Co-expression of Cytokeratins and Neurofilament Proteins in a Permanent Cell Line: Cultured Rat PC12 Cells Combine Neuronal and Epithelial Features

Werner W. Franke,* Christine Grund,* and Thomas Achtstätter‡

*Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany; and ‡Center of Molecular Biology, University of Heidelberg, D-6900 Heidelberg, Federal Republic of Germany

Abstract. The cytoskeleton of the rat cultured cell line PC12, which is widely used in cell biology as a model system for neuron-like differentiation, displays an unusual combination of intermediate-sized filaments (IFs). As determined by electron microscopy, immunolocalization, and biochemical analyses, these cells contain, in addition to neurofilaments, an extended meshwork of bundles of cytokeratin IFs comprising cytokeratins A and D, equivalent to human cytokeratin polypeptides Nos. 8 and 18, irrespective of whether they are grown in the presence or absence of nerve growth factor. The two IF systems differ in their fibrillar arrays, the neurofilaments being concentrated in perinuclear aggregates similar to those found in certain neuroendocrine tumors of epithelial origin. We conclude that PC12 cells permanently co-express IFs of both the epithelial and the neuronal type and thus present an IF combination different from those of adrenal medulla cells and pheochromocytomas, i.e., the putative cells of origin of the line PC12. The IF cytoskeleton of PC12 cells resembles that of various neuroendocrine tumors derived from epithelial cells. The results show that the development of a number of typical neuronal differentiation features is compatible with the existence of an epithelial type IF cytoskeleton, i.e., cytokeratins. The implications of these findings concerning the validity of the PC12 cell line as a model for neuronal differentiation and possible explanations of the origin of cells with this type of IF co-expression are discussed.

INTERMEDIATE-SIZED filaments (IFs)[1] represent a large portion of the cytoskeleton of most vertebrate cells and are formed by five major classes of proteins of a large multigene family which are expressed in patterns related to major routes of cell differentiation (Franke et al., 1982; Lazarides, 1982; Osborn et al., 1982*b*). Of these five classes of IF proteins the cytokeratins are characteristic of epithelial cell differentiation and are generally maintained in epithelium-derived tumors and cultured cells (Franke et al., 1978*b*, *c*, 1979*a–d*, 1982; Sun and Green, 1978; Sun et al., 1979; Osborn and Weber, 1983; Steinert et al., 1985). While most epithelia in situ express only IF of the cytokeratin type, some special epithelia co-express cytokeratin and vimentin IF in the same cell, e.g., certain cell types of human mesothelium and ovary, amnion epithelium, endometrium, thyroid gland, and fetal kidney (Holthöfer et al., 1984; LaRocca and Rheinwald, 1984; Czernobilsky et al., 1985; Regauer et al., 1985; Achtstätter et al., 1986*b*; Franke et al., 1986) as well as cells of parietal endoderm of mouse embryos (Lane et al., 1983; Lehtonen et al., 1983). Expression of vimentin IF in addition to cytokeratin IF has also been found in several epithelium-derived tumors (Caselitz et al., 1981; Krepler et al., 1982; Herman et al., 1983; Holthöfer et al., 1983; Miettinen et al., 1984; Vogel et al., 1984; Blobel et al., 1985b; McNutt et al., 1985) and in a number of epithelium- or carcinoma-derived cell lines growing in culture (Franke et al., 1979*a–c*, 1981*a–c*, 1982; Virtanen et al., 1981; Connell and Rheinwald, 1983).

More recently, co-existence of cytokeratin with IF proteins other than vimentin have been noticed in specific cells. For example, glial filaments and cytokeratins co-exist in certain cells of human parotid gland and in pleomorphic adenomas (Nakazato et al., 1985; Achtstätter et al., 1986*b*). Immunohistochemical observations on certain neuroendocrine tumors of epithelial origin have indicated the co-existence of cytokeratin IF and neurofilaments in the same cell (Höfler et al., 1984; Van Muijen et al., 1984, 1985; Moll and Franke, 1985; Miettinen et al., 1985*a*,*b*; McNutt et al., 1985; Merot et al., 1986). In the present study we report on the consistent co-expression of neuronal and epithelial IFs in a permanent cultured cell line, the rat PC12 cell, which is assumed to have originated from a non-epithelial tumor, i.e., a transplantable pheochromocytoma, and is widely used as an in vitro model system for studies of neuronal cell differentiation (e.g., Greene and Tischler, 1976; Schubert and Klier, 1977; Lander

