expression in developing and adult pancreas. (a and b) Immunoperoxidase detection of N-CAM (a) and PDX1 (b), a marker for pancreatic endoderm, on consecutive transversal sections of 9.5 dpc pancreas. N-CAM is expressed in the pancreatic endoderm and in the mesenchyme, which surrounds both the ventral (vb) and dorsal (db) pancreatic buds. Each pancreatic bud is indicated by a broken line. (c and d) Double immunofluorescence staining of a transversal section of an 11.5 dpc pancreas with polyclonal antibodies against N-CAM (c) and glucagon (d). N-CAM is expressed in mesenchymal cells (m) and in clusters of α cells (arrows), but not in primitive epithelial cells (ep) which are indicated by a broken line. (e and f) Double immunofluorescence staining of 17.5 dpc pancreas with polyclonal antibodies against N-CAM (e) and insulin (f). At this stage N-CAM is selectively expressed in islet-like clusters of endocrine cells. Exocrine tissue is indicated by e. (g) Immunofluorescence staining of adult pancreas with polyclonal antibodies against N-CAM, showing specific expression in pancreatic islets. Exocrine tissue is indicated by e. (h) Immunoblotting analysis of extracts from pancreatic islets (C57) and the insulinoma cell line βTC4 (βTC; Efrat et al., 1988). The major N-CAM isoform expressed in islets is N-CAM-120. Bars, 20 μm.

age. In further support of N-CAM's involvement in islet cell type segregation, the extent of inter-mixing of cells within mixed islets of N-CAM-deficient mice was markedly higher as compared with the few mixed islets of control mice. Thus, the percentage of α cells positioned centrally to the three most peripheral cell layers was significantly higher in N-CAM-deficient animals than in control mice (20 +/−, 21 −/− vs. 14 +/+; Table I). Taken together these data suggest that N-CAM regulate cell type segregation during the development of islet of Langerhans. There are, however, several potential explanations for these observations.

Either the number of islet cells has changed, or the segregation of islet cells is affected in N-CAM-deficient mice. However, the fact that the number of α cells and pancreatic insulin and glucagon levels exhibits no marked difference between the different genotype mice (Table I and II), suggests that N-CAM is, indeed, a key regulator of islet cell sorting. Besides α cells and β cells, islets consist of two other endocrine cell types, somatostatin-producing D cells and pancreatic polypeptide-producing PP cells. Similarly to α, D, and PP cells are also preferentially confined to the outer rim of islets in control mice, however, to a much lower
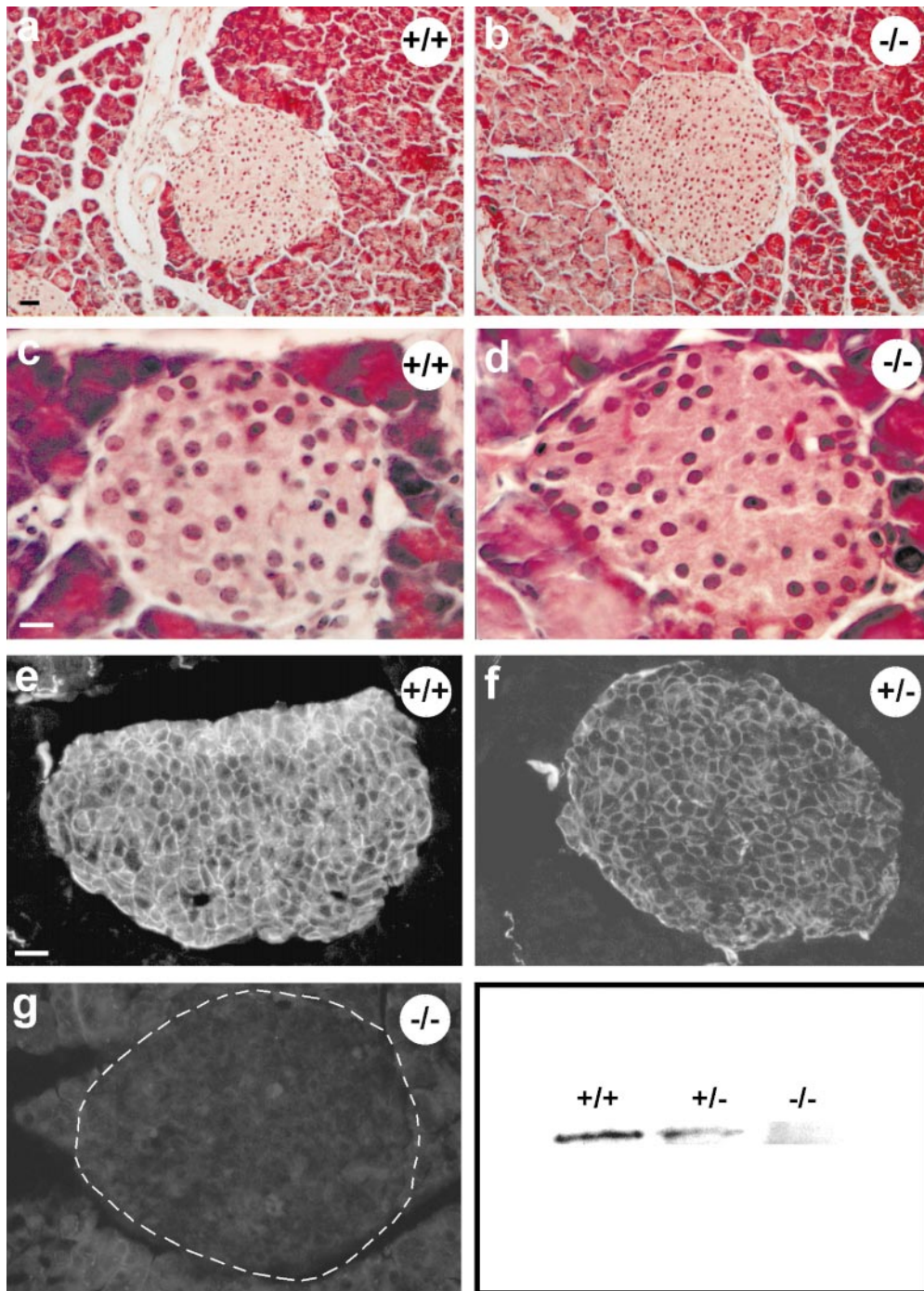
Figure 2. Histological analysis of adult wild-type and N-CAM-deficient pancreata. (a–d) Hematoxylin-eosin stainings of paraffin sections of control (a and c, +/+) and N-CAM −/− (b and d, −/−) mice. Based on hematoxylin-eosin stainings, pancreata of N-CAM-deficient mice appear normal (data not shown for N-CAM +/− pancreata). (e-g) Immunofluorescence stainings of sections from adult wild-type (e, +/+), N-CAM +/− (f, +/−), and N-CAM −/− (g, −/−) pancreata with anti-N-CAM polyclonal antibodies. (h) Immunoblotting analysis of pancreatic islet extracts from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) animals, using anti-N-CAM polyclonal antibodies. While N-CAM is completely absent in N-CAM −/− islets, N-CAM +/− islets express ~50% of wild-type islets. Bars: (a, b, and e–g) 20 μm; (c and d) 10 μm.

