Identification of a Calcium-regulated Insulinoma Cell Phosphoprotein as an Islet Cell Keratin

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ABSTRACT This report describes the cytoskeleton nature of a 60,000-mol-wt protein, P_{60}, previously shown to undergo Ca^{2+} influx–induced phosphorylation concomitant with insulin release in hamster insulinoma cells. Four lines of evidence suggest that P_{60} is an intermediate filament protein of the keratin class. (*a*) As previously described (Schubart, U.K., 1982, *J. Biol. Chem.* 257:12231–12238), Triton X-100–insoluble cytoskeletons are enriched for P_{60}; (*b*) these cytoskeletons contain 7–11-nm filaments as determined by negative staining; (*c*) immunoblot analysis revealed that all proteins detected in the insulinoma cell cytoskeletons are recognized by a monoclonal antibody that interacts with a common determinant in all intermediate filament proteins; and (*d*) P_{60} was shown, by its identical migration on two-dimensional electrophoresis and by its immunologic relatedness, to be analogous to a known keratin present in HeLa cells. An antibody specific for P_{60}, as judged by immunoblotting, was developed in a rabbit. In indirect immunofluorescence studies on insulinoma cells, this anti-P_{60} antibody produced a filamentous staining pattern. The antibody also permitted the identification of P_{60} in normal pancreatic islets as determined both by immunoblotting of hamster islet proteins resolved by two-dimensional electrophoresis and by indirect immunofluorescence microscopy on cryostat sections of hamster pancreas. In addition, the antibody recognized an antigen in the epithelial layer of pancreatic exocrine ducts, as determined by indirect immunofluorescence. The data have implications for the embryonic origin of pancreatic islets. Together with the phosphorylation data, these findings suggest that this islet cell cytokeratin may be involved in the regulation of insulin release.

The critical role of Ca^{2+} in insulin release is well established (for review, see reference 1). To gain insights into the mechanism by which Ca^{2+} activates the secretory process, we are exploring the molecular events that occur in pancreatic beta-cells during Ca^{2+}-mediated insulin release. These studies have utilized cells isolated from a transplantable hamster insulinoma, which release insulin in response to depolarization-induced Ca^{2+} influx (2). The current hypothesis assumes that protein phosphorylation may be involved in Ca^{2+}-mediated insulin release (3). In this regard, we have demonstrated that these insulinoma cells contain a Ca^{2+}-activated protein kinase activity that also requires calmodulin (4). In addition, we have shown that depolarization-induced Ca^{2+} influx stimulates phosphorylation of a 60,000-mol-wt protein, P_{60}, concomitant with insulin release in intact insulinoma cells (5). Recent studies have suggested that this protein is associated with the cytoskeleton of these cells (6).

To explore the potential role of P_{60} in Ca^{2+}-mediated insulin release, we have directed the current studies toward identifying the nature of this protein in greater detail. As there are distinct differences between insulinoma cells and normal pancreatic beta-cells, it was also of importance to assess whether P_{60} is a protein specific to insulinoma cells or whether it is also expressed in normal islets.

The studies reported here demonstrate that P_{60} is a structural component of 10-nm filaments, which are generally referred to as intermediate-sized filaments (for review, see references 7 and 8). Based on electrophoretic and immunologic properties of P_{60}, it was possible to assign insulinoma cell intermediate filaments to the keratins, one of the five intermediate filament classes currently distinguished (7, 8). In addition, studies will be described that have shown, with the help of a polyclonal antiserum specific for P60, this protein to be present not only in insulinoma cells but also in normal pancreatic islets and in the epithelium that shares a common embryonic origin with islet cells.

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MATERIALS AND METHODS

Materials: $Na^{125}I$ (500 mCi/ml) was obtained from Amersham Corp. (Arlington Heights, IL); Triton X-100, phenylmethanesulfonyl fluoride, and DNase I were from Sigma Chemical Co. (St. Louis, MO); urea from Schwarz/Mann, (Orangeburg, NY); protein A from Pharmacia Inc., (Piscataway, NJ); nitrocellulose sheets (0.45 μm thick) from Schleicher and Schuell Inc., (Keene, NH); Iodobeads (*N*-chloro-benzene sulfonamide-derivatized nonporous polystyrene beads) from Pierce Chemical Co. (Rockford, IL); and polyvinylchloride microtitration plates from Dynatech Laboratories, Inc. (Alexandria, VA).

Preparation of Cytoskeletons: Hamster insulinomas were maintained by serial subcutaneous transplantation in female Syrian hamsters and insulinoma cells isolated by collagenase digestion as previously described (9). Cytoskeletons were prepared according to the procedure described by Franke et al. (10) with slight modifications. Cells were lysed for 3 min at 0–4°C in cell lysing buffer (140 mM NaCl, 1% Triton X-100 (vol/vol), 5 mM EDTA, 0.4 mM phenylmethanesulfonyl fluoride, 10 mM Tris-HCl, pH 7.6) and the nuclei and cytoskeletons were collected by low speed centrifugation (3,000 *g* for 5 min). The pellet was then extracted at 0–4°C with 1.5 M KCl in cell lysing buffer, to which DNase I and $MgCl_2$ were added to final concentrations of 1 mg/ml and 10 mM, respectively, to digest viscous chromatin material apparent at this step. This required ~10 min at 0–4°C. The cytoskeletons were then pelleted as described above and reextracted with the same high salt buffer (without the other additions) for 15 min at 0–4°C and washed twice with PBS. The final pellets were stored at −20°C or used directly for the studies described.

Electron Microscopy: Cytoskeletons resuspended in PBS were applied to carbon-coated electron microscope grids. After 1 min the preparations were negatively stained with 2% uranyl acetate. The grids were examined in a Siemens 101 electron microscope.

Two-dimensional Electrophoresis: Cytoskeleton pellets were dissolved in lysis buffer (11) and subjected to two-dimensional electrophoresis according to the method of O'Farrell (11) using minor modifications as previously described (6). The second dimension SDS polyacrylamide slab gels (8.5% acrylamide) were either stained with Coomassie Blue as previously described (4) or used for immunoblotting studies (see below).

