Ectopic Synthesis of Epidermal Cytokeratins in Pancreatic Islet Cells of Transgenic Mice Interferes with Cytoskeletal Order and Insulin Production

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Abstract. The members of the multigene family of intermediate filament (IF) proteins are expressed in various combinations and amounts that are specific for a given pathway or state of differentiation. Previous experiments in which the cell type-specific IF cytoskeleton was altered by introducing foreign IF proteins into cultured cells or certain tissues of transgenic animals have shown a remarkable tolerance, without detectable interference with cell functions. To examine the importance of the cell type-specific cytokeratin (CK) IF pattern, we have studied the ectopic expression of CK genes in different epithelia of transgenic mice. Here we report changes observed in the β cells of pancreatic islets expressing the genes for human epidermal CKs 1 and/or 10 brought under control of the rat insulin promoter. Both genes were efficiently expressed, resulting in the appearance of numerous and massive bundles of aggregated IFs, resembling those of epidermal keratinocytes. While the synthesis of epidermal CK 10 was readily accommodated and compatible with cell function, mice expressing CK 1 in their β cells, alone or in combination with CK 10, developed a special form of diabetes characterized by a drastic reduction of insulin-secretory vesicles and of insulin- and CK 1-producing cells. In many CK 1-producing cells, accumulations of fibrous or granular material containing CK 1 were also seen in the nucleus. This demonstration of functional importance of the specific CK-complement in an epithelial cell indicates a contribution of cell type-specific factors to cytoplasmic IF compartmentalization and that the specific CK complement can be crucial for functions and longevity of a given kind of epithelium.

Of the three major cytoskeletal filament systems, the actin microfilaments, the microtubules, and the intermediate-sized filaments (IFs),[1] the latter are still enigmatic as, up to date, no distinct cellular functions can be ascribed to them. While microfilaments and microtubules are ubiquitous elements also involved in general ("housekeeping") cell functions, IFs and IF proteins are not imperative components and obviously do not serve the same functions in different cells. (*a*) The more than 40 members of this large multigene family are expressed in different combinations in relation to cell differentiation pathways. (*b*) Most puzzling, several cultured cell lines and tumors are totally devoid of IF proteins (e.g., 33, 40, 54, 78). (*c*) The type of IF protein present in the same cell type can grossly differ from one species to another (for review, see 30). (*d*) Intracellular immunoprecipitation of IFs by injected antibodies does not result in detectable functional damage (e.g., 32, 46, 47, 55).

Elucidation of the possible functions of IFs has been attempted in various laboratories along three major lines of experimental research as follows. The most rigorous approach, i.e., the targeted elimination of a specific IF protein gene by homologous recombination techniques, has recently been taken in several laboratories. The only result reported so far has been negative (7): Mouse embryo cells in which the gene encoding the only type II cytokeratin (CK) expressed, i.e., CK 8, has been disrupted, can no longer form IFs, but nevertheless differentiate to a well organized polar epithelium.

In a second line of research, cells or animals have been transfected with genes encoding an experimentally mutated IF protein, resulting in structural alterations of the endogenous IF system. Using this approach, it has been shown that expression of a gene encoding a CK defective in the central "rod" domain (for reviews see 2, 75) leads to dramatic changes in the IF system, notably aggregate formation, the formation of which correlates, in transgenic mice, with severe defects of intercellular coherence (3, 4, 15, 57, 77, 78, 82). The similarity of these effects in transgenic mice carrying and expressing a defective CK gene to the inheritable hu-

1. *Abbreviations used in this paper*: CK, cytokeratin; IDDM, insulin-dependent diabetes mellitus; IF, intermediate-sized filaments; RIP, rat insulin promoter.

man blistering disease *Epidermolysis bullosa simplex* (EBS) has recently led to the discovery that human EBS is associated with certain mutations in the rod portion of CK 14 or CK 5 (12, 16; see also 43). When similarly defective desmin molecules were introduced into cultured fibroblasts or myoblasts, aggregate formation and extensive disorganization of the desmin/vimentin IF system was also observed but this did not affect cell function and sarcomeric differentiation (73).

In a third kind of experimentation, the cell's IF system has been disturbed by the forcefully induced "ectopic" or dysregulated expression of one or several IF protein genes, either by microinjection of the IF protein (79) or the corresponding mRNA (28, 41, 49), or by transfecting cultured cells or mice with IF protein genes brought under control of promoters directing their expression to different kinds of cells, including cases of ectopic expression and overexpression (6, 8, 13, 18, 19, 34, 51, 53, 57, 64, 66). These experiments have shown that foreign or overexpressed IF proteins are well accommodated in the recipient cultured cells as well as in the transgenic animals, without noticeable effects on function or phenotype, the only exception reported being cataract formation in eye lenses filled with overexpressed IFs of various kinds (vimentin, desmin, neurofilament protein; 13, 19, 64).

All these results have led to the general view that, in many different kinds of cells and tissues, neither the type nor the amount of IFs really matters for cell function and morphology. Even when ectopically synthesized epidermal CKs were expressed in mammary gland-derived cells in great excess over the endogenous CKs, the resulting permanent cell lines were perfectly viable and did not reveal major shape changes (8).

To examine whether the cell's IF composition might be important for certain special functions and/or differentiation processes we targeted, in transgenic mice, the ectopic expression of certain IF protein genes to some cell types that are known for distinct, very sensitively balanced functions. Here we report the specific results obtained after disturbing the cytoskeleton of the β cells of pancreatic islets by the expression of CKs 1 and 10, i.e., hallmarks of suprabasal differentiation of epidermis (e.g., 23, 24, 31, 71). We show that these cells do not tolerate a specific "wrong" CK, i.e., CK 1, and that both their insulin-secretory function and their lifetime is drastically reduced, resulting in diabetes, whereas another foreign CK, i.e., CK 10, is well accepted, even when present in large amounts.

Materials and Methods

Genes and Constructs

A genomic λ-clone (λKH1) for the human CK 1 gene was isolated from a genomic library using oligonucleotide probes corresponding to various parts of the sequence published by Johnson et al. (44). The clone was characterized and identified by restriction enzyme mapping and by partial sequence analysis. The genomic clone for the human CK 10 gene (λKH10[5]) has been previously described (69). The construct RIP-Tag carrying the SV-40 T-antigen under the control of the rat insulin gene promoter (see reference 36) was a kind gift of Dr. D. Hanahan (University of California, San Francisco, CA), the construct pSV2β-globin is a derivative of pSV2 (52).

The genes for CKs 1 and 10 were brought either under the control of the rat insulin gene promoter (RIP-H1 and RIP-H10) or the SV-40 promoter (SV-H1 and SV-H10) as summarized in Fig. 1.

Cell Cultures and Transfections

3T3-L1 preadipocytes (ATCC CL 173; 29) and HeLa cells (ATCC CCL 2) were cultured in DME supplemented with 10% FCS. Transfections were performed using the calciumphosphate-DNA coprecipitation technique (see reference 8).

Generation of Transgenic Mice

Transgenic mice were generated by pronuclear injection of an equimolar mixture of the 10-kb EcoRI fragment of RIP-H1 and of the 5.18-kb XhoI–NotI fragment of RIP-H10. In total, 21 offspring were born and subsequently analyzed for the presence of the transgene by Southern blotting. Two male animals were detected carrying the RIP-H10 construct alone (Tg1-RIP-H10 and Tg2-RIP-H10). In addition, two females were found which carried either the RIP-H1 construct alone (Tg-RIP-H1) or both constructs (Tg-TIP-H1/10). All four founder animals transmitted the transgenes to offspring, and breeding of line Tg-RIP-H1/10 confirmed that the constructs RIP-H1 and RIP-H10 were integrated on the same chromosome, most likely at the same integration site. Since RIP-H1 transgene-positive animals become hyperglycemic and sick early in life (6–10 wk of age) only positive males were used for further breeding immediately upon sexual maturation. All experiments with mice were performed on F1 and F2 mice of a cross between C57BL/6 and SJL mice.

During the study, a total of 69 transgenic mice of line Tg-RIP-H1, 47 of line Tg-RIP-H10, and 40 of line Tg-RIP-H1/10 were examined.