degree. Their distribution was not detectably affected in N-CAM mutant mice (data not shown).

Islet Cell Polarity Is Affected in N-CAM Mutant Mice

To elucidate the molecular mechanisms behind the altered cell type segregation within islets of Langerhans in N-CAM-deficient mice, the subcellular distribution of molecules that are known to affect morphogenetic behaviors of cells or act as molecular markers for cell polarity were examined. Thus far, three members of the classic cadherins have been found to be expressed in islets, E-, N-, and R-cadherin (Begemann et al., 1990; Moller et al., 1992; Hutton et al., 1993; Dahl et al., 1996; Esni, F., and H. Semb, unpublished observations). Only N-cadherin and E-cadherin localize to cell–cell contacts (Begemann et al., 1990; Dahl et al., 1996), whereas R-cadherin distribute mainly in the cytoplasm (Dahl, U., F. Esni, and H. Semb, unpublished observations). Analysis of N-CAM-deficient mice revealed striking changes in the subcellular distribution of N-cadherin and E-cadherin, whereas R-cadherin was unaffected. As mentioned previously, these cadherins normally localize to all regions engaged in cell–cell adhesion without any apparent clustering (Begemann et al., 1990; Dahl et al., 1996;
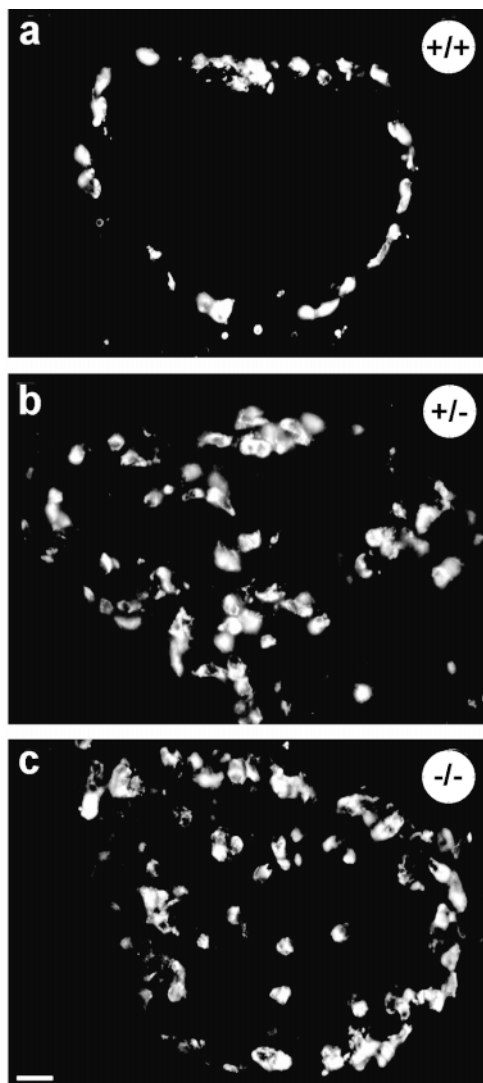
Figure 3. Distribution of glucagon-producing α cells in pancreatic islets of control and N-CAM-deficient mice. Immunofluorescence staining of adult control (a, +/+), N-CAM +/− (b, +/−), and N-CAM −/− (c, −/−) mice with polyclonal antibodies against glucagon. In control animals the majority of α cells are localized in the periphery of islets. However, in N-CAM +/− and N-CAM −/− mice the α cells become more randomly distributed within the islets, referred to as mixed islets in Table I. Bar, 20 μm.

Fig. 4 a). In N-CAM-deficient mice, N-cadherin (Fig. 4, a–d) but also E-cadherin (data not shown) accumulate in discrete regions of cell–cell contacts, suggesting that N-CAM prevents clustering of cadherins within islets of Langerhans in control mice. Because cadherins are capable of affecting cortical F-actin organization and cell polarity, the subcellular distribution of F-actin and Na$^+$/K$^+$-ATPase (Hammerton et al., 1991; Drubin and Nelson, 1996), apical and basolateral epithelial cell polarity markers, respectively, were investigated in N-CAM-deficient mice. In control islets F-actin's preferential distribution is adjacent to cell–cell contacts (Fig. 4 e), whereas in N-CAM-deficient mice it accumulates together with the cadherins (Fig. 4, f–h). The visualization of the redistribution of N-cadherin, E-cadherin, and F-actin appears as multicellular rosette