Antibodies: The anti-P_{60} antibody was raised in a New Zealand rabbit. P_{60} was obtained after two-dimensional electrophoresis by excising the corresponding protein spots from multiple second dimension SDS polyacrylamide slab gels stained with Coomassie Blue. The gel slices were minced with scissors and liquified in a tightly fitting stainless steel Dounce homogenizer. The resultant liquid was mixed in a plastic syringe with an equal volume of complete Freund's adjuvant and an emulsion was prepared by sonication. After preimmune serum was collected, the rabbit was injected at multiple intradermal and subcutaneous dorsal sites with an estimated total amount of 50–100 μg protein. Subcutaneous booster injections (~25–50 μg protein) were administered 1 and 2 mo later employing emulsions of gel slices prepared as described above except that incomplete Freund's adjuvant was used.

The rabbit was bled multiple times and the antibody titer was determined by a solid-phase radioimmunoassay as described below. All studies reported utilized serum from a single bleed.

Rabbit antivimentin antibody (12) was provided by Dr. Richard O. Hynes, Massachusetts Institute of Technology, and a mouse monoclonal antibody against a common antigenic determinant in intermediate filament proteins (13) by Dr. Rebecca M. Pruss, National Institutes of Health. A mixture of rat monoclonal antibodies specific for mouse kappa and lambda light chains, respectively, biosynthetically labeled with [^{14}C]Arg and [^{14}C]Lys (14) was a gift from Dr. Matthew D. Scharff (Albert Einstein College of Medicine). Rhodamine-labeled goat anti-rabbit immunoglobulin was from Miles Laboratories, Inc. (Elkhart, IN).

Antibody Titration by Solid-phase Radioimmunoassay: The titer of anti-P_{60} antibody was determined by a solid-phase radioimmunoassay similar to the one described by Pierce and Klinman (15). Polyvinylchloride microtitration plates were coated at 0–4°C with 0.1-ml aliquots of insulinoma cell cytoskeletons, which had been solubilized in 8 M urea, 25 mM beta-mercaptoethanol, 10 mM Tris-HCl, pH 9.0, and diluted 50-fold to 50 μg/ml with PBS. The wells were rinsed three times with PBS and loaded with diluent (1% BSA, 1% fetal calf serum, 10 mM sodium azide in PBS), which, after 30 min at room temperature (RT),[1] was replaced by 0.1-ml aliquots of immune serum serially diluted in diluent. After incubation for 1 h at 37°C, rinsing with PBS was repeated as above. Diluent containing ^{125}I-labeled protein A (5×10^4 cpm/0.1 ml) was then added to each well followed by incubation for 30 min at 25°C. After three additional washes with PBS, the amount of ^{125}I-protein A bound to individual wells was determined in a gamma counter. Background radioactivity was determined by using serial dilutions of preimmune serum. The anti-P_{60} antiserum used in the studies described bound, under the conditions of this assay, 10% of the total radioactivity added (approximately three times background) at a dilution of 10^{-4}.

Radioiodination of Protein A: For radioiodination of protein A, 50 μl of 0.1 M sodium phosphate buffer, pH 7.0, one Iodobead, and 0.5 mCi $Na^{125}I$ (13–15 mCi/μg iodine) were added to a plastic test tube. After preincubation for 1 min at RT, 2 μl of protein A (5 mg/ml) was added. Following incubation for 1 min at RT, the reaction was terminated by transferring the reaction mixture to another test tube leaving the Iodobead behind. Under these conditions, 50–70% of the radioactivity was incorporated into trichloroacetic acid–insoluble protein. The reaction mixture was diluted by addition of 250 μl of diluent (1% BSA, 1% fetal calf serum, 10 mM sodium azide in PBS), and applied to a 10-ml Sephadex G-25 column that had been prewashed with 10 ml of diluent and equilibrated with PBS. The column was eluted with PBS and the fraction containing the initial peak of radioactivity (>95% protein bound) was mxied with diluent, aliquoted, and stored at −20°C for up to 3 wk without appreciable loss in immunoglobulin binding activity.

Immunoblotting Methods: Immunoblotting was carried out according to the method of Towbin et al. (16) with modifications. After two-dimensional electrophoresis, the proteins were transferred to a nitrocellulose sheet. Prior to electrophoretic transfer, the SDS polyacrylamide slab gel could be stored in a closed container at −20°C for several weeks without effect on the results obtained. After thawing, the gels were soaked briefly in 0.5% SDS. Electrophoretic transfer was carried out for 4 h at 50 V in a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Richmond, CA) filled with 25 mM Tris-192 mM glycine/methanol, 4:1 (vol/vol). The blot was stained briefly with amido black and destained as described (16) and notched at the margins to mark the position of reference proteins. It could then be stored in 10 mM sodium azide at 4°C for at least 2 wk. Before incubation with antibody, the blot was immersed in diluent (1% BSA, 1% fetal calf serum, 10 mM sodium azide in PBS) and incubated for 1 h at RT. The antibody to be tested was then directly added to obtain the desired dilution. Incubation was continued first for 30–60 min at RT with gentle shaking and then at 4°C overnight. The blot was then rinsed twice for 10 min with 1% (vol/vol) Triton X-100 in PBS and once with PBS.

Two methods were used for detecting the localization of the bound antibody. For identification of rabbit immunoglobulin, ^{125}I-protein A (5×10^5 cpm/ml diluent) was used employing a 1-h incubation at RT. The mouse monoclonal antibody (13) was localized using a ^{14}C-labeled rat monoclonal antibody (2.8×10^5 cpm/ml diluent) specific for mouse immunoglobulin light chains (14) in an overnight incubation at 0–4°C. The blots were then washed with mechanical shaking in four changes of 1% Triton X-100 (vol/vol) in PBS (each wash lasting at least 20 min). After a brief rinse in PBS, the blots were dried between filter papers and mounted on cardboard. The bound radioactive tracer was localized by autoradiography using Kodax SB-5 X-ray film. Additional details are provided in the legends to Fig. 2, 3, 5, and 8.

Immunofluorescence Microscopy: For indirect immunofluorescence microscopy, smears of freshly isolated cells (hamster insulinoma cells), cells grown on glass coverslips (HeLa cells), or cryostat sections (10 μm) were allowed to dry at RT for 1 h. They were then fixed for 30 s with 1% acetic acid/99% ethanol at −20°C and rinsed extensively with PBS. After a brief application of diluent (1% BSA, 1% fetal calf serum, 10 mM sodium azide in PBS), the sections were covered with antiserum in diluent and incubated in a moist chamber for 45 min at RT. After rinsing with PBS, diluent was again applied briefly and replaced by rhodamine-labeled goat anti-rabbit immunoglobulin diluted 1:300 in diluent. After 30 min at RT, the sections were rinsed three times with PBS, fixed with 5% acetic acid/95% ethanol for 30 s at −20°C, and rinsed extensively with PBS. The slides were mounted in glycerol/PBS, 1:1 (vol/vol) and examined on a Zeiss standard microscope equipped with epifluorescent UV illumination, selective filters for fluorescein or rhodamine, and alternate phase-contrast illumination as previously described (17). For photography, Kodak Tri-X film was used. For additional details see the legends to Fig. 4, 6, and 7.