Antibodies

Monoclonal CK1 antibody EAB-904 was from Enzo Biochem (Neckargemünd, FRG). RKSE60, a monoclonal murine antibody specifically reacting with CKs 10 and 11, and guinea pig antibodies to insulin were obtained from Bio-Science Products (Emmenbrücke, Switzerland). A monoclonal antibody reacting with CKs 1, 10, and 11 (K_K8.60, from Bio-Makor, Rehovot, Israel) has been described in detail (42). For the visualization of glucagon we used a rabbit antiserum (Bio Trend, Köln, FRG) or monoclonal murine antibody 23.6B4 (35). Synaptophysin antibody SY38 (80) served as a general neuroendocrine marker. Murine CKs ortholog to CKs 8 and 18 were detected by specific guinea pig antibodies (available from Progen, Heidelberg, FRG) or with mAbs TROMA 1 and TROMA 2 (11). A broad range monoclonal CK antibody, lu-5, was obtained from Hoffmann-La Roche Diagnostica (Basel, Switzerland). For identification of lymphocytes and macrophages we applied antibodies recognizing Thy-1, CD2, CD45, and α,β-TCR (all from Dianova, Hamburg, FRG). FITC- and Texas red–labeled secondary antibodies were also from Dianova.

Measurement of Blood Glucose

Blood obtained from the tail was allowed to clot for 1 h. After centrifugation the serum was collected and glucose levels were determined using a Beckman glucose analysator (the kind help of Dr. M. Zorn, Krehl Clinic for Internal Medicine, University of Heidelberg, is acknowledged).

Gel Electrophoresis and Protein Identification

Cytoskeletal preparations, two-dimensional gel electrophoresis (2-D GE) and immunoblotting tests were performed essentially as described (1). Immunoblot reactions of nitrocellulose-bound polypeptides with monoclonal murine antibodies were visualized by secondary reaction with ^{125}I-labeled goat antibodies to mouse IgGs (Amersham-Buchler, Braunschweig, FRG), followed by autoradiography.

Light Microscopy and Immunolocalization

Tissue samples were routinely fixed and processed for paraffin-embedding and hematoxylin-eosin (H&E) staining (the cooperation of Dr. F. Amelung, this Center, is gratefully acknowledged). For immunolocalization, cultured cells were fixed 40 h after transfection with cold (−20°C) methanol/acetone for 5 min and frozen sections (5 μm) of tissue samples with acetone for 10 min. Single- and double-label immunofluorescence microscopy were performed as described (28).

Electron and Immunoelectron Microscopy

Tissue samples were fixed in 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.4, containing 50 mM KCl and 2.5 mM $MgCl_2$ for 20 min, washed in buffer, "postfixed" for 2 h with 2% osmium tetroxide in 25

mM plain cacodylate buffer, and blockstained overnight with 0.5% uranyl acetate (in water). Alternatively, samples were fixed simultaneously with 2.5% glutaraldehyde (same buffer) for 20 min plus 1% osmium tetroxide. After a second fixation with 2% OsO_4 solution in 25 mM cacodylate buffer, samples were block-stained overnight in 0.5% uranyl acetate (in water). All further procedures were as described (28).

Cryosections (5 μm) mounted on coverslips were fixed as described above, incubated with CK antibodies for 30 min in a wet-chamber, washed with PBS three times for 5 min each, and incubated overnight with ~5 μm colloidal gold particles coated by conjugated IgG (Amersham-Buchler). After three washes with PBS, samples were fixed with 2.5% glutaraldehyde for 15 min, washed, postfixed with 2% OsO_4 for 30 min, dehydrated, and flat-embedded in Epon 812 (see reference 28).

Alternatively, the bound antibodies were reacted with ~1-nm-diam gold particles coated with anti-mouse IgG (Bio Trend, Aurion Gold Reagents) overnight, washed three times with PBS, fixed first with glutaraldehyde, and then with OsO_4 (see above), and finally washed with distilled water. For "silver enhancement," sections were then exposed for 3 min to a mixture of one part "Developer" and one part "Enhancer" (Bio Trend, Aurion) mixed immediately before use.

When CK antibody lu-5 was used, sections were first fixed with 2% freshly prepared formaldehyde (in PBS) for 20 min, then permeabilized with 0.1% saponin (in PBS) for 10 min, and incubated as described above.

Results

Gene Constructs and Their Expression in Cultured Cells

For studies of the synthesis and assembly of human epidermal differentiation CKs 1 and 10 in cells in which they normally are absent, we have brought the human genes encoding thee proteins (see references 44, 69) under the control of different promoters. Here we specifically describe the results obtained with the rat insulin promoter (RIP; see reference 36), for cell type-specific expression in pancreatic β cells, and with the SV-40 promoter, for permissive expression in several different cell types. The constructs used are schematically shown in Fig. 1, *a* and *b*.

Transcription of the transfected genes, translation of the encoded mRNA, and the structural assembly of the resulting protein(s) were examined by transient transfections of cultured cells of several lines, and in some examples also in stably transfected cells selected for introduced neomycin resistance (see reference 8). Using antibodies which react only with human CK 1 or CK 10 or both, the structures formed in the transfected cells were routinely studied by immunofluorescence microscopy.

These epidermal CKs encoded by the constructs shown in Fig. 1 were able to integrate into a pre-existing CK fibril meshwork or to form CK fibrils de novo, as demonstrated by transient transfections of different cell culture lines. For example, upon transfection of diverse epithelial cell lines, including HeLa cells (Fig. 2 *a*), with one or both of the SV-40 promoter-driven constructs (Fig. 1 *b*), we noticed the formation of meshworks of CK 1- or CK 10-positive cytoplasmic fibrils. In double-label immunofluorescence microscopy, far-reaching colocalization of these foreign CKs with the endogenous CKs 7, 8, 17, and 18 (60) was seen, confirming the co-assembly of the forcefully synthesized epidermal CKs and the endogenous CKs into a common IF system, as previously shown for various other CKs and cell lines (8, 28, 34, 51).

After double transfection of nonepithelial cells devoid of any endogenous CK IFs with both human CK genes, the de novo formation of CK 1- and CK 10-positive fibrillar structures or aggregates was observed (Fig. 2 *c*, murine 3T3-L1

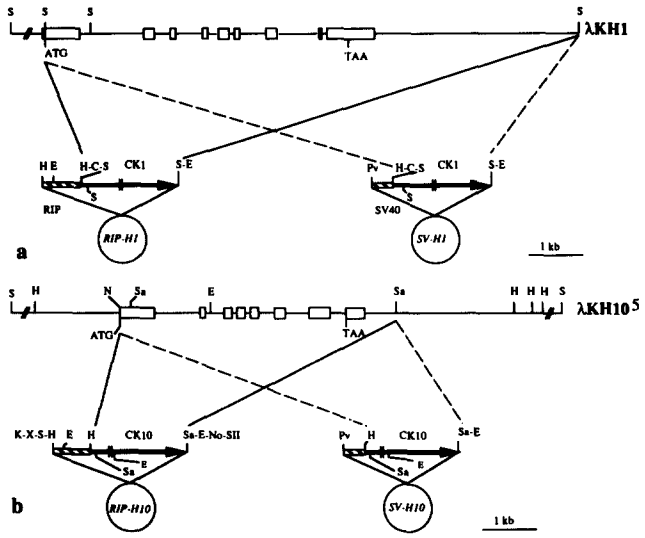


Figure 1. Construction of plasmids containing the genes for human CKs 1 (*a*) and 10 (*b*) under the control of either the SV-40 promoter or the RIP. Exons are represented by open rectangles, promoter fragments by hatched rectangles, introns and flanking sequences as lines. *C*, ClaI; *E*, EcoRI; *H*, HindIII: *K*, KpnI; *N*, NlaIV; *No*, NotI; *P*, PstI; *Pv*, PvuII; *S*, SalI; *SII*, SacII; *Sa*, SacI; *Sp*, SphI; and *X*, XhoI. (*a*) A genomic λ-clone (*λKHI*) containing the whole CK 1 gene and several kilobases of flanking regions were used. A 10-kb fragment generated by partial SalI digest and ranging from the second codon to ~3 kb downstream from the polyadenylation site, was cloned in the correct orientation into the SalI site of a derivative of pUC8 in which the PstI site has been destroyed by treatment with Klenow DNA polymerase and a ClaI site has been introduced with an octameric ClaI linker, thereby restoring the ATG start codon in the correct frame. In the resulting plasmid, H1-MC, the codon for serine (AGT) following the start codon was changed to a glycine codon (GGT; not shown). The gene was then coupled either to the SV-40 promoter by cloning the 10-kb HindIII/EcoRI insert of H1-MC into the pSV2β-globin construct opened with HindIII/EcoRI to yield SV-H1, or to RIP by inserting the 750-bp BamHI/HindIII fragment to RIP-Tag into the HindIII site of H1-MC, using HindIII linkers, to yield RIP-H1. (*b*) The 180-bp NlaIV/SacI fragment of λKH10⁵, a genomic clone encoding human CK 10, was inserted into pUC 18 cleaved by SphI/SacI double digestion. Then the 5-kb SacI fragment was added in the correct orientation to yield pH10. The 5.18-kb insert of pH10 was excised by HindIII/EcoRI partial digestion and cloned into Bluescript opened by HindIII/EcoRI double digestion. The resulting plasmid H10-MC contains the CK 10 gene from the ATG site to ~0.5 kb downstream of the polyadenylation site (not shown). The gene was then coupled either to the SV-40 promoter by cloning the 5.18-kb HindIII/EcoRI partial fragment of H10-MC into pSV2β-globin opened with HindIII/EcoRI to yield SV-H10, or to RIP as described in (*a*) to yield RIP-H10.

preadipocytes; for vimentin IFs in these cells see reference 29). This confirms previous observations made after double transfections of gene constructs encoding other type I and II CKs (e.g., 6, 8, 34, 50, 57).