structures, which are composed of all endocrine cell types, and are reminiscent of the apical enrichment of E-cadherin and F-actin in exocrine acinar cells (insets in Fig. 4, d and h). We propose to refer to these structures as endocrine acini. The striking changes in the organization of cadherins and actin filaments in islets of N-CAM-deficient mice suggest that cell polarity may also be affected. In further support for alterations in islet cell polarity, the subcellular localization of the basolateral epithelial cell polarity marker Na$^+$/K$^+$-ATPase and the nuclei within islets of N-CAM-deficient is reminiscent of their distribution within fully polarized exocrine acinar cells (Fig. 5, compare a with c and d, and e with g and h). In normal control islets, Na$^+$/K$^+$-ATPase colocalizes with N-cadherin and E-cadherin in cell contact regions (Fig. 5 b, and data not shown), whereas in islets of N-CAM-deficient mice Na$^+$/K$^+$-ATPase does not accumulate in regions with increased cadherin and F-actin clustering (Fig. 5, c and d). Furthermore, while islet cell nuclei are randomly distributed in control islets (Fig. 5 f), cell nuclei are preferentially localized to the basal regions of those islet cells that are organized in endocrine acini in N-CAM-deficient mice (Fig. 5, g and h). Similar to the effect on cell type segregation, changes in the subcellular localization of cadherins and F-actin in N-CAM-deficient mice were first observed at 4–5 wk of age. To examine whether transdifferentiation of endocrine cells into exocrine acinar cells could explain the appearance of the endocrine acinar structures, expression of acinar markers, such as amylase or carboxypeptidase, was investigated. However, none of these exocrine markers is expressed in islets of N-CAM +/− and N-CAM −/− mice, indicating that the appearance of acinar structures in islets of Langerhans does not resemble a transdifferentiation process (data not shown).

Taken together, our data suggest that N-CAM is required for islet cell type segregation. Moreover, N-CAM may play a functional role in the regulation of islet cell polarity, probably by modulating the organization of cadherins and F-actin within islet cells.

Ultrastructural Changes in Islet Cells of N-CAM Mutant Mice

Changes in cell–cell interactions and in cellular sorting may also affect intracellular organization and possibly physiological functions of a cell. To investigate this possibility we analyzed islets of Langerhans of control mice and N-CAM-deficient mice by transmission electron micros-

Table II. Pancreatic Insulin and Glucagon Are Not Altered in N-CAM-deficient Mice

	Insulin	Glucagon
	*μg/mg protein**	*ng/mg protein‡*
N-CAM +/+	11 ± 1	99 ± 13
N-CAM +/−	10 ± 0.6	85 ± 6
N-CAM −/−	9.8 ± 0.3	70 ± 8

Values are mean ± SEM.
*Insulin was measured in total pancreatic extracts. Statistical analysis showed no significant difference between any of the groups ($P = 1$).
‡Glucagon was measured in total pancreatic extracts. Statistical analysis showed no significant difference between any of the groups ($P = 0.3$).
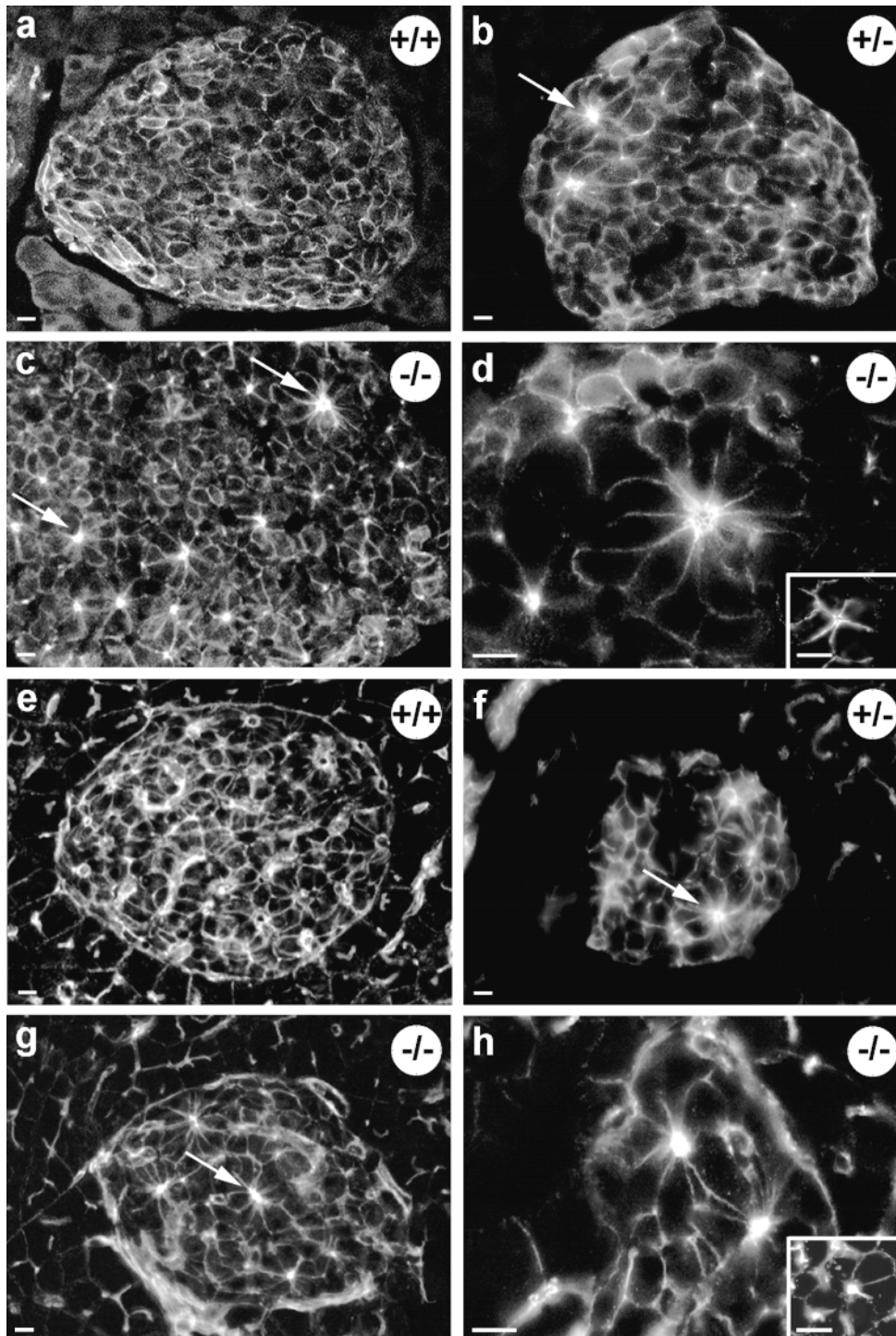
Figure 4. Subcellular organization of N-cadherin and F-actin is altered in pancreatic islets of N-CAM mutant mice. (a–d) Immunofluorescence stainings of sections from adult control (a, +/+), N-CAM +/− (b, +/−), and N-CAM −/− (c and d, −/−) pancreata with anti-N-cadherin mAb. Inset in d shows E-cadherin distribution in an exocrine acinus. (e–h) Staining of F-actin on sections from adult control (e, +/+), N-CAM +/− (f, +/−), and N-CAM −/− (g and h, −/−) pancreata with rhodamine-phalloidin. Inset in h shows F-actin distribution in an exocrine acinus. Arrows indicate rosette-like structures, or endocrine acini. The subcellular localization of N-cadherin and F-actin is reminiscent of the distribution of E-cadherin and F-actin in exocrine acini. Bars, 10 μm.