Pancreatic islets were isolated from female Syrian hamsters using the method of Lacy and Kostianovsky (18).

HeLa cells were obtained from Dr. Shu-Hui Yen (Albert Einstein College of Medicine) and grown with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

Protein was determined by a modification (19) of the method of Lowry et al. (20) using BSA as standard.

RESULTS

Demonstration of the Presence of Intermediate Filaments in Insulinoma Cell Cytoskeletons

Previous studies on the subcellular localization of P_{60} have demonstrated that the protein is highly enriched in a Triton

[1] *Abbreviation used in this paper:* RT, room temperature.

X-100–insoluble cytoskeleton residue (6). To identify the structural elements present in such preparations, we examined cytoskeletons by electron microscopy using negative staining. As shown in Fig. 1, such preparations contain two apparent size classes of filaments. The predominant structures are unbranched filaments of nearly uniform diameter (7–11 nm). Filaments of this appearance found in cytoskeletons of many cells are generally referered to as intermediate-sized filaments (7, 8). In addition, thinner filamentous elements (2–4 nm diam) were noted (Fig. 1). These may represent intermediate filaments partially disassembled in the course of the preparation of cytoskeleton residues. Similar structures observed in preparations of disassembled intermediate filaments from various other sources have been referred to as protofilaments (21–24).

These findings demonstrate that the predominant structural elements of insulinoma cell cytoskeleton preparations closely resemble intermediate filaments of other cell types. Since P_{60} is one of the four major proteins that are present in these preparations (see below), it is likely that it is a structural component of intermediate filaments.

Immunologic Identification of P_{60} as an Insulinoma Cell Intermediate Filament Protein

The proteins present in the Triton X-100–insoluble cytoskeleton residue obtained from insulinoma cells were resolved by two-dimensional electrophoresis and analyzed by immunoblotting. A representative gel stained with Coomassie Blue is shown in Fig. 2*a*. As demonstrated in previous studies, in which cytoskeletons obtained from ^{32}P-labeled cells were analyzed by two-dimensional electrophoresis and peptide mapping (6), these preparations contain, in addition to P_{60}, three other major proteins, all present in a partially phosphorylated state. To illustrate this, the unphosphorylated form of P_{60}, molecular weight 60,000, isoelectric pH 6.0, is indicated by the upward arrow (Fig. 2*a*, darker spot). The unphosphorylated forms of the other three proteins are indicated by arrowheads. Their apparent molecular weight and isoelectric pH are 59,000 and 6.3, 46,000 and 5.6, and 40,000 and 5.2, respectively. The proteins migrating on the acidic side of all four proteins are their phosphorylated derivatives (6). A minor protein also present in this preparation (oblique arrow, Fig. 2*a*) was identified as vimentin as shown in the immunoblot in Fig. 2*b*). A gel run identically to the one depicted in Fig. 2*a* was processed for immunoblotting using a vimentin-specific antibody (12). As evident in the corresponding autoradiogram (Fig. 2*b*), the only protein spot recognized by the antivimentin antibody was this minor protein (oblique arrow).

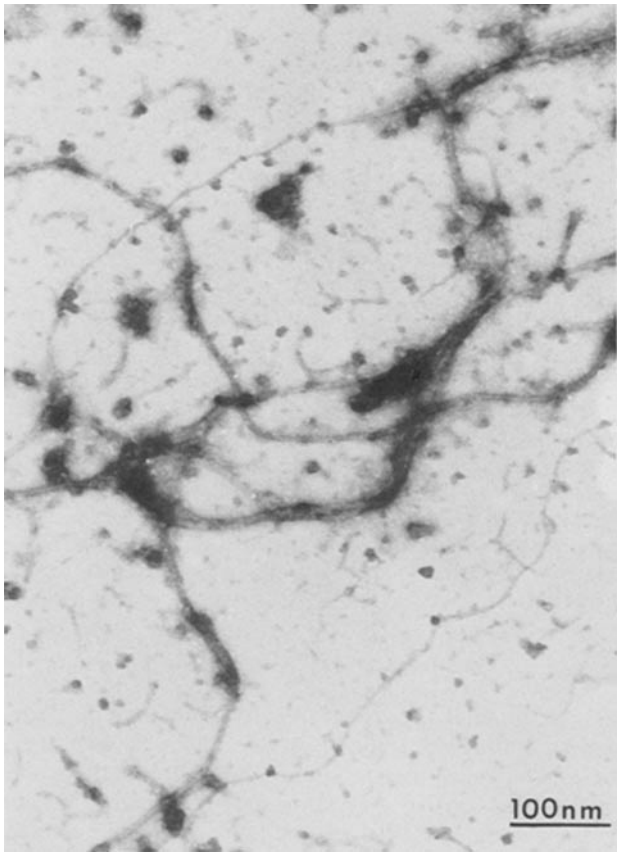

FIGURE 1 Ultrastructure of intermediate filaments isolated from hamster insulinoma cells. Triton X-100–insoluble cytoskeletons were prepared from freshly isolated insulinoma cells and negatively stained as described in Materials and Methods. Bar, = 0.1 μm. × 120,000.