In some experiments showing the fibril-forming competence of CKs 1 and 10, we occasionally also noticed cells in which CK-positive material was concentrated in "dots" or "whiskers" around the nuclear surface or even in the nuclear interior (Fig. 2, *b* and *d*).

Transgenic Mice and Ectopic Cytokeratins

Both acinar and islet cells of the mammalian pancreas con-

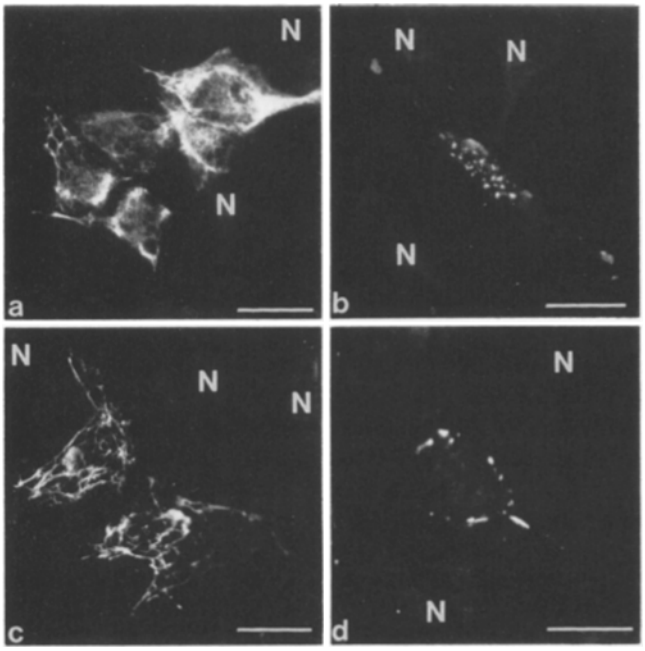


Figure 2. Immunofluorescence microscopy of structures formed by CKs 1 and 10 in HeLa cells (*a* and *b*) and murine preadipoyctes of line 3T3-L1 (*c* and *d*) after transient transfection with construct SV-H1 alone (HeLa cells, *a* and *b*) or with both SV-H1 and SV-H10 (preadipocytes, *c* and *d*). mAb K_K8.60 specifically recognizing both human CKs 1 and 10 but not reactive with endogenous HeLa CKs has been used. Note that in the cytoplasm of both cell types the newly synthesized epidermal CK1 can form filamentous structures, either with an endogenous type I CK (*a*) or with the cotransfected epidermal pair-partner, human CK 10 (*c*). In a number of such transfected cells, we have also observed intranuclear or juxtanuclear (*b* and *d*) CK dot- or rod-like structures. *N*, nuclei of cells not showing CK expression. Bars, 25 μm.

tain typical, albeit small desmosomes and bundles of IFs primarily composed of CK 8 and CK 18 and enriched in the cell periphery, mostly at desmosomes. The frequency of both structures, however, is much lower in islet cells (e.g., 45, 61, 62, 81). The extremely low content of CKs (and thus of IFs) in all types of islet cells characteristically results in a very weak, sometimes even negative-appearing CK immunolocalization of islets (data not shown). Reactions with antibodies specific for human epidermal CKs 1 and 10 were consistently negative on all pancreatic cells.

Transgenic mice containing one or both of the human genes for epidermal CKs 1 and 10 under RIP control developed normally until ~2 wk after birth, without changes detectable in islets stained with H&E, but their further fate differed dramatically: all mice expressing the transgene for CK 1 in the β cells of their pancreatic islets developed insulin-dependent diabetes mellitus (IDDM), whereas those expressing only the CK 10 gene appeared normal.

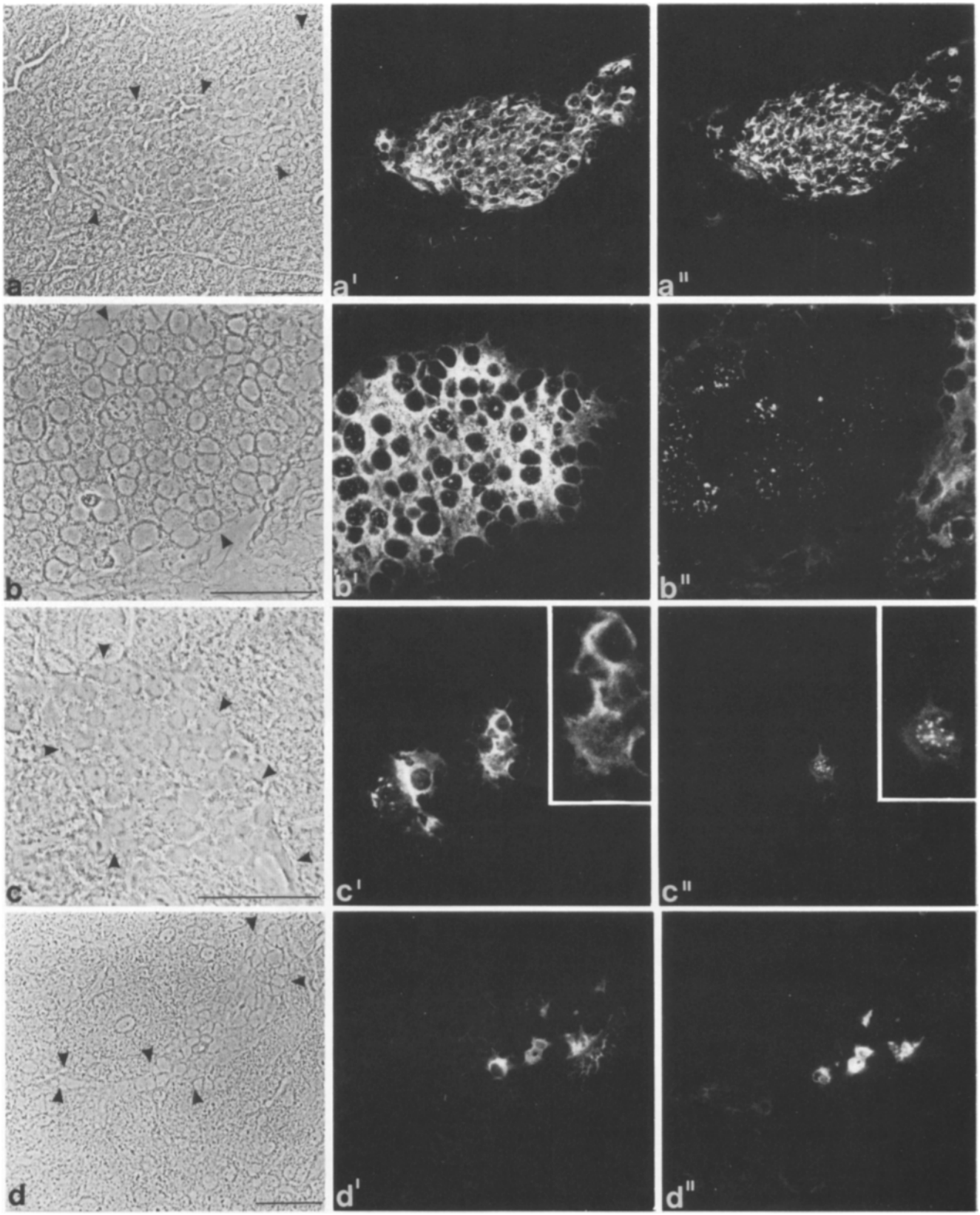
Mice of line Tg-RIP-H10 containing only the human CK 10 gene (two different founder lines, Tg1 and Tg2, and a total of 47 animals) showed synthesis of human epidermal CK 10 in β cells of pancreatic islets which appeared in abundant and relatively large fibrillar structures (Figs. 3, *a*–*a*″, and 4, *a* and *a*′). In double-label immunofluorescence microscopy, most of the CK 10-positive fibrils were also positive for the endogenous CKs 8 and 18 (Fig. 4, *a* and *a*′). The occurrence of both ectopic human CK 10 and endogenous murine CKs 8 and 18 in the massive fibrils, which are not seen in islets of normal mice, was also evident from step-sections immunostained with CK antibodies of the Troma series (not shown). EM showed that this additional CK 10 was contained in massive bundles of normal-looking but densely fasciated IFs (see below). The CK 10-positive cells showed normal intensity of immunostaining for insulin (Fig. 3 *a*′) and general neuroendocrine markers such as synaptophysin (not shown; see references 68, 81). These mice were healthy and their life span was normal. Only very occasionally, cells with sparse nuclear CK 10-immunofluorescence were also seen.