copy. Although in both N-CAM +/− and N-CAM −/− animals the majority of endocrine cells appeared morphologically normal, a significant fraction of β cells and α cells appeared ultrastructurally perturbed. The reorganization of cadherins and F-actin as seen by light microscopy (Fig. 4) appears to correlate with the accumulation of cell–cell junctions in multicellular endocrine structures (Fig. 6, b and c). Although each of these junctions, including desmosomes and adherens type junctions, are found scattered in islet cell contacts of control mice, they appear to accumulate towards the center of groups of cells in islets of

N-CAM-deficient mice (Fig. 6, b and c). Moreover, a significant fraction of β cells contained a diminished number of secretory granules (Fig. 6, compare a to b) together with an increased number of residual bodies that are frequently associated with the plasma membrane (Fig. 6, b–d). Secretory granules were occasionally observed within residual bodies (Fig. 6 d), suggesting increased autophagy. Dilation of the rough endoplasmic reticulum was seen in both β cells and α cells (Fig. 6 c, and data not shown). As the corresponding changes were not observed in control mice, it appears that N-CAM-mediated cell–cell interactions are
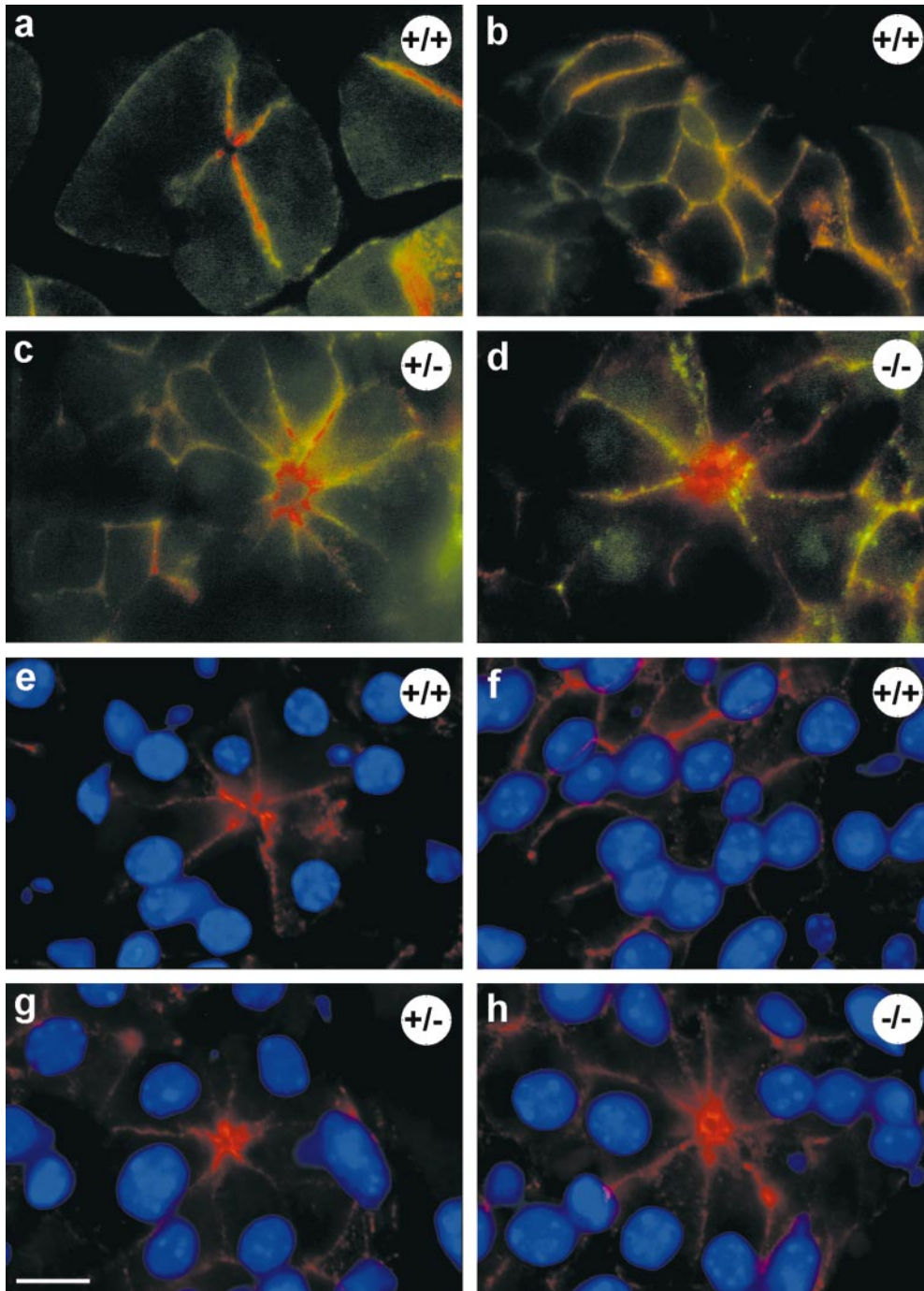
Figure 5. Subcellular localization of cell polarity markers suggests altered cell polarity in pancreatic islets of N-CAM-deficient mice. (a) Double immunofluorescence staining of an exocrine acinus in section of adult control (+/+) pancreas with anti-Na$^+$/K$^+$-ATPase polyclonal antibody (FITC) and anti-E-cadherin mAb (Cy3). While Na$^+$/K$^+$-ATPase colocalizes with E-cadherin in lateral cell–cell contacts, the molecule does not accumulate together with E-cadherin in adherens junctions. (b–d) Double immunofluorescence stainings of islet cells in sections from adult control (b, +/+), N-CAM +/− (c, +/−), and N-CAM −/− (d, −/−) pancreata with anti-Na$^+$/K$^+$-ATPase polyclonal antibody (FITC) and anti-N-cadherin mAb (Cy3). Normally Na$^+$/K$^+$-ATPase colocalizes with N-cadherin in most islet cell–cell contacts (b). However, in N-CAM-deficient mice Na$^+$/K$^+$-ATPase does not accumulate together with N-cadherin in the apical regions of endocrine acini (c and d). (e) Exocrine acinus in section of adult control (+/+) pancreas immunostained with anti-E-cadherin mAb. Nuclei were stained with DAPI. E-cadherin is clustered in the apical region of lateral cell–cell contacts, while nuclei are preferentially distributed in the basal region of the cells. (f–h) Islet cells in sections of adult control (f, +/+), N-CAM +/− (g, +/−), and N-CAM −/− (h, −/−) pancreata immunostained with anti-N-cadherin mAb. Nuclei were stained with DAPI. In contrast to in control mice, the redistributions of N-cadherin and nuclei in endocrine acini of N-CAM +/− (g) and N-CAM −/− (h) mice are reminiscent of the localization of E-cadherin and nuclei in exocrine acini (e). Bar, 10 μm.