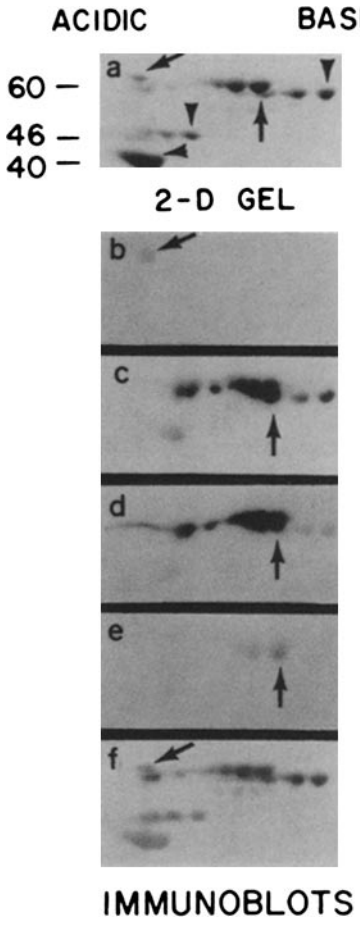

FIGURE 2 Immunoblot analysis of insulinoma cell cytoskeleton proteins resolved by two-dimensional electrophoresis. Aliquots (15 μg protein) of the same cytoskeleton preparation were subjected to two-dimensional electrophoresis (11) and either stained with Coomassie Blue as shown in *a*, or processed for immunoblotting as shown in the autoradiograms depicted in *b–f*. The antibodies used and their respective dilutions were rabbit antivimentin (12), 1:250 (*b*); rabbit anti-P_{60} (see Materials and Methods), 1:25 (*c*); 1:500 (*d*); and 1:5000 (*e*); and mouse monoclonal anti-intermediate filament antibody (13), 1:250 (*f*). The numbers at the left border represent molecular weight × 10^{-3}. Autoradiographic exposure was 6 h (*c*), 16 h (*b*, *d*, and *f*), and 3 d (*e*), respectively. The area of the gel (*a*) or autoradiograms (*b–f*) shown contains all proteins detected by staining or autoradiography, respectively.

An antibody against P_{60} was raised in a rabbit and its titer was determined by a solid-phase radioimmunoassay (see Materials and Methods). The specificity of this antibody was assessed by immunoblotting using the same insulinoma cytoskeleton preparation. Fig. 2 shows the results obtained with increasing dilutions of this antiserum. At lower dilutions (Fig. 2, *c* and *d*) some cross-reactivity was noted with the slightly more basic protein migrating near P_{60}. This cross-reactivity probably resulted from the presence in the immune serum,

although in lower titer than that of the anti-P_{60} antibody, of a second antibody that was inadvertently raised against this protein owing to the partial co-migration of the two proteins on two-dimensional electrophoresis (Fig. 2*a*, upward arrow). The additional minor spot (smaller and more acidic than P_{60}) also visible in Fig. 2, *c* and *d*, most likely represents a proteolytic fragment of P_{60} since it does not coincide with any of the other proteins present in the preparation. At the highest dilution utilized (1:5000), the antiserum recognized only P_{60} (upward arrow) and its phosphorylated derivatives (Fig. 2*e*).

The anti-P_{60} antiserum was also reacted with whole insulinoma cell proteins resolved by two-dimensional electrophoresis. The resulting gel stained with Coomassie Blue and the corresponding immunoblots are shown in Fig. 3. At a low dilution (1:25, Fig. 3*b*), the antibody detected a few minor spots in addition to P_{60} (upward arrow) and its more acidic forms. At a dilution of 1:100, P_{60} and its phosphorylated derivatives were the only antigens identified by the antiserum (Fig. 3*c*). The data demonstrate that this antiserum has a high degree of specificity for P_{60}.

These studies have also utilized a monoclonal antibody that is known to interact with an epitope common to all intermediate filament proteins (13). As shown in Fig. 2*f*, this antibody recognized all proteins present in this cytoskeleton preparation as detected by Coomassie Blue staining (Fig. 2*a*). Although it has not been unequivocally established that this antibody interacts exclusively with intermediate filament proteins, the data suggest that all proteins identified in the insulinoma cell cytoskeleton preparation may be subunits of intermediate filaments.

As some cells have been shown to express vimentin in addition to their cell type–specific intermediate filament protein(s) (for review, see references 7, 8, 25, and 26), the presence of a small amount of vimentin in the insulinoma cell cytoskeletons suggested that these cells might represent another example of co-expression of two intermediate filament types within the same cell. To explore this possibility, we examined insulinoma cells by indirect immunofluorescence microscopy. Fig. 4 shows the results obtained with anti-P_{60} antiserum. When the immunofluorescence (Fig. 4*a*) and corresponding phase-contrast (Fig. 4*b*) images are compared, it is evident that the insulinoma cells yielded strong fluorescence in a staining pattern reminiscent of intracellular filaments. No staining was seen without prior permeabilization of the cells (not shown) indicating that P_{60} is not expressed on the cell surface. Slight background fluorescence was observed when preimmune serum was used (Fig. 4*c*). In contrast to the findings obtained with the anti-P_{60} antibody, antivimentin antibody produced a selective immunofluorescence staining pattern. It bound to larger cells that accounted for <1% of the total cell population but did not interact with the insulinoma cells (Fig. 4, *e* and *f*). We conclude, therefore, that the small amount of vimentin detected in the insulinoma cytoskeleton preparations is not present in these insulinoma cells but in another cell type contaminating the cell preparation. Because of their coarse granular inclusions, these cells were tentatively identified as macrophages.

Presence of P_{60} in Normal Islet Cells and in the Epithelium of Pancreatic Exocrine Ducts

Pancreatic islets obtained from hamster pancreas by collagenase digestion (18) were examined for the presence of P_{60}.