By contrast, mice that contained and expressed the human CK 1 gene, either alone (Tg-RIP-H1; total number of animals studied: 40), or together with that for CK 10 (Tg-RIP-H1/10; total number: 69), invariably developed, mostly at 2–10 wk of age, symptoms characteristic of IDDM with polydipsia and polyuria, typical diabetic behavior, and serum glucose concentrations of a mean value of 760 mg/dl (range: 634–990 mg/dl; nontransgenic controls and Tg-RIP-H10 mice presented mean values of 181 mg/dl). IDDM was also seen in the F1 offspring of matings between Tg-RIP-H1 males and Tg-RIP-H10 females which carried the RIP-H1 transgene, either alone or together with the maternally inherited RIP-H10 transgene. The disease usually progressed only moderately in both sexes but, in the absence of dietary and therapeutic interventions, invariably led to death within several months.

Histological examination of the pancreatic islets of CK 1-positive mice showed, with increasing time, some disorder of tissue architecture with an increased frequency of irregularly contoured, shrunken islets, and sparse, scattered β-cells (see Fig. 3, *b*–*d*″). On immunofluorescence microscopy with antibodies to neuroendocrine marker proteins and to CKs, extensive alterations of the IF cytoskeleton were noticed in these mice. Most notable was the appearance of small dot-like CK 1-positive structures, usually both in the nucleus and in the cytoplasm, which were also seen in Tg-RIP-H1/10 double-transgenic mice (Fig. 3, *b*–*b*″).

Double-label immunofluorescence microscopy revealed that most β-cells could routinely be identified by their positive reactions for synaptophysin and insulin as well as for human CK 1 and, in Tg-RIP-H1/10 mice, CK 10 (Fig. 3, *b*–*d*″). The positive CK 1 or CK 10 reactions often appeared as dots, rods, or whiskers, some of which could be related to the cytoplasm, whereas others were clearly located at or within

Figure 3. Expression of human epidermal CKs in pancreatic islet cells of transgenic mice and progressive changes of islet organization and/or the frequency of β cells. Phase contrast (*a*–*d*) and double label immunofluorescence (*a*′–*d*″) microscopy of frozen sections through pancreata of transgenic mice of lines Tg1-RIP-H10 at the age of 15 mos (*a*, *a*′ and *a*″), and Tg1-RIP-H1 of 2 (*b*, *b*′ and *b*″) and 10 (*c*, *c*′, and *c*″) weeks, and in double-transgenic mice of line Tg1-RIP-H1/H10 at postnatal week 10 (*d*, *d*′, and *d*″), immunostained with guinea pig antibodies to insulin (*a*′, *b*′, *c*′, and *d*′) and mAb K_K8.60 specific for CKs 1 and 10 (*a*″, *b*″, *c*″, and *d*″). The contours of the islets



are demarcated by arrowheads. Note that most, probably all, β cells of Tg-RIP-H10 mice are positive for (pro-)- insulin (*a'*) and that extensive fibrous structures containing human CK 10 are seen in these cells (*a''*). Islets of mice expressing the transgenic CK 1 (*b–d*) show remarkable changes: in the islet shown in (*b'–b''*) many of the β cells (*b'*) produce human epidermal CK in small whisker- or dot-like structures, some of them apparently in nuclei (*b''*). With increasing age (*c–d''*) the number of cells positive for insulin (*c'* and *d'*) and CK (*c''* and *d''*) is drastically reduced (the insert in *c''* shows the intranuclear location of punctate CK deposits in an insulin-positive β-cell; see *c'*). Bars, 50 μm.

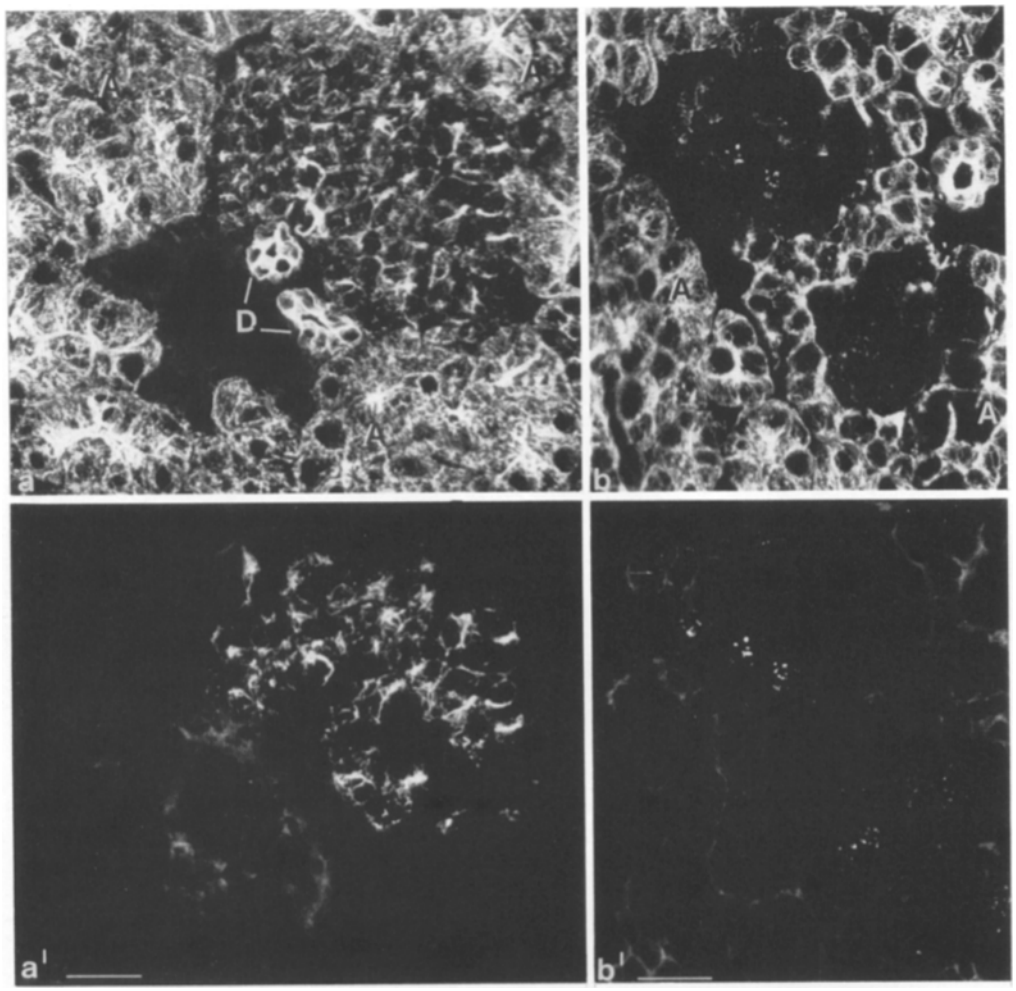


Figure 4. Colocalization of transgenic human CKs and complementary endogenous murine CKs 8 and 18 by double-label immunofluorescence microscopy of sections through pancreatic islets of transgenic female mice (3-mos-old) of lines Tgl-RIP-H10 (*a* and *a'*) and Tgl-RIP-Hl (*b* and *b'*), using guinea pig antibodies for CKs 8 and 18 (*a* and *b*) in combination with CK 10-detecting monoclonal murine antibody K_K8.60 (*a'*) or CK 1-specific antibody EAB-904 (*b'*). Note the intense reaction of CKs 8 and 18 on acinar (*A*) and ductal (*D*) structures compared with a less intense but fibrous reaction on the cells of the islets (*a* and *b*). In contrast, the reaction for the ectopic epidermal-type CKs 10 (*a'*) and 1 (*b'*) is exclusive for β-cells of islets where it appears predominantly fibrillar in (*a'*) or predominantly granular in (*b'*). In the latter situation, a number of the CK 1-positive "dots" are located within nuclei. In normal control mice, these islet cells show only very sparse, small and faint structures immunostained for CKs 8 and 18. Bars, 25 μm.

cell nuclei (Figs. 3, *b'* and *b''*, and 4, *b* and *b'*). Double-label immunofluorescence microscopy with additional antibodies reactive with murine CK 8 or CK 18 showed coincident immunostaining in many but not all CK 1-positive structures, indicating that most of them also contained endogenous CKs and that endogenous and ectopic CKs mix (Fig. 4, *b* and *b'*).