required for organizing islet cell–cell contacts, as well as for maintaining normal activity and turnover of organelles within islet cells.

N-CAM Affects the Epithelial Cell Morphology of Cadherin-expressing L Cells

To examine whether N-CAM's effects on pancreatic endocrine cell morphology also applies to other cell types, and to assess more directly whether N-CAM can influence cadherin function, we performed a series of cell transfection experiments. It has previously been demonstrated that expression of cadherins in fibroblast cell lines results in the transition from a mesenchymal to an epithelial cell morphology (Nose et al., 1988). To determine whether N-CAM affects the epithelial phenotype of cadherin-expressing L cells, we transiently transfected parental L cells and L cells that expressed either E-cadherin (LE; Nose
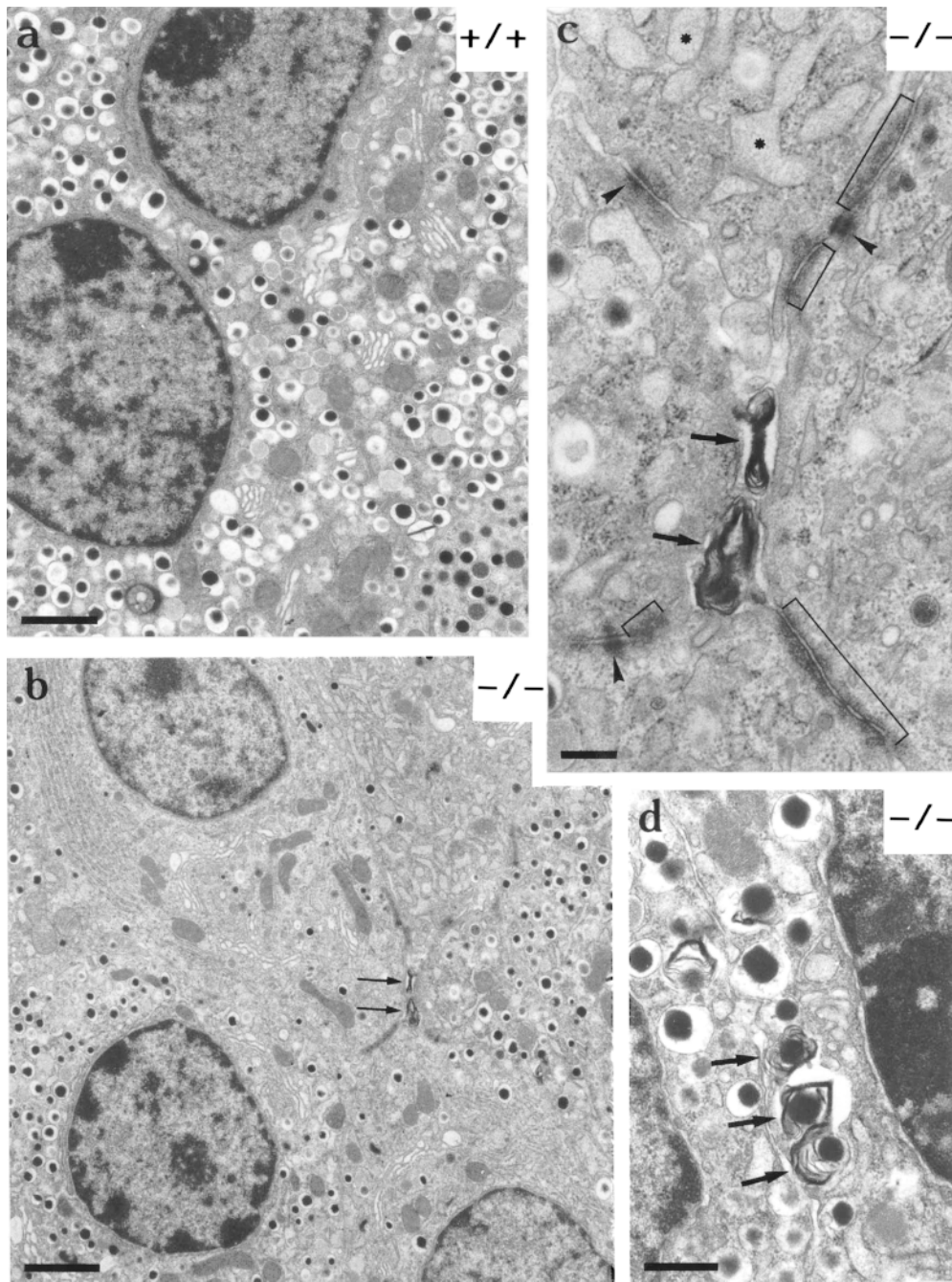
Figure 6. N-CAM-deficient mice exhibit ultrastructural alterations in islet cells. Electron photomicrographs of islets from control (a, +/+) and homozygous (b–d, −/−) animals. Because the ultrastructural changes were the same in heterozygous and homozygous mutants, only the data from the homozygous mice is shown. In b, and at higher magnification in c, clustering of cell–cell junctions, including desmosomes (arrowheads in c) and adherens type junctions (brackets in c), between four β cells are shown. In b three β cells contain a diminished number of secretory granules. Arrows in b-d indicate accumulation of residual bodies. In d residual bodies contain secretory granules. Dilation of rough endoplasmic reticulum was observed in β cells (asterisks in c) and α cells (data not shown). Bars: (a) 1 μm; (b) 2 μm; (c and d) 0.5 μm.