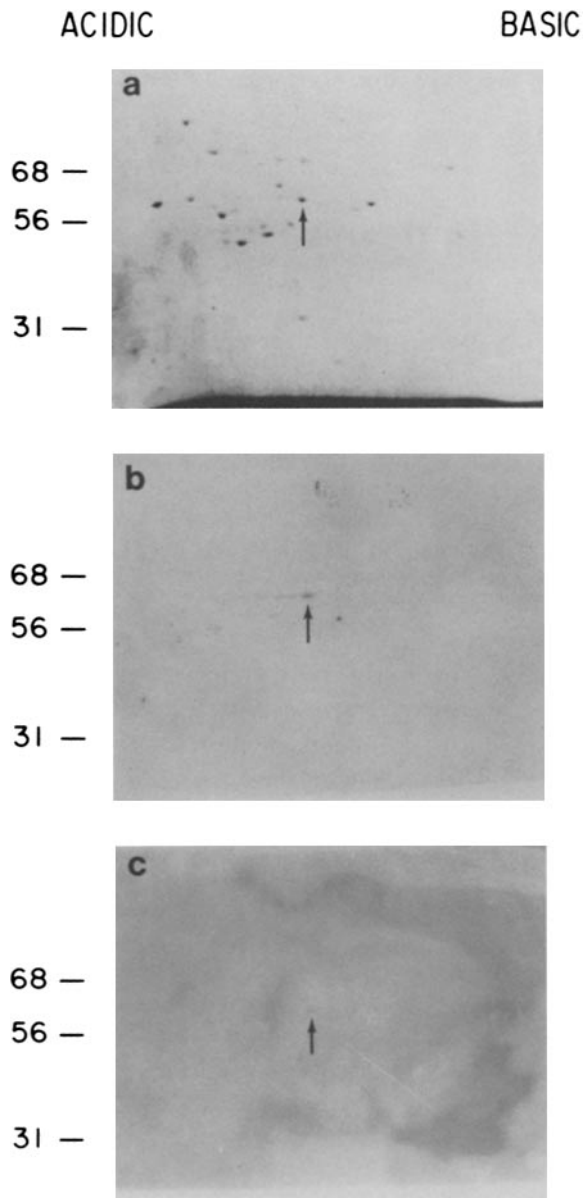

FIGURE 3 Immunoblot analysis of whole insulinoma cell proteins resolved by two-dimensional electrophoresis using anti-P_{60} antibody. Hamster insulinoma cells (150 μg protein) were directly dissolved in lysis buffer (11) and analyzed as in Fig 2. (a) Gel stained with Coomassie Blue; (*b* and *c*) immunoblots using anti-P_{60} antiserum diluted 1:25 (*b*) and 1:100 (*c*), respectively. Autoradiographic exposure: *b*, 2 d; *c*, 6 d. The numbers at the left border correspond to the positions of standard proteins (BSA, glutamate dehydrogenase, and carbonic anhydrase, respectively) applied adjacent to the isoelectric focusing gel on the SDS slab gel and represent molecular weight × 10^{-3}.

The islet proteins were resolved by two-dimensional electrophoresis and stained with Coomassie Blue as shown in Fig. 5*a*. For reference, note the most prominent protein spot, which is BSA carried over from the buffer used in the islet isolation. An identical gel was processed for immunoblotting and the corresponding autoradiogram is shown in Fig. 5*b*. The anti-P_{60} antibody interacted with a single group of closely migrating islet proteins exhibiting a migration behavior on two-dimensional electrophoresis identical to that of insulinoma cell P_{60} and its phosphorylated derivatives. The corre-

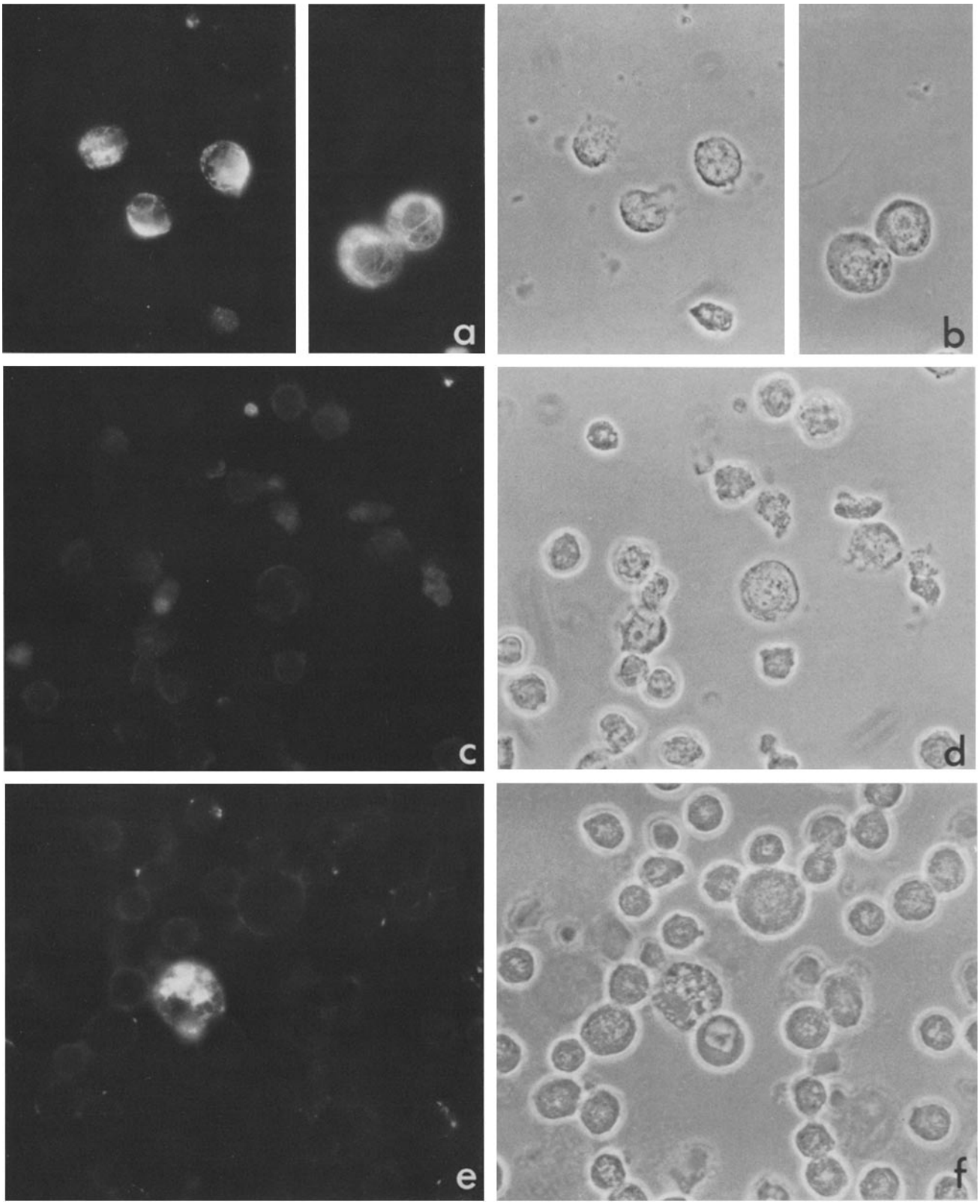

FIGURE 4 Indirect immunofluorescence microscopy on hamster insulinoma cells. Phase-contrast images are shown in the right panels and the corresponding red fluorescence images in the left panels. The antisera used and their respective dilutions were rabbit anti-P_{60} antibody; 1:50 (*a* and *b*); rabbit preimmune serum, 1:50 (*c* and *d*); rabbit antivimentin antibody (12), 1:100 (e and *f*). Fluorescence exposure, 10 s. × 900.

sponding predominant protein spot on the gel is indicated by the arrow (Fig. 5 *a*).