With progressing IDDM, the number of insulin-positive cells decreased drastically and the distribution of the residual β cells was also altered. Some of the few residual and scattered insulin-containing cells also showed punctate CK 1 and/or CK 10 immunofluorescence, which was often additionally, or even exclusively, in the nucleus (Fig. 3, *c–c''*). Essentially similar situations were found in pancreatic islets of Tg-RIP-Hl and Tg-RIP-Hl/10 mice (e.g., Figs. 3, *d–d''*, and 4 *b'*). Glucagon-positive α cells were still present at near-normal frequency and were generally negative for CK 1 and/or CK 10 (not shown).

The onset and the rate of development of IDDM symptoms in mice carrying the human CK 1 gene under RIP control varied considerably from mouse to mouse, even in the same litter. While some Tg-RIP-Hl/10 mice showed the first symptoms at postnatal weeks 2–4, others appeared symptom-free until weeks 8–10.

There was also remarkable variability between islets of the same pancreas and between β cells of the same islet. Normal-

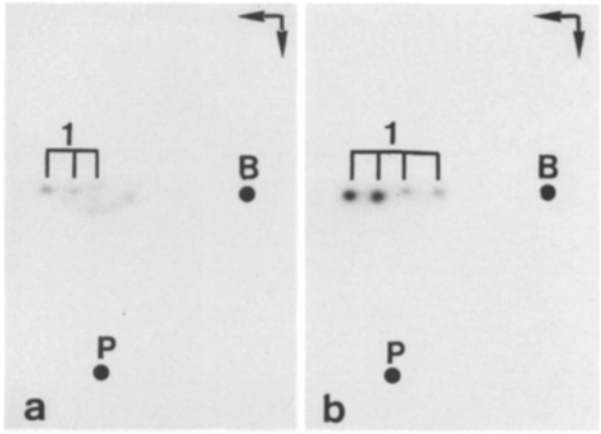


Figure 5. Autoradiofluorograph showing gel electrophoretic identification of transgenic gene product by immunoblot analysis of cytoskeletal proteins of pancreata from 10 transgenic mice of line Tgl-RIP-Hl (*a*) and four transgenic mice of line Tgl-RIP-Hl/10 (*b*) after separation by 2-D GE (*horizontal arrow*, direction of non-equilibrium pH gradient electrophoresis; *downward arrow*, SDS-PAGE), using antibody EAB-904 specific for CK 1 (isoelectric variants are denoted by *brackets*). Positions of reference proteins used for coelectrophoresis have been determined by Poinceau S staining. *B*, BSA; *P*, phosphoglycerokinase.

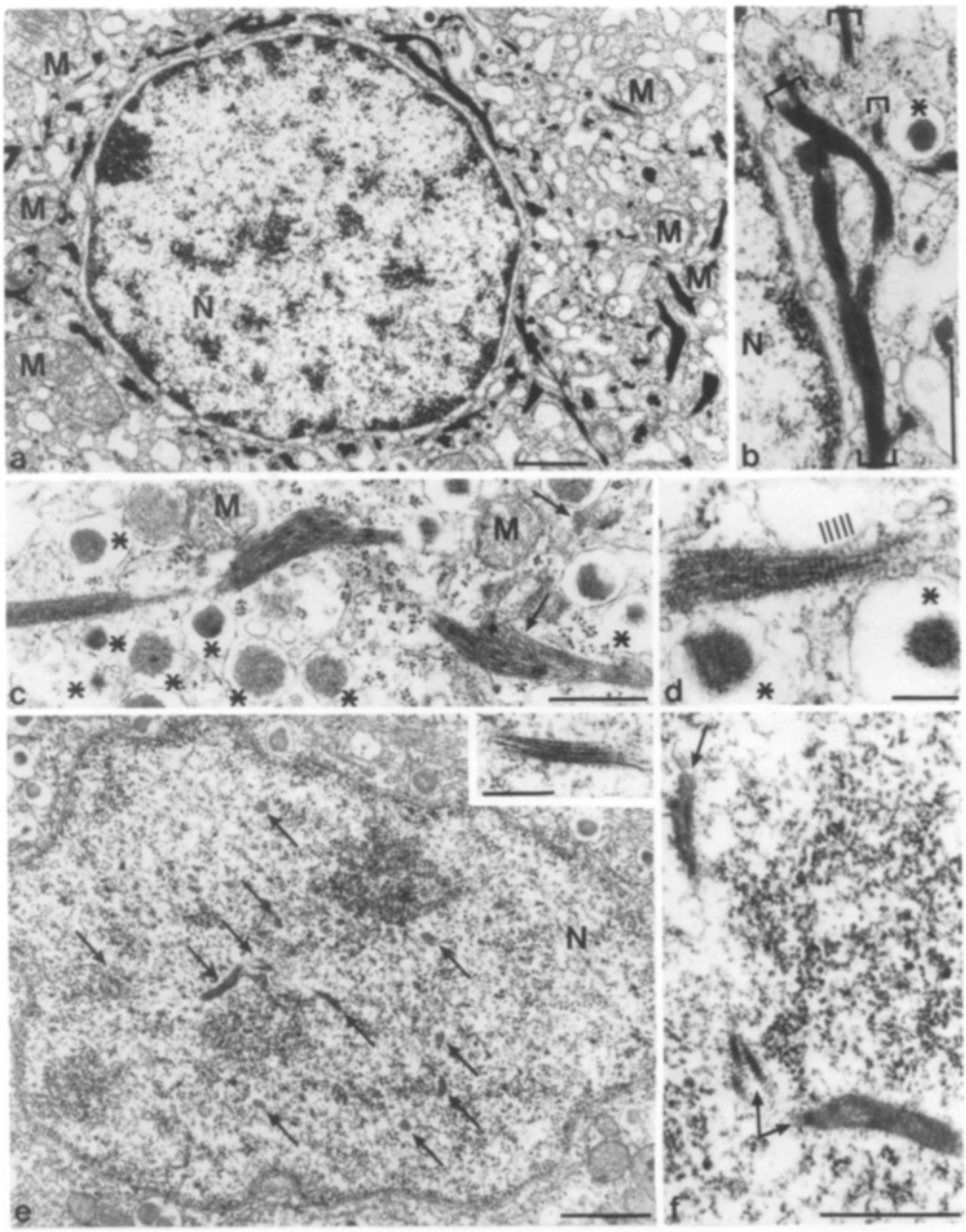


Figure 6. Electron micrographs showing the appearance of massive CK filament bundles in murine pancreatic β cells expressing human epidermal CK 1 and/or 10 alone or in combination. Pancreata of mice of lines Tgl-RIP-H1/10 (*a* and *b*; 8 wk old mice), Tgl-RIP-H10 (*c*, *d*; 8 wk) and Tgl-RIP-H1 (*e*, *f*; 5 wk) were fixed, embedded, ultrathin sectioned, and observed. Many β cells of islets containing numerous dense-core vesicles (some are denoted by *asterisks* in *b–d*) accumulate human epidermal CK 1 and/or 10 assembled into numerous and densely stained bundles (brackets in *b* and *c*) of tightly packed filaments (*a*, survey picture; *b*, partial magnification) in the cytoplasm (*a–d*) and/or the nucleoplasm (*arrows* in *e* and *f*; *e*, survey, *f*, partial magnification). Individual IFs are locally resolved in some cytoplasmic (*d*) and nuclear (*inset* in *e*) bundles. *N*, nucleus; *M*, mitochondria; *lines* in *d* denote the ~22-nm periodicity. Bars: (*a* and *e*) 1 μm; (*b*, *c*, and *f*) 0.5 μm; (*d*, and *inset* in *e*) 0.2 μm.

sized islets with normal morphology were seen next to others showing extensive structural distortion with only few scattered insulin-positive cells left. Immunofluorescence microscopy using a panel of markers for macrophages (monocytes) and lymphocytes (see Materials and Methods) did not reveal any significant inflammatory infiltration which would be indicative of autoimmune insulitis (not shown).

The expression of human CK 1 and CK 10 in murine pancreatic islets was confirmed by 2-D GE of cytoskeletal proteins residual after extractions with detergent and high-salt buffers (see reference 60), followed by immunoblotting with antibodies specific for human CK 1 and/or CK 10. Fig. 5 presents, for example, the identification of human CK 1 in pancreatic tissue of Tg-RIP-H1 (Fig. 5 *a*) and Tg-RIP-H1/10 (Fig. 5 *b*) mice.