et al., 1988) or N-cadherin (LN; Miyatani et al., 1989) with cDNA constructs encoding N-CAM-120, N-CAM-140, and N-CAM-180. Both N-CAM-140 and N-CAM-180, but not N-CAM-120, induced extensive neurite-like extensions or filopodia on the cells, regardless whether the L cells expressed cadherins or not (Fig. 7, e–g). Notably, the majority of the N-CAM-expressing cells left the monolayer and migrated on top of neighboring cells (Fig. 7, e and f). Furthermore, thick protrusions were observed on the dorsal side of N-CAM-expressing cells (data not shown).

Next, we examined N-CAM's effect on the subcellular localization of E-cadherin and N-cadherin. In the majority of N-CAM-expressing cells cadherins were diffusely distributed on the cell surface, in particular in cells with neu-

rite-like extensions (Fig. 7, g and h). However, in N-CAM-expressing cells that remained within the cell monolayer, the distribution of E-cadherin and N-cadherin was not significantly changed (Fig. 7, i and j). Expression of either eGFP or another cell adhesion molecule, N-cadherin, did not result in morphological changes in any of the cell lines (Fig. 7, c and d, and data not shown). The failure of N-CAM-120 to induce morphological changes is probably due to the fact that, although expressed at significant levels, N-CAM-120 was not localized to the cell membrane. Thus, the results with L cells in vitro are consistent with our findings in N-CAM-deficient mice in vivo. Together, the results suggest that N-CAM-mediated changes in cell morphology are dominant over cadherin function.
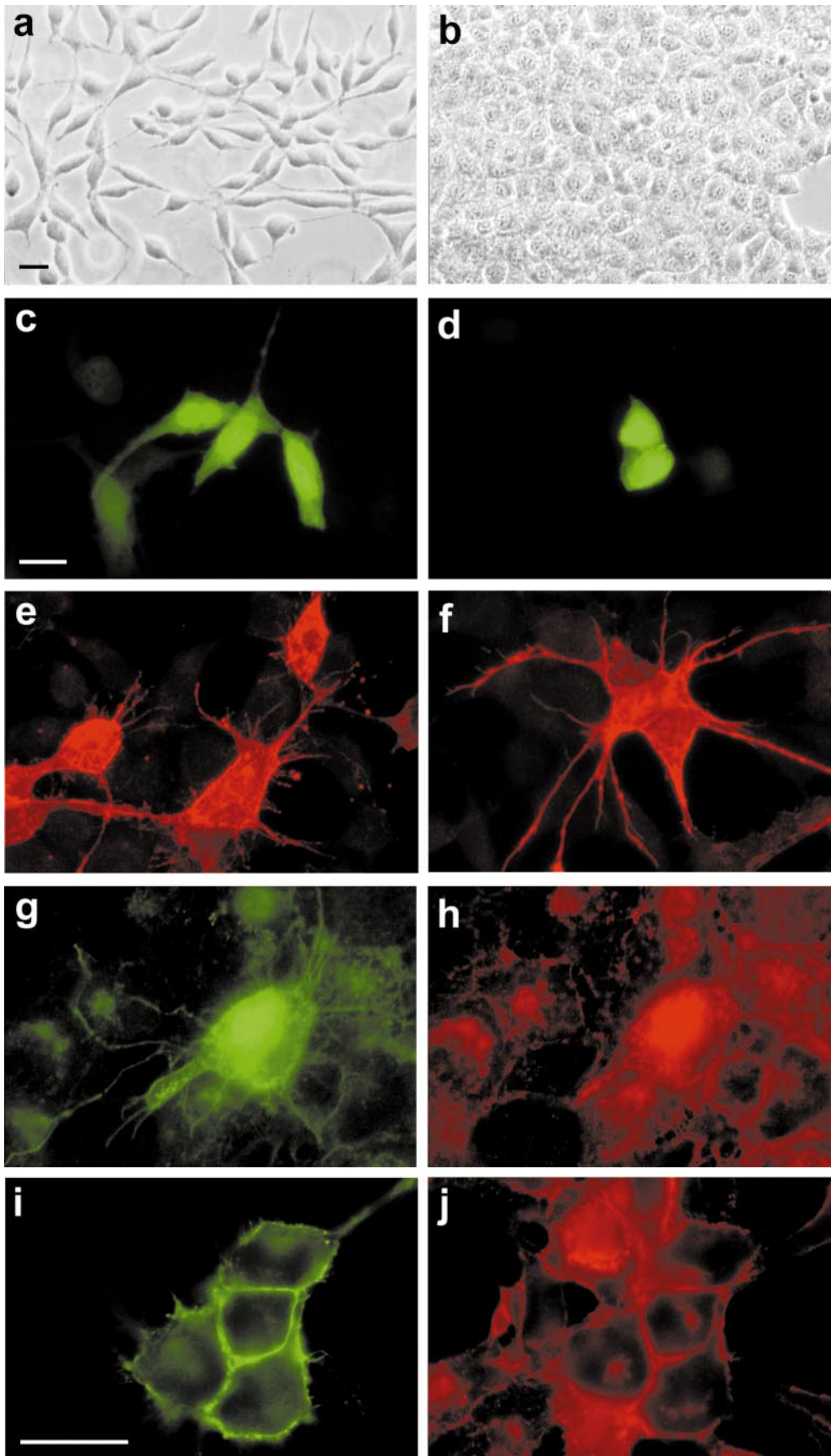
Figure 7. N-CAM affects the epithelial cell morphology of cadherin-expressing L cells. Parental L cells and L cells transfected with cDNAs for murine E-cadherin (LE; Nose et al., 1988) and N-cadherin (LN; Miyatani et al., 1989) were transiently transfected with cDNAs encoding murine N-CAM-120, N-CAM-140, and N-CAM-180. Except for N-CAM-120, which did not localize to the cell surface, all N-CAM isoforms exhibited similar effects on L cell morphology, independent of cadherin expression. Results from parental L cells and LE cells transfected with N-CAM-140 are shown. (a and b) Phase-contrast images of L cells (a) and LE cells (b). (c and d) Fluorescence of eGFP in L cells (c) and LE cells (d) transfected with eGFP alone. No change in cell morphology was observed. (e and f) Immunofluorescence staining of L cells (e) and LE cells (f) transfected with N-CAM-140. Expression of N-CAM conferred similar effects on cell morphology independently of whether the transfected cells exhibited a fibroblast-like or epithelial-like cellular phenotype. N-CAM induced extensive neurite-like extensions or filopodia on the cells, regardless of the mesenchymal or epithelial phenotype of the cell lines. In LE cells the majority of the transfected cells left the monolayer and migrated on top of neighboring cells. (g–j) Double immunofluorescence stainings of N-CAM-140 transfected LE cells with anti-N-CAM polyclonal antibody (g and i) and anti-E-cadherin mAb (h and j). The majority of the transfected cells lost their typical epithelial phenotype upon N-CAM expression (g and h), however, occasionally, transfected cells remained within the monolayer (i and j). While the polar distribution of E-cadherin was lost in the former cells, it remained unchanged in the latter cells. Bars, 20 μm.