To confirm the presence of P_{60} in pancreatic islets, we examined cryostat sections of hamster pancreas by indirect immunofluorescence microscopy employing the anti-P_{60} antiserum. Immunofluorescence staining of an islet is shown in

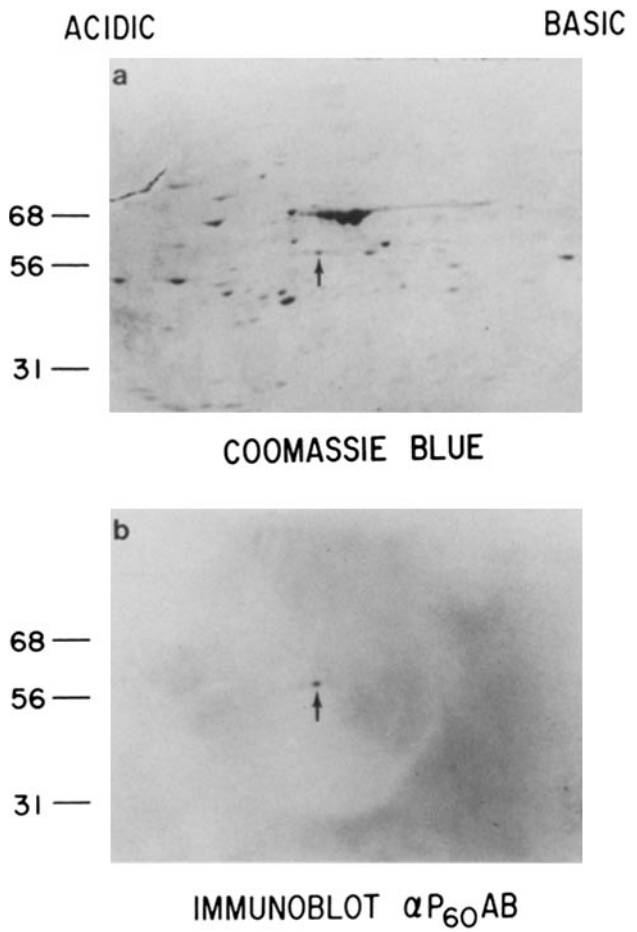

FIGURE 5 Demonstration of the presence of P_{60} in pancreatic islets by immunoblotting. Microdissected hamster islets (75 islets) were analyzed as in Fig. 3. (*a*) Gel stained with Coomassie Blue; (*b*) immunoblot using rabbit anti-P_{60} antiserum diluted 1:100. Autoradiographic exposure, 2 d. (Numbers at left border, molecular weight $\times 10^{-3}$.)

Fig. 6*a*. This staining was specific, as it was not obtained with preimmune serum (Fig. 6 *c*). Some staining of exocrine pancreatic tissue surrounding the islet was also observed (Fig. 6*a*). At least in part, this was not due to specific antibody binding inasmuch as similar staining was observed when preimmune serum was used (Fig. 6*c*). Bright staining of the epithelial layer of pancreatic exocrine ducts was also noted (Fig. 6*b*) and this staining was not reproduced by preimmune serum (Fig. 6*d*). Thus, the data suggest that P_{60} is not only present in hamster insulinoma cells but also in normal pancreatic islets and in the epithelium of pancreatic exocrine ducts.

Demonstration That P_{60} Is Analogous to a Keratin-type Intermediate Filament Protein Present in HeLa Cells

The migration of P_{60} on two-dimensional electrophoresis resembles that of a previously described keratin present in HeLa cells and some other epithelial cells (10, 28–30). To test whether P_{60} is related to this keratin, we examined HeLa cells by indirect immunofluorescence microscopy using the anti-P_{60} antiserum. As shown in Fig. 7, the resultant fluorescence staining pattern is characteristic of keratin-type intermediate filaments. This staining was not reproduced with preimmune serum.

To examine which protein(s) in these filaments reacted with the anti-P_{60} antibody, we prepared Triton X-100–insoluble cytoskeletons from HeLa cells and analyzed them by two-dimensional electrophoresis and immunoblotting (Fig. 8). The gel stained with Coomassie Blue (Fig. 8*a*) shows that these preparations contain, in addition to vimentin (*v*, bracket), three other proteins, which were previously identified as cytokeratins (10, 29, 30). An additional minor keratin described in earlier reports (10, 28–30) was not detected in our preparation by staining with Coomassie Blue. The positions of the three keratins on the two-dimensional electrophoresis gel resemble closely those of three of the insulinoma cell intermediate filament proteins (compare Fig. 8*a* and Fig. 2*a*). These are P_{60} (Fig. 8, arrow), and the two other insulinoma cytoskeleton proteins, 59,000 and 46,000 mol wt, respectively (Fig. 8*a*, arrowheads). It appears that the latter protein overlaps partially with residual actin, also present in the preparation.

For the immunoblots shown in Fig. 8, *b* and *c*, two different dilutions of the anti-P_{60} antiserum were used. The antibody reacted with two proteins that correspond to P_{60} and the 59,000-mol-wt insulinoma cell intermediate filament protein migrating near P_{60}, respectively. These findings are analogous to those obtained with insulinoma cell proteins (Figs. 2 and 3). The anti-P_{60} antibody bound preferentially to the HeLa cell keratin that shows a migration on two-dimensional electrophoresis identical to that of P_{60}. The data suggest that P_{60} is the hamster analog to this human keratin.

DISCUSSION

The present report describes the cytoskeletal nature of a 60,000-mol-wt insulinoma cell protein, P_{60}, which was previously shown to undergo calcium influx–stimulated phosphorylation in intact insulinoma cells, concomitant with insulin release (5). P_{60} was shown to be a structural component of intermediate filaments using several criteria: (*a*) it was found in Triton X-100–insoluble cytoskeletons, a cell residue rich in intermediate filaments, as demonstrated previously in numerous other studies (for review, see references 7 and 8); (*b*) examination of insulinoma cell cytoskeletons by electron microscopy identified intermediate-sized filaments (Fig. 1); and (*c*) P_{60} and the other three proteins found in insulinoma cell cytoskeletons were all recognized by a monoclonal antibody known to interact with an epitope shared by all intermediate filament proteins (Fig. 2*f*) (13).