Islet Pathogenesis As Seen in the Electron Microscope

In β cells, the new cytoskeletal structures comprising human epidermal CK 1, CK 10, or both were readily recognized as bundles of densely fasciated IFs throughout the cytoplasm, although often with a marked accumulation in the vicinity of the nucleus (Fig. 6, *a* and *b*). These abundant and thick epidermal CK-containing IF bundles, which often appeared in close association with the dense-core vesicles containing insulin (Fig. 6, *a–d*), differed strikingly from the sparse, short, and loose-packed tufts of the endogenous CK 8- and CK 18-containing IFs characteristic of normal islet cells.

The very dense packing of these new IF bundles containing epidermal CKs, up to 0.25 μm in diameter and sometimes showing local ~22-nm cross-striation (Fig. 6 *d*),

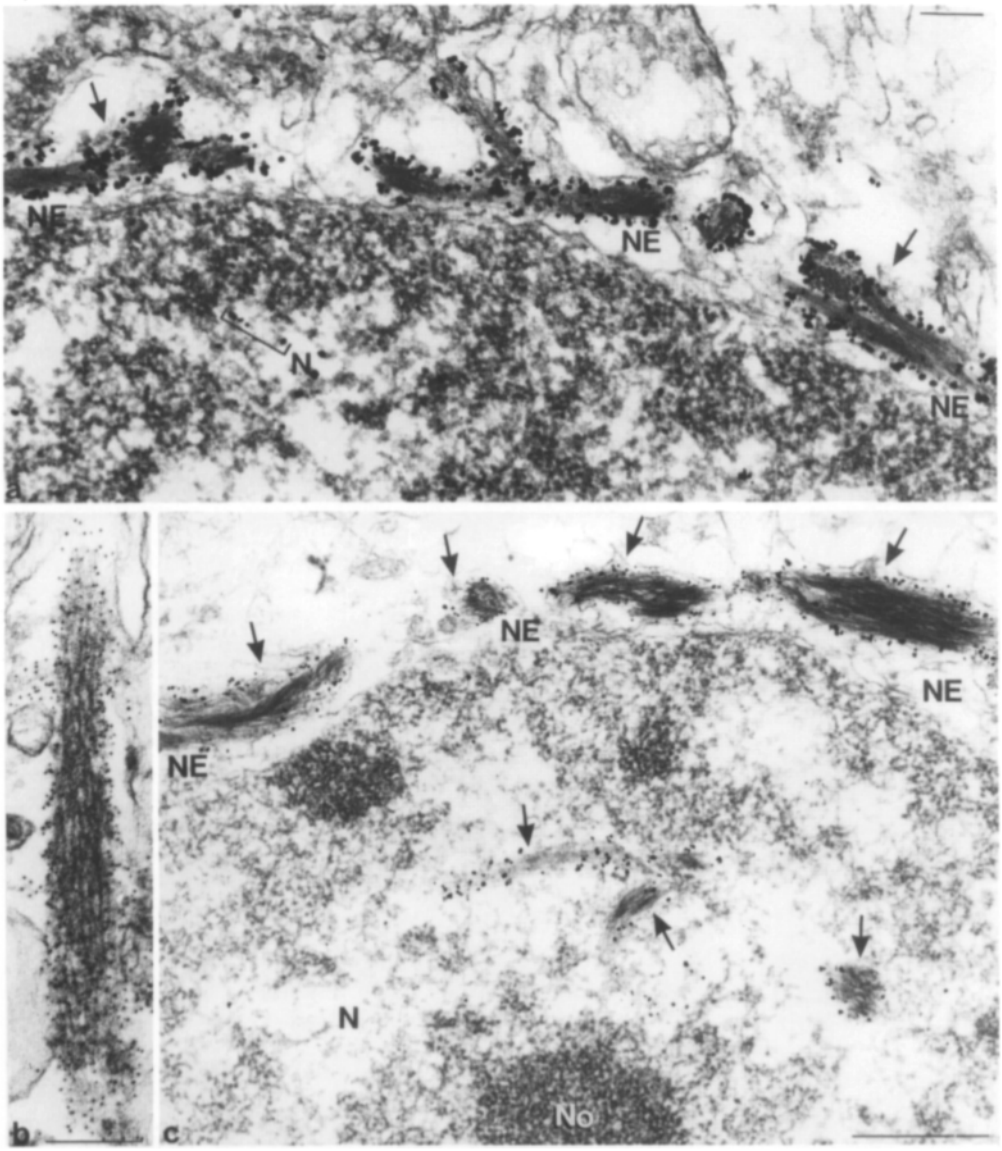


Figure 7. Electron microscopic immunolocalization of human epidermal CKs in pancreatic islet β cells of transgenic mice. An intensive immunogold reaction of mAbs EAB-904 and RKSE60, specific for human CKs 1 and 10, is seen in Tg-RIP-H1/10 mice, with (*a* and *c*) or without (*b*) silver enhancement of antibody-coated colloidal gold particles, showing the specific decoration of IF bundles with antibodies to CKs 1 and 10. Note intense labeling of juxtanuclear (10–30-nm-large silver grains in *a* and *c*, 5-nm gold particles in *b* and *c*; individual IFs are resolved in *b*) as well as intranuclear (*arrows* in *c* denote both cytoplasmic and nucleoplasmic structures) IF bundles. *N*, nucleus; *C*, cytoplasm; *NE*, nuclear envelope; *No*, nucleolus. The bracket in *a* indicates a nuclear reaction site not obviously related to an IF bundle. Bars: (*a* and *b*) 0.2 μm; (*c*) 0.5 μm.

strikingly resembled the IF bundle aggregates typical of differentiating epidermal keratinocytes, notably in the granular layer (e.g., 17, 63).

In a number of islet cells, densely packed and darkly stained bundles of IF structures were also detected in the nucleoplasm (Fig. 6 *e*), often at high frequency, and these also occasionally revealed local cross-striation (Fig. 6 *f*). Nuclear bundles of filaments or other CK structures were only rarely detected in Tg-RIP-H10 transgenic mice but were very frequent in the two lines carrying CK 1.

That these unusually thick and dense bundles of IFs indeed contained the human epidermal CKs 1 and/or 10 was shown by immunoelectron microscopy (Fig. 7, *a–c*). Besides the immunogold particle decoration of these bundles, we also occasionally found small clusters of gold particles elsewhere in the nucleus (e.g., Fig. 7 *a*), indicating that some of the foreign CK material may exist in non-IF states.

In addition to fibrillar CK assemblies, we also noted, in Tg-RIP-H1 mice, accumulations of variously sized, irregularly shaped and densely stained amorphous CK material, in the cytoplasm and/or in the nucleus, which by immunogold labeling showed intense CK 1-label (not shown).

During IDDM development, we noticed gross structural changes in CK 1-expressing β cells. In early stages, the cells containing bundles of the ectopic CK did not display any other conspicuous alterations and their cytoplasm was still crowded with the typical dense-cored, insulin-containing secretory vesicles (see Fig. 6, *a–d*). However, in more advanced stages, a number of β cells were seen that contained dense CK bundles in the cytoplasm (and often also in the nucleoplasm) but were drastically depleted in dense-core vesicles (Figs. 8, *a–d*, and 9, *a–d*).

In the electron microscope, such β cells which appeared "light" due to lower frequency of dense-core vesicles, were best identified at low magnification, often in the immediate vicinity of normal-looking islet cells with numerous dense-core vesicles (Figs. 8, *a* and *b*, and 9, *a* and *b*). In the same "light cells," we often found, on careful examination, cytoplasmic CK bundles together with aggregates of CK filament material throughout the nucleus. These intranuclear CK aggregates did not show regular associations with blocks of dense chromation and/or the nucleolus (Fig. 9, *b–d*).