1. *Abbreviations used in this paper*: IFs, intermediate-sized filaments; NGF, nerve growth factor.

et al., 1983; Mallet et al., 1983; O'Malley et al., 1983; Alemà et al., 1985; Bar-Sagi and Feramisco, 1985; Nose et al., 1985; Richter-Landsberg et al., 1985; Togari et al., 1985; Friedlander et al., 1986; Green et al., 1986; Hagag et al., 1986). Our results show that the IF cytoskeleton of PC12 cells differs profoundly from that of neurons and adrenal medulla cells and their tumors but is similar to that of certain neuroendocrine tumors of epithelial origin, thus calling for a reconsideration of the general relevance of PC12 cells as a model system for the differentiation of neuronal cells.

Materials and Methods

Cells and Tissues

PC12 cells examined included early passages (passages 30–32) which were kindly provided by Dr. H. Thoenen (Max-Planck-Institute for Psychiatry, Martinsried, FRG; the cooperation of Mrs. Heide Thorun of that institute is gratefully acknowledged) who originally had received them from Dr. L. A. Greene (cf. Greene and Tischler, 1976). They were routinely grown in culture medium consisting of "Eutroph" powder (7.86 g/liter; "Eutroph" is Dulbecco's minimal essential medium without glucose; Laboratories Eurobio, Paris V, France) plus 3.6 g/liter $NaHCO_3$, 1 g/liter D-glucose, 2 mM glutamine, 1 mM sodium pyruvate, and supplemented with 10% horse serum and 5% fetal calf serum. Another subline studied in detail displaying somewhat greater adherence to the substratum was subline PC12-51 developed from passage 51 in the laboratory of Dr. H. Betz (Center of Molecular Biology, University of Heidelberg). In some experiments cells were grown in the presence of 25 or 50 ng/ml nerve growth factor (NGF; kindly provided by Drs. H. Betz and H. Rehm, Center of Molecular Biology, Heidelberg) for 12–36 h.

For comparison, cytoskeletal material from rat spinal cord (Achtstätter et al., 1986*b*), rat liver tissue, and cultured rat hepatoma cells of line MH_1C_1 (cf. Franke et al., 1981*a–c*; Achtstätter et al., 1986*a*) were used.

Cytoskeleton Preparations, Gel Electrophoresis, and Peptide Map Analysis

Cytoskeletal material enriched in IFs was prepared from cell cultures by lysis and extraction in buffers containing Triton X-100 and high salt buffer essentially as described previously (Franke et al., 1981*c*; Achtstätter et al., 1986*a*). Polypeptides were separated by one- or two-dimensional gel electrophoresis and were stained with Coomassie Brilliant Blue or with silver staining (for minor modifications see Moll et al., 1982; Achtstätter et al., 1986*a*). Polypeptides were identified by immunoblotting (Towbin et al., 1979; Achtstätter et al., 1986*a*) using antibodies to various IF proteins (see below) or by excision of polypeptide spots and tryptic peptide mapping according to Elder et al. (1977), with modifications described (Schiller et al., 1982).