A small amount of vimentin, the intermediate filament protein of fibroblasts and other mesenchymally derived cells (7, 8), was also identified in insulinoma cell cytoskeleton preparations. However, in contrast to certain other cells (e.g., some cultured cells, glial cells, and vascular smooth muscle cells), which contain vimentin in addition to their cell type–specific intermediate filament protein(s) (7, 8, 25, 26), vimentin is not present in these insulinoma cells but resides in contaminating cells tentatively identified as macrophages (Fig. 4, *e* and *f*).

In addition to insulinoma cells, P_{60} appears also to be present in normal islets, as demonstrated by immunoblotting (Fig. 5) and indirect immunofluorescence microscopy (Fig. 6). With the aid of the rabbit anti-P_{60} antibody, the tissue distribution of P_{60} is currently being studied in this laboratory. The protein appears not to be restricted to islet cells because

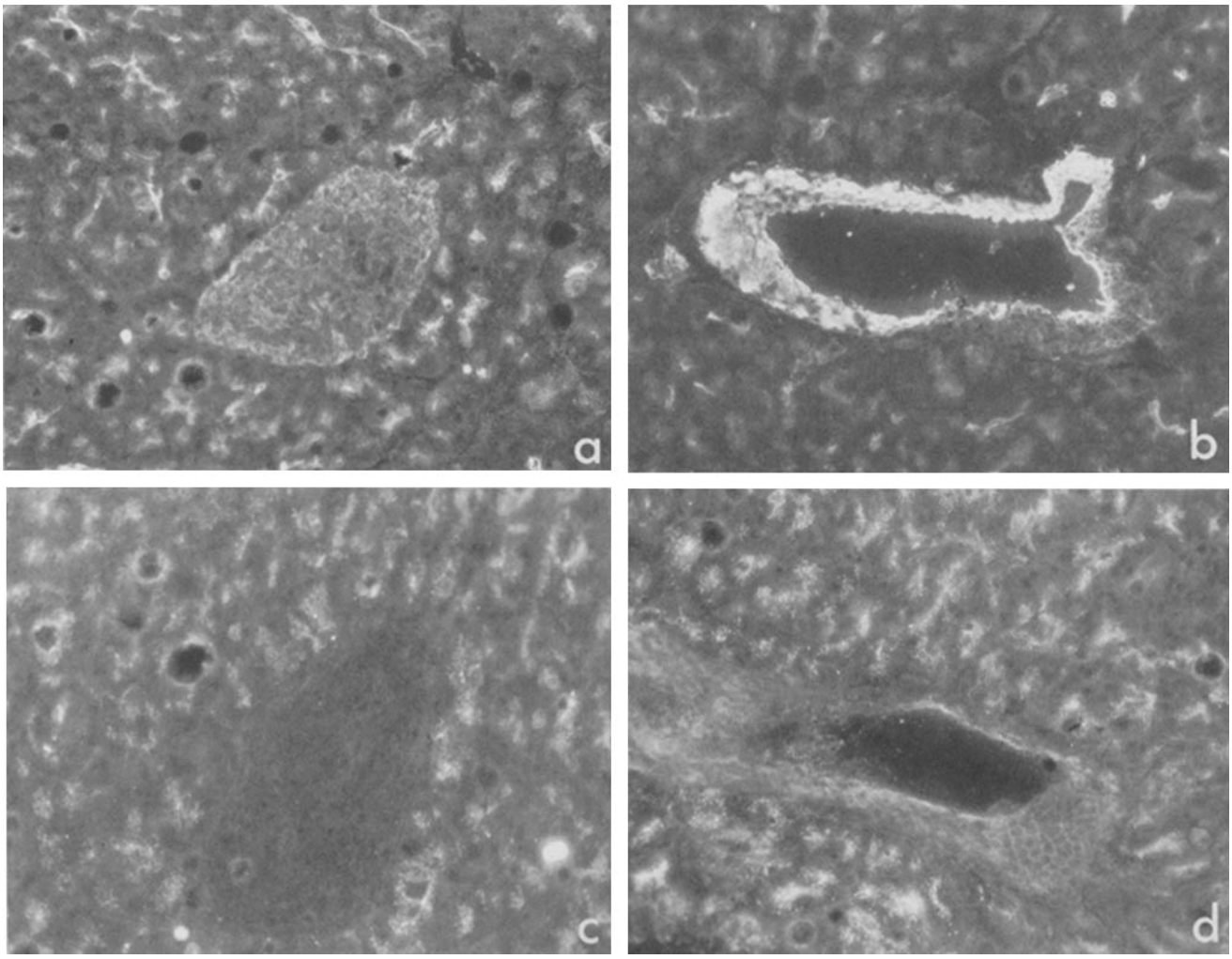

FIGURE 6 Demonstration of the presence of P_{60} in hamster pancreas by indirect immunofluorescence microscopy on cryostat sections. The section shown in the upper panels (*a* and *b*) was stained with anti-P_{60} antibody diluted 1:50; the section in the lower panels (*c* and *d*) with preimmune serum (1:50 dilution). Areas containing an islet (*left panels*) or an exocrine duct (*right panels*), respectively, are shown. Fluorescence exposure: left panels, 2 min; right panels, 30 s. × 530.

the anti-P_{60} antibody was able to detect an antigen in epithelial cells of pancreatic exocrine ducts, which, however, has not yet been shown to be structurally identical to P_{60}. The present studies also suggest that islet cells contain intermediate filaments of the epithelial type, which are the most diverse of five classes of intermediate filaments currently distinguished (7, 8, 10, 25–27). Based on their molecular weight and isoelectric pH as determined by two-dimensional electrophoresis, the four insulinoma cell intermediate filament proteins are distinct from vimentin, desmin, glial fibrillary acidic protein, and neurofilament proteins. In contrast, the insulinoma cell proteins closely resemble certain intermediate filament proteins of epithelial cells. This class of proteins has been extensively studied in a number of laboratories and referred to as prekeratins, keratins, keratin-like proteins, or cytokeratins (for review, see references 7, 8, and 25–27). A map of the two-dimensional electrophoretic migration of 19 distinct human cytokeratins that are found in various combinations characteristic for different human epithelia was recently published by Moll et al. (25). Indeed, the electrophoretic migration of three of the insulinoma cell intermediate filament proteins appears to be identical to three cytokeratins represented on this map. Thus, the proteins with molecular weights of 59,000, 46,000, and 40,000 may be analogues of cytokeratins (8, 18, and 19 respectively, according to the nomenclature of Moll et al. (25). Studies are currently in progress to examine the relationship of these proteins more closely by comparing the peptide maps of the respective proteins of epithelial cells and of insulinoma cells.