Otherwise, most CK 1-positive β cells, whether depleted in dense-core vesicles or not, did not reveal major altera-

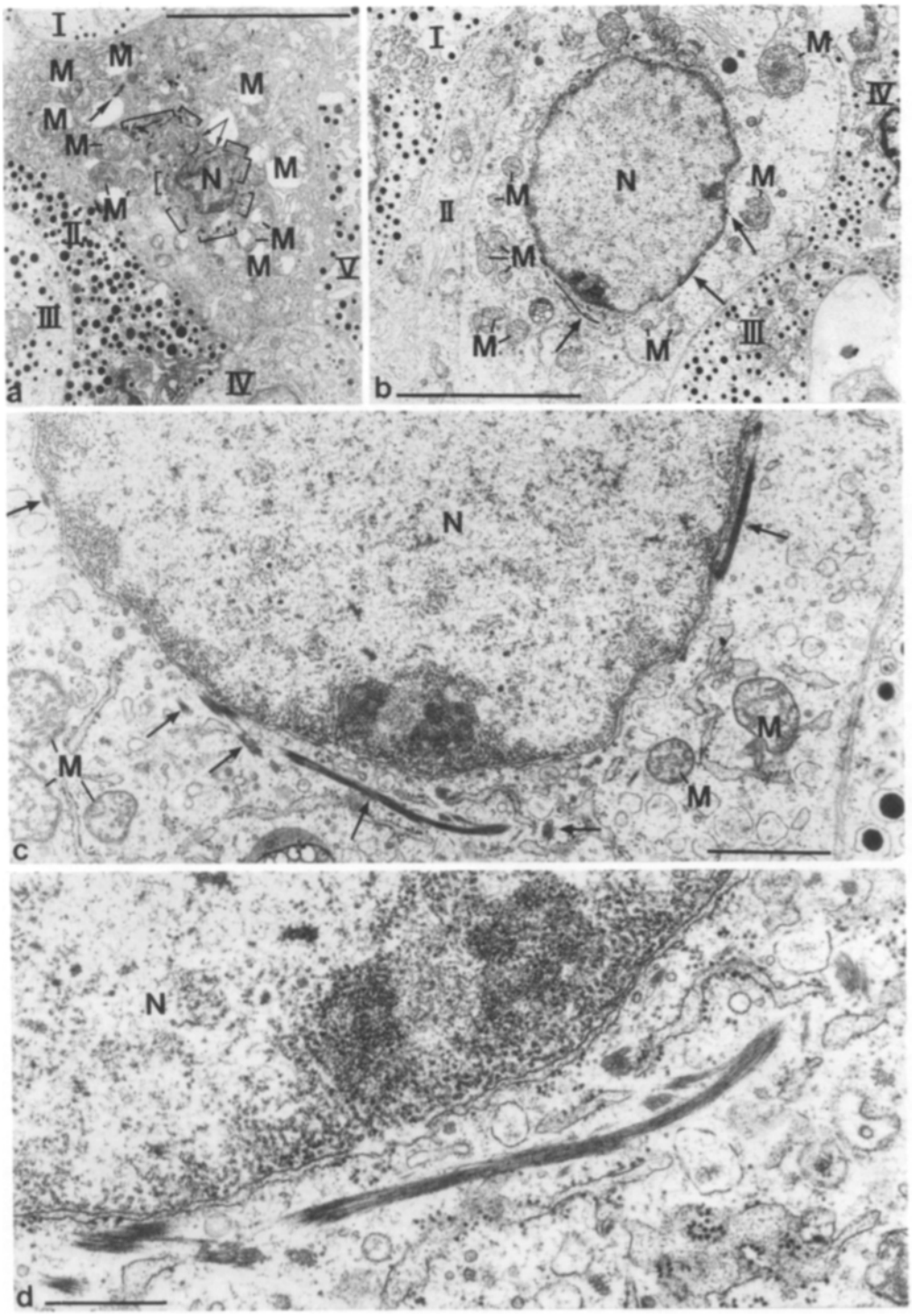


Figure 8. EM of "light cells" in pancreatic islets of transgenic mice characterized by the appearance of massive CK bundles and a reduction of dense-core variables. Islets of mice of line Tg-RIP-H1/10 with beginning IDDM contain a number of β cells the cytoplasm of which, at low magnification, appears "light" in overall electron density, due to the drastic reduction in the number and frequency of dense-core vesicles. These cells also contain extensive CK IF bundles typical of those containing epidermal CK 1 and/or 10. (*a* and *b*) Survey micrographs showing light cells with very few dense-core vesicles, surrounded by normal-looking cells rich in dense-core vesicles (denoted by roman numerals); another light-appearing cell is designated II in *b*. On closer inspection, these light cells are seen to contain many bundles of densely packed CK IFs (denoted by *brackets* and *arrows*). (*c* and *d*) Successively higher magnifications of a region of (*b*), showing details of the distribution and organization of these CK bundles which often are concentrated in the juxtanuclear region, more or less parallel to the nuclear envelope (*arrows* in *c*; individual filaments can be recognized in *d*). *N*, nuclei; *M*, mitochondria. Bars: (*a* and *b*) 5 μm; (*c*) 1 μm; (*d*) 0.5 μm.

tions. Only rarely did we observe nuclear pyknosis, extensive (auto-)phagocytosis or other features characteristic of apoptotic or necrotic damage.

Discussion

Obviously, for the secretory activity and the viability of pancreatic islet β cells the specific composition and/or arrangement of its IF system does matter. The drastic changes of the cytoskeleton and of insulin secretory vesicle frequency, together with IDDM development, that we have observed in these cells upon ectopic expression of a certain kind of CK, i.e., CK 1, show that the correct IF cytoskeleton can be of critical importance for the function and stability of a given cell type. This observation contrasts a series of previous studies reporting that the advent of nonorthotypic IF proteins in various cultured cell lines, be it spontaneous, induced by drug treatment, or resulting from gene transfection, does not result in demonstrable changes of cell shape, function, or proliferation (e.g., 6, 8, 34, 51, 57, 67, 73).

The remarkable specificity of transgenic IDDM induction observed for CK 1, but not for CK 10, excludes several possible simple explanations of regulation and pathogenesis. Obviously, the structural changes in, and losses of, β cells and