Discussion

Previous analyses of N-CAM-deficient mice showed that mutant mice appear healthy and are fertile, demonstrating that N-CAM is not essential for embryogenesis (Tomasiewicz et al., 1993; Cremer et al., 1994). However, close examination of the development and function of the nervous system in these mice have revealed interesting new insights into the role of N-CAM in the nervous system (Cremer et al., 1997; Shen et al., 1997; Krushel et al., 1998; Moscoso et al., 1998).

Based on a series of reaggregation experiments of dissociated rat islets in vitro, it was proposed that N-CAM is also involved in pancreatic islet morphogenesis by regulating islet cell type segregation (Cirulli et al., 1994). We have now employed N-CAM knockout mice to investigate N-CAM's functional role in pancreas organogenesis in vivo. Normally, glucagon-producing α cells are preferentially distributed in the periphery, while insulin-producing β cells are found in the center. Both in N-CAM $+/-$ and N-CAM $-/-$ mice, islet cell type segregation was affected, resulting in a more random distribution of α cells within islets. To our knowledge this is the first demonstration of the involvement of N-CAM in developmental cell sorting in vivo. Concomitant with these changes, the subcellular distribution of epithelial cell polarity markers, cadherins, F-actin, and cell–cell junctions were altered, suggesting that N-CAM may influence cell polarity and cadherin-mediated adhesion in islet cells. Finally, the diminished number of secretory granules in β cells of N-CAM mutant mice suggests that N-CAM-mediated cell–cell interactions are crucial for the normal turnover of insulin.

Interestingly, all our findings in the N-CAM-deficient mice were seen to the same extent in heterozygous and homozygous animals, suggesting that N-CAM protein levels are critical for the processes described above. This is in accordance with the recent demonstration that one functional N-CAM allele is not sufficient to compensate for the effects on aggressive behaviors and tumor cell dissemination in N-CAM-deficient mice (Stork et al., 1997; Perl, A.-K., U. Dahl, P. Wilgenbus, H. Cremer, H. Semb, and G. Christofori, manuscript submitted for publication), indicating that a distinct dosage of N-CAM expression is crucial for several cellular processes.

How could N-CAM's involvement in islet cell type segregation be explained in molecular terms? Initially, the mechanisms for cell rearrangement during development such as tissue spreading movement and segregation of unlike cells were explained by Holfreter as differences in tissue affinities (Townes and Holfreter, 1955). Steinberg and Takeichi (1994) showed that two motile cell types differing only in the levels of expression of a single adhesion system, will not only segregate from one another but also arrange themselves in the form of an envelope of less cohesive cells surrounding a core of more cohesive cells. The differential adhesion hypothesis could thereby be attributed to forces generated by intercellular adhesions within and between migrating cell populations (Steinberg, 1996). Steinberg and coworkers then went on to explain in physical terms that tissue surface tensions determine the arrangement of cells that are free to rearrange within tissues (Foty et al., 1996; Davis et al., 1997). According to the differen-

tial adhesion hypothesis, β cells, forming the core of islets of Langerhans, should be more cohesive than peripheral non-β cells. Is there any evidence that islet cell type segregation could be explained by differences in adhesion properties? In rat, Ca^{2+}-dependent adhesion appears to be similar in β cells and non-β cells, whereas non-Ca^{2+}-dependent adhesion, including that mediated by N-CAM, is more pronounced in non-β cells (Rouiller et al., 1990, 1991). In mouse, the picture is not as clear, since similar immunohistochemical methods failed to reveal any differential expression of N-CAM within mouse islets (Fig. 1 g). The only CAM that has been shown to be differentially expressed in mouse islets is R-cadherin. However, this cadherin appears to be differentially expressed in β cells at very low levels. Most importantly, it is predominantly localized to the cytoplasm (Dahl, U., F. Esni, and H. Semb, unpublished observations). In addition to R-cadherin, E-cadherin and N-cadherin are also expressed in islets (Begemann et al., 1990; Moller et al., 1992; Hutton et al., 1993; Dahl et al., 1996). However, in the mouse neither E-cadherin nor N-cadherin appears to be differentially expressed by the various cell types within islets (Begemann et al., 1990; Rouiller et al., 1991; Esni, F., and H. Semb, unpublished observations). Thus, the existence of a CAM that by virtue of its differential expression would make β cells more adhesive than non-β cells remains elusive. Nonetheless, it is important to consider the fact that the cell surface expression level of a CAM may not be a reliable indicator of a cell's adhesiveness.