Antibodies and Immunofluorescence Microscopy

The following antibodies were used: (*a*) Monoclonal murine antibody SY38 to synaptophysin as a marker for neuroendocrine vesicles (available from Boehringer Mannheim GmbH, Mannheim, FRG; Wiedenmann and Franke, 1985; Rehm et al., 1986; Wiedenmann et al., 1986); (*b*) monoclonal murine antibody PKK1 against simple epithelium-type cytokeratins (from Labsystems Oy, Helsinki, Finland; for characterization see Holthöfer et al., 1983); (*c*) monoclonal murine antibodies LE61 and 65 (generously provided by Dr. Birgitte Lane, Imperial Cancer Research Fund, London, UK; for references see Lane et al., 1985); (*d*) monoclonal murine antibody VIM.9 against vimentin (from Biochrom, West Berlin); (*e*) monoclonal antibodies against neurofilament polypeptides NF-H (M_r ~200,000), NF-M (M_r ~170,000), NF-L (M_r ~68,000), all from Boehringer Mannheim GmbH (Debus et al., 1983); (*f*) monoclonal antibodies to glial filament protein such as GFI2.24 (from PROGEN GmbH, Heidelberg, FRG; Achtstätter et al., 1986*b*) and anti-GFP (from Boehringer Mannheim GmbH); (*g*) monoclonal antibody DPI&2-2.15 (available from Boehringer Mannheim GmbH; Cowin et al., 1985); (*h*) monoclonal antibody to vinculin (kindly provided by Dr. B. Geiger, Weizmann Institute of Sciences, Rehovot, Israel); (*i*) guinea pig antibodies raised against cytokeratin component D from murine liver which is equivalent to human cytokeratin No. 18 (Franke et al., 1981*a*; Moll et al., 1982; Schiller et al., 1982); (*j*) guinea pig antibodies against purified cytokeratins Nos. 8 and 18 from human MCF-7 cells which cross-react with rat liver cytokeratins; (*k*) guinea pig antibodies to vimentin (cf. Franke et al., 1979*d*); (*l*) guinea pig antibodies raised against the gel electrophoretically purified neurofilament polypeptide NF-H and NF-L (Achtstätter et al., 1986*b*); (*m*) guinea pig antibodies to desmoplakins (see Cowin et al., 1985); (*n*) guinea pig antibodies against plakoglobin, an adherens junction plaque-associated protein (Cowin et al., 1986).

As secondary antibodies we used fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-coupled goat antibodies to immunoglobulins of guinea pig or mouse (from Dianova, Hamburg, FRG). For double label immunofluorescence microscopy the appropriate primary antibody combinations of mouse and guinea pig were simultaneously applied and processed as previously described, using the corresponding secondary antibodies (Geiger et al., 1983; Moll and Franke, 1985).

Electron Microscopy

Cells were fixed as cell clusters loosely adherent to the bottom of Falcon dishes or coverslips or as pellets for 20 min with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4) containing 50 mM KCl and 2.5 mM $MgCl_2$ at room temperature. After several washes in cold (~4°C) buffer the cells were postfixed with 2% OsO_4 in cold buffer for 2 h, washed, dehydrated, flat-embedded in Epon, and sectioned as described (Franke et al., 1978*a*).

For immunoelectron microscopy, the cells were fixed in 2% formaldehyde freshly made from paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 20 min at room temperature. After brief rinsing in cold PBS the cells were rinsed three times in PBS containing 50 mM NH_4Cl for 10 min each, then once in PBS containing NH_4Cl and 0.1% saponin for 10 min, and finally washed again in cold PBS. The thus permeabilized cells were then incubated with antibodies (e.g., 0.5 μg/ml in the case of SY38) at room temperature for 30 min, washed three times in PBS, and then incubated for 16 h with secondary antibodies that had been coupled with 5-nm colloidal gold particles (from Janssen Pharmaceutica, Beerse, Belgium). After three washes in cold PBS, specimens were fixed first in buffer containing 2.5% glutaraldehyde (see above) and then in buffer containing 2% OsO_4 and processed as previously described for a similar pre-embedding procedure (Kartenbeck et al., 1984).

Results

Light and Electron Microscopy

The PC12 cell cultures from the various sources all grew in clusters of cells and showed a very similar appearance, one of the sublines (PC12-51) displaying somewhat greater adhesion to the plastic dish substratum. They contained numerous vesicles of the "dense-core granule" type (Fig. 1), ranging in diameter from ~0.08 to 0.3 μm, which are typical of cells actively storing and secreting neuroendocrine substances and have been described as a characteristic feature of PC12 cells (Greene and Tischler, 1976; Schubert and Klier, 1977; Tischler and Green, 1978; Luckenbill-Edds et al., 1979). In addition, the cytoplasm, including the NGF-induced processes, was scattered with very small (30–80-nm diam) vesicles without an electron-dense content (Fig. 1 *b*, arrows) and occasional coated pits and vesicles (cf. Greene and Tischler, 1976; Tischler and Greene, 1978).

The cells also contained numerous IFs which occurred in relatively tightly fasciated bundles (Fig. 2, *a–d*) as they are typical of cytokeratin IF bundles (e.g., Franke et al., 1978*a*, 1982). The frequency and diameter of such IF bundles were somewhat variable from cell to cell within the same culture, and these structures were generally more prominent in the PC12-51 subline. In addition, most cells contained large aggregates of loosely and haphazardly arranged IF, the latter being particularly frequent in the perinuclear cytoplasm