Inasmuch as the migration of P_{60} on two-dimensional electrophoresis suggested that this protein may be analogous to cytokeratin 7 (25), the relationship of these two proteins was examined with the aid of the anti-P_{60} antibody. Using HeLa cells that are known to contain cytokeratin 7 (10, 28), we have shown that the anti-P_{60} antibody, which has a high degree of specificity, produces, on indirect immunofluorescence microscopy, a staining pattern characteristic of keratin-type filaments (Fig. 7). In addition, it was demonstrated by immunoblotting that anti-P_{60} antibody binds to cytokeratin 7 (Fig. 8). The different estimates of the molecular weight of this protein which are 54,000 (10), 50,000 (28), and 60,000 (this study), respectively, are likely the result of technical differences. Thus, based not only on the identical migration of the two proteins on two-dimensional electrophoresis (compare Figs. 2*a* and 8*a*) but also on their immunologic relatedness, the data suggest that P_{60} and cytokeratin 7 are analo-

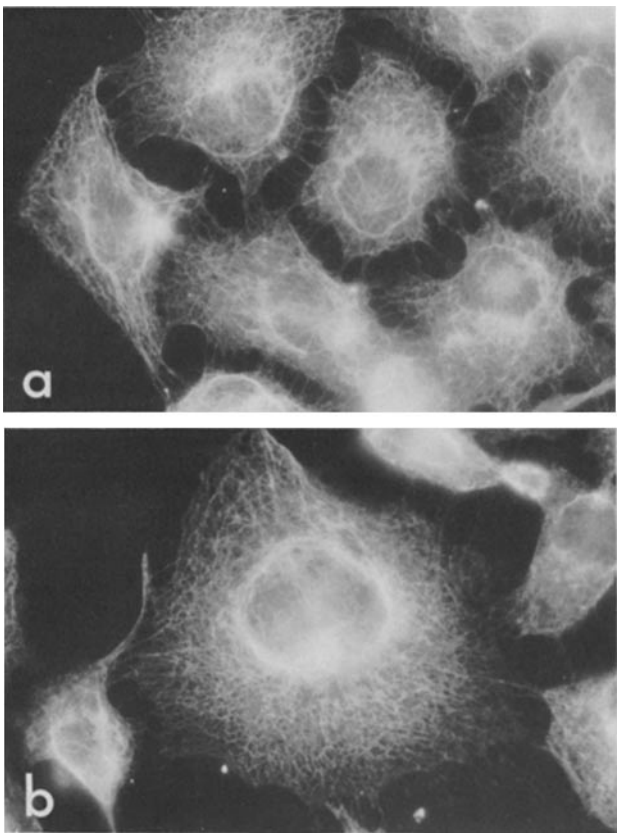

FIGURE 7 Indirect immunofluorescence staining of HeLa cells using anti-P_{60}. HeLa cells were grown on glass coverslips and processed for indirect immunofluorescence microscopy using anti-P_{60} antibody at dilutions of 1:50 (*a*), and 1:100 (*b*). Fluorescence exposure: (*a*) 1 min; (*b*) 2 min. × 880.

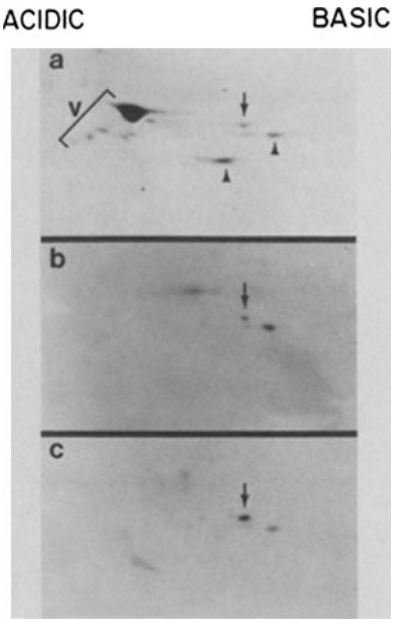

FIGURE 8 Immunoblot analysis of HeLa cell intermediate filament proteins resolved by two-dimensional electrophoresis. Aliquots (15 μg) of Triton X-100–insoluble cytoskeleton proteins obtained from HeLa cells were analyzed as in Fig. 3. (*a*) Gel stained with Coomassie Blue; (*b* and *c*) immunoblots using anti-P_{60} antiserum diluted 1:100 (*b*) and 1:500 (*c*). Autoradiographic exposure, 2 d. *V*, vimentin.

gous proteins. Taken together, the evidence presented in this report suggests that islet cell intermediate filaments are of the keratin type.

The composition of intermediate filament proteins in islet cells has not been previously reported. It is now well established that intermediate filament proteins are cell type–specific and thus provide information regarding cellular origin (7, 8, 25–27). In this regard, the finding of keratins in pancreatic islets attests to their epithelial nature. Numerous histologic studies have suggested that iselts are of endodermal origin and are derived from the primitive pancreatic duct (for review, see reference 31). In contrast, the demonstration that islet cells have amine precursor uptake and decarboxylation characteristics led Pierce to suggest that islets may be derived from the neural crest (32). This notion was further supported by Schmechel et al. who provided evidence for the presence of neural-specific enolase in islets (33). However, developmental studies have established conclusively that islets are not of neural crest origin (for review, see reference 34). Nevertheless, the cytologic properties that islet cells share with other "neuroendocrine" cells remain an intriguing enigma of cytodifferentiation. The findings in the present report are most consistent with an endodermal origin of islet cells based on the demonstration that islet cell intermediate filaments are of the keratin type and that at least one of the islet cell keratins (P_{60}) appears also to be expressed in the epithelium of pancreatic exocrine ducts.

The function of intermediate filaments is still poorly understood. It is also not known if and how the properties of intermediate filament proteins may be altered by phosphorylation, even though many of them have been shown to contain phosphate (7, 8). In the case of P_{60}, this protein was shown previously to undergo Ca^{2+}-dependent phosphorylation in insulinoma cells (5, 6). Inasmuch as Ca^{2+} plays a critical role in insulin release, the findings presented in this report suggest the possibility that intermediate filaments may be involved in the process of exocytosis in pancreatic beta cells. Although such a role is speculative at present, further studies should provide interesting insights regarding the possible interaction of these cytoskeletal structures with elements of the secretory apparatus.

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