Alternatively, islet cells may express molecules, which negatively influence the basic adhesion machinery, which in islets is primarily mediated by cadherins. Our results suggest that N-CAM might fulfil the criteria for a molecule that negatively influences the basic adhesion mechanisms. Differential expression of N-CAM in non-β cells, or at least in α cells, would make them less cohesive than β cells, resulting in their peripheral distribution within islets.

Concomitant with the cell sorting defect, the subcellular distribution of cadherins and F-actin were altered in N-CAM $-/-$ and N-CAM $+/-$ mice. Recent data suggest that one prerequisite for strong cadherin adhesion is the clustering of cadherin molecules (Adams et al., 1996; Yap et al., 1997). During the development of cell–cell contacts in epithelial cells it is thought that E-cadherin and actin filaments form localized clusters, designated "puncta" by Adams et al. (1996), which gradually merge in order to strengthen adhesion in cell–cell junctions. This is presumably the mechanism by which E-cadherin concentrates to the cell–cell junctional region of acinar cells in the exocrine pancreas. However, in normal pancreatic islets no apparent clustering of N-cadherin and E-cadherin is apparent. In contrast, in islets of N-CAM $-/-$ and N-CAM $+/-$ animals, cadherins appear to cluster more efficiently, even forming acinar structures, suggesting that cadherin-mediated adhesion becomes stronger. This notion is further supported by ultrastructural studies showing an accumulation of cell–cell junctions in areas reminiscent of areas with increased clustering of cadherins and F-actin. These observations, together with the polarized localization of cell nuclei and the basolateral cell polarity marker, Na^+/K^+-ATPase, within rosette-like structures, provide rather compelling evidence for changes in islet cell polarity.

To explain a possible attenuating influence of N-CAM on cadherin-mediated adhesion, we speculate that N-CAM, either directly or indirectly, interferes with the clustering of cadherins through changes in cell morphology. It is known that N-CAM isoforms, which contain polymers of α-2,8-linked polysialic acid (PSA-N-CAM), have strong anti-adhesive properties (Rutishauser et al., 1988; Acheson et al., 1991; Rougon, 1993). Although conflicting evidence exists regarding the expression of PSA-N-CAM in rat islets (Moller et al., 1992; Kiss et al., 1994), the molecule was not detected in mouse islets by the use of a PSA-N-CAM-specific monoclonal antibody (5A5; Dodd et al., 1988; data not shown).

To further elucidate the mechanism for a possible connection or cross-talk between N-CAM and cadherins, and to examine if it applies to other cell types, we performed a series of L cell transfection experiments. These in vitro experiments reveal that N-CAM exhibits a dominant effect over cadherins on cell morphology. They also indicate that our observations in pancreatic endocrine cells may apply to other cell types as well. However, expression of N-CAM-120 and N-CAM-140 in fully polarized epithelial MDCK cells did not result into changes of cell morphology, raising the possibility that different cell types may exhibit varying responses to N-CAM expression (Powell et al., 1991). Future experiments will have to identify the mechanisms by which N-CAM affects the subcellular organization of cadherins.

Regarding N-CAM's involvement in F-actin organization, it is unlikely that this involves a direct physical interaction with the actin cytoskeleton, since the major N-CAM isoform expressed in the islets is the glycosylphosphatidylinositol-linked N-CAM-120. Alternatively, N-CAM could indirectly regulate actin filament organization either through the observed rearrangement of cadherins or through the activation of intracellular signaling cascades. There is now considerable evidence that N-CAM can activate the FGF receptor through a direct physical interaction (Doherty and Walsh, 1994; Saffell et al., 1997). The possible involvement of N-CAM in actin filament rearrangement through the FGF receptor signaling pathway is further supported by the fact that N-CAM-mediated activation of the FGF receptor leads to activation of GAP-43 (Walsh et al., 1997), an intracellular protein that is involved in the organization of the actin cytoskeleton.

Finally, the question remains whether cell–cell contacts and correct islet cell organization are required for normal islet function. One line of evidence suggests that this could, indeed, be the case. When β cells are separated, insulin biosynthesis and secretion decrease, especially in response to glucose concentrations that physiologically stimulate pancreatic islets. However, these changes are rapidly corrected after cell reaggregation (Lernmark, 1974; Halban et al., 1982; Salomon and Meda, 1986; Bosco et al., 1989; Philippe et al., 1992). It is interesting to note that perturbation of islet cell organization in diabetic patients, as well as in animal models of the disease, suggests that the inability to organize endocrine cells may explain the hyperglycemic phenotype (Gepts and Lecompte, 1981; Gomez Dumm et al., 1990; Tokuyama et al., 1995). Therefore, the altered cell type segregation in islets of N-CAM −/− and N-CAM +/− animals seemed appropriate for testing this hypothesis. However, even though occasional glucose-intolerant individuals were found among N-CAM mutant mice, no statistically significant alterations in glucose-tolerance of N-CAM −/− and N-CAM +/− mice were observed (data not shown). This was rather surprising in light of the ultrastructural changes observed in islet cells of N-CAM-deficient mice, which indicated that function could be impaired in at least a fraction of the endocrine cells. Maybe the absence of a grossly impaired islet function is due to the fact that the reported effects are only found in a fraction of the islet cells. However, these data warrant further studies to elucidate whether N-CAM or other CAMs are directly involved in glucose-mediated insulin secretion and, if so, whether the expression of any of these CAMs is affected in diabetic patients and in animal models of the disease.

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