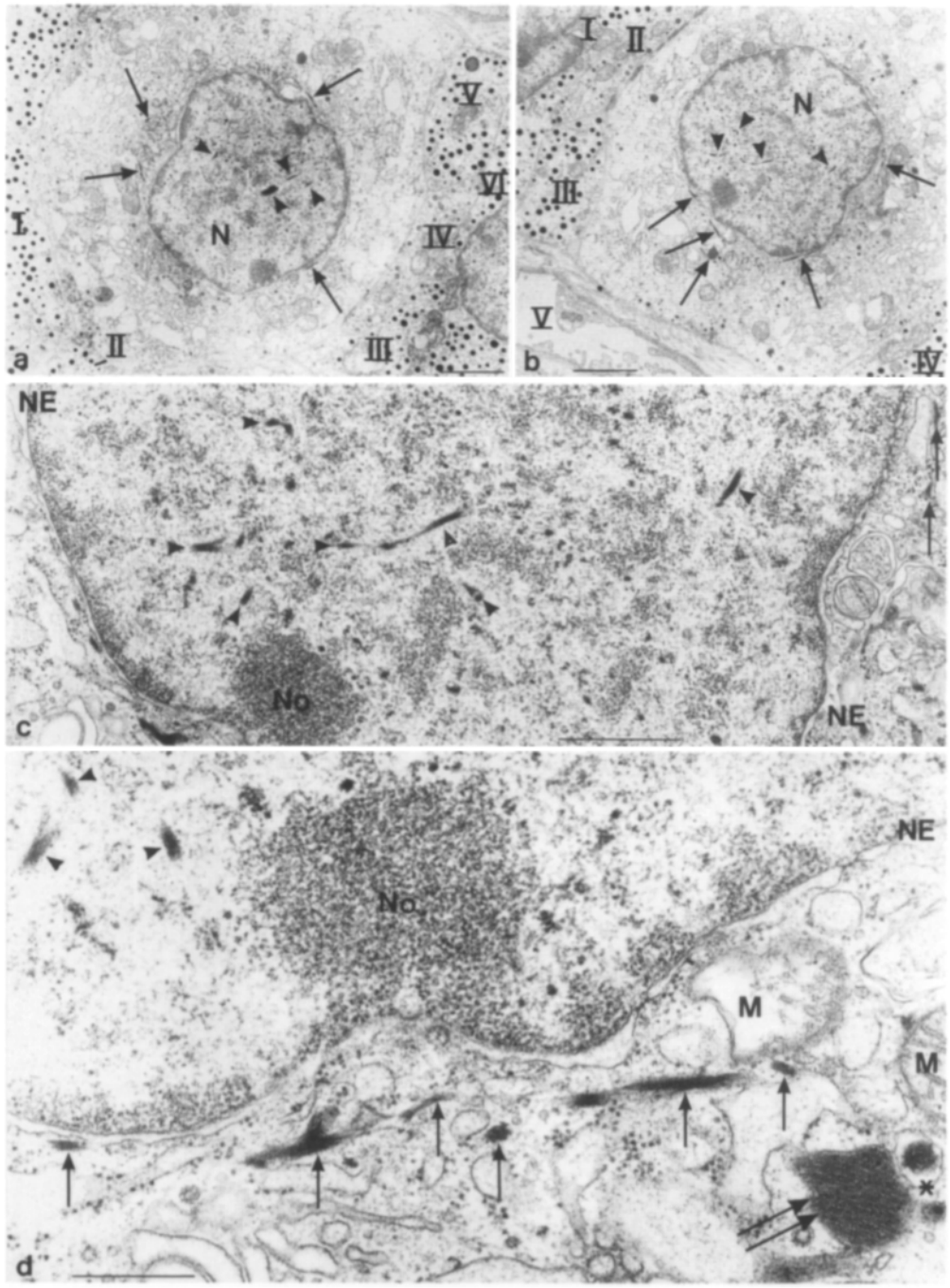


Figure 9. EM showing intranuclear filament bundles of human epidermal cytokeratins ectopically expressed in transgenic mice. "Light" pancreatic islet β cells (*N*, nuclei) of transgenic mice (same as described in Fig. 7) also contain densely stained intranuclear filament bundles or deposits of tightly packed CK material (*a* and *b*, survey micrographs; *c* and *d*, partial magnifications of regions shown in *b*) that is not seen in the surrounding normal-looking "dark" cells rich in dense-core vesicles (Roman numerals; *V* in *b* denotes a vascular lumen of a capillary). These intranuclear IF protein structures are denoted by arrowheads, cytoplasmic IF bundles by arrows, and a particularly massive paracrystalline IF array (in *d*) by a double arrow. The presence of some residual dense-core vesicles in these cells identifies them as endocrine cells (see, e.g., the enrichment at the basal membrane of the "light cell" shown in *b* and the vesicle indicated by an asterisk at the right margin in *d*. *NE*, nuclear envelope; *No*, nucleolus; *M*, mitochondria. Bars: (*a* and *b*) 2 μm; (*c*) 1 μm; (*d*) 0.5 μm.

the development of hyperglycemia are not merely the result of the occurrence of a foreign CK, a generally greater abundance of unusually massive IF bundles, or the competition for RIP-binding transcription factors. At present, we cannot explain why the β cells tolerate the type I CK 10 but not the type II CK 1, even when the latter is synthesized together with its "natural" partner, CK 10. It is clear, however, that the "toxic" and diabetogenic principle is associated with CK 1.

CK IFs are obligatory heteropolymers of type I and II CK polypeptides. As CK polypeptides of one type alone would not form IFs unless they can bind to a CK of complementary type (e.g., 38, 39, 48, 51, 53), it is evident that the epidermal CK 1 or CK 10 additionally synthesized in β cells must be stabilized by stoichiometric amounts of complementary type murine CK(s). The results of our double-immunolabel experiments, showing colocalization of transgenic CK 1 or CK 10 with endogenous CK 8 (in the case of Tg-RIP-H10) or CK 18 (in Tg-RIP-H1 mice), suggest that here the synthesis of the specific complementary endogenous CK is upregulated, to contribute to the formation of the new heterotypic and heterologous CK filaments.

In our transgenic experiments, the synthesis in β-islet cells of large amounts of CKs that normally are synthesized only during suprabasal differentiation of epidermal keratinocytes results in the accumulation of bundles of IFs packed as tightly and stained as densely as the CK "macrofibrils" typical of the upper strata of epidermis and other stratified

epithelia (17, 31). It is widely thought that, in the granular cell layer of epidermis, this dense IF fasciation involves specific aggregation-promoting molecules such as filaggrin (17) or other IF-associating proteins (IFAPs; for reviews see 17, 75). However, in the β-islet cells containing IF bundles of transgenic CKs 1 and/or 10 we have not detected, immunohistochemically, any filaggrin (data not shown). Therefore, although we cannot formally exclude the contribution of a β cell–specific IFAP, we take this as an indication that tight macrofibril-like IF bundling is a potential intrinsic to CKs 1 and/or 10 (for a high aggregation tendency, although unoriented, of IFs formed from purified CKs 1 and 10 see reference 24). An unusually high intrinsic stability and resistance to solubilization and denaturation for IFs containing CKs 1 and 10 has also been reported (see references 23, 27), suggesting that in the β cells the IFs containing transgenic CKs 1 and/or 10 have structural properties different from those of the sparse, loose and short IFs comprising only endogenous CKs 8 and 18.

Completely unexpected was our observation that fibrillar structures containing the foreign CK 1 frequently appear not only in the cytoplasm but also in the nucleus. In some cells, it even looked as if the majority, if not the total, of the ectopic CK was deposited within the confinements of the nuclear envelope. Recent observations of nuclear IF accumulations of several CKs with deleted carboxyterminal ("tail") domains (6) as well as tail-truncated vimentin (20) indicate that this part of the molecule normally contributes to its correct, i.e., cytoplasmic compartmentalization. Our present results now show that nuclear accumulation of CK IFs is not simply a consequence of tail truncation but can also be observed with certain intact CK molecules appearing in the "wrong cell type." This points to the possible importance of IF-protein interaction with other cell-specific elements to restrict IF distribution and secure their correct compartmentalization to the cytoplasm. The mechanisms by which such relatively large molecules reach (and accumulate in) the interphase nucleus are unclear. Apparently, their nuclear uptake does not depend on a special nuclear "accumulation" sequence (for review see reference 74).

Whether the unusual accumulation of ectopic CK 1 in nuclei of β cells contributes to IDDM pathogenesis also remains to be examined. At any rate, our study provides a clear example to show that the synthesis of a "wrong" or of "too much" IF protein in a cell can result in dysfunction and death, be it by its interference with cytoplasmic functions or due to its incorrect, i.e., nuclear location. In future transgenic experiments we will study the specific mechanisms of nuclear IF deposition and functional consequences.

Development of IDDM as a consequence of the transgenic introduction of ectopic proteins into β cells of pancreatic islets has not been noticed with a large number of gene constructs (22, 37) but has been reported only for certain apparently unrelated genes, including those encoding Ha-*ras* protein (21, 22), calmodulin (26), γ-interferon (IFN-γ; 72), several viral antigens (65, 70), and class I or II histocompatibility complex (MHC) polypeptides (5, 9, 37, 56, 58, 59, 72). In most of these studies, IDDM was associated with reduced insulin secretion and/or a loss of β cells. Obviously, the β cell and its secretory function is sensitive to a diversity of disturbances of cellular architecture and protein composition.

However, while for some of these situations β cell pathogenesis was ascribed to autoimmune reaction and inflammatory destruction resulting in histological insulitis (e.g., 65, 70, 72), such an immunological basis of cell dysfunction disease is considered unlikely in the other studies. Recently, autoantibodies to peripherin, a minor IF protein of islet β cells, have been reported to be characteristic for the insulitits and IDDM of nonobese diabetic mice (10). In our study we have not noticed the occurrence of significant antibodies to β-cell antigens or to other pancreatic structures in the sera of transgenic mice expressing the RIP-CK 1 gene, nor any signs of insulitis, including infiltration of lymphocytes and/or macrophages/mononucleocytes. This suggests that autoimmune reactions are not causally involved in IDDM development of mice expressing CK 1 in their β cells. Consequently, this form of IDDM should not yet be regarded as typical "type I IDDM" (25) but rather as an IDDM form sui generis. In this respect, the CK 1–induced β-cell dysfunction and depletion seems to be similar to certain other transgenic IDDM models from which it, however, differs in several other characteristics: in the CK 1-induced IDDM we have not noted a preference of pathogenesis for males as in MHC (59; see reference 56) and Ha-*ras* (21, 22) transgenic animals. While irregular organization and some vesicle reduction ("degranulation") of β cells have also been described for mice expressing "foreign" MHC genes (e.g., 5, 56, 59, 72) or overexpressing calmodulin (26), the markedly decreased frequency of secretory vesicles described in this study has only been reported for the male-specific Ha-*ras* situation (22) in which the defect appeared late, i.e., after ~5 mos.

At present, we cannot decide which pathogenic mechanism relates the advent of CK 1 and CK 1–containing fibrils to the disappearance of secretory vesicles and, finally, to cell death. As IFs can associate intimately with various membranous structures (14), a direct interaction with the membranes of the secretory apparatus is one possibility to consider but an interference with much earlier functions in the chains of syntheses, such as inhibitory effects in the cell nucleus by the intranuclear CK 1–containing aggregates is likewise possible. Hopefully, future experiments, using other CK gene constructs, including CK 1 deletion mutations and some that will result in overproduction of endogenous CK 8 or 18, will help to identify the specific pathogenic principles and domains in CK 1 and to understand why transgenic changes in the cellular complement of certain proteins result in an impairment of β-cell functions and survival, whereas other transgenically introduced proteins are well tolerated.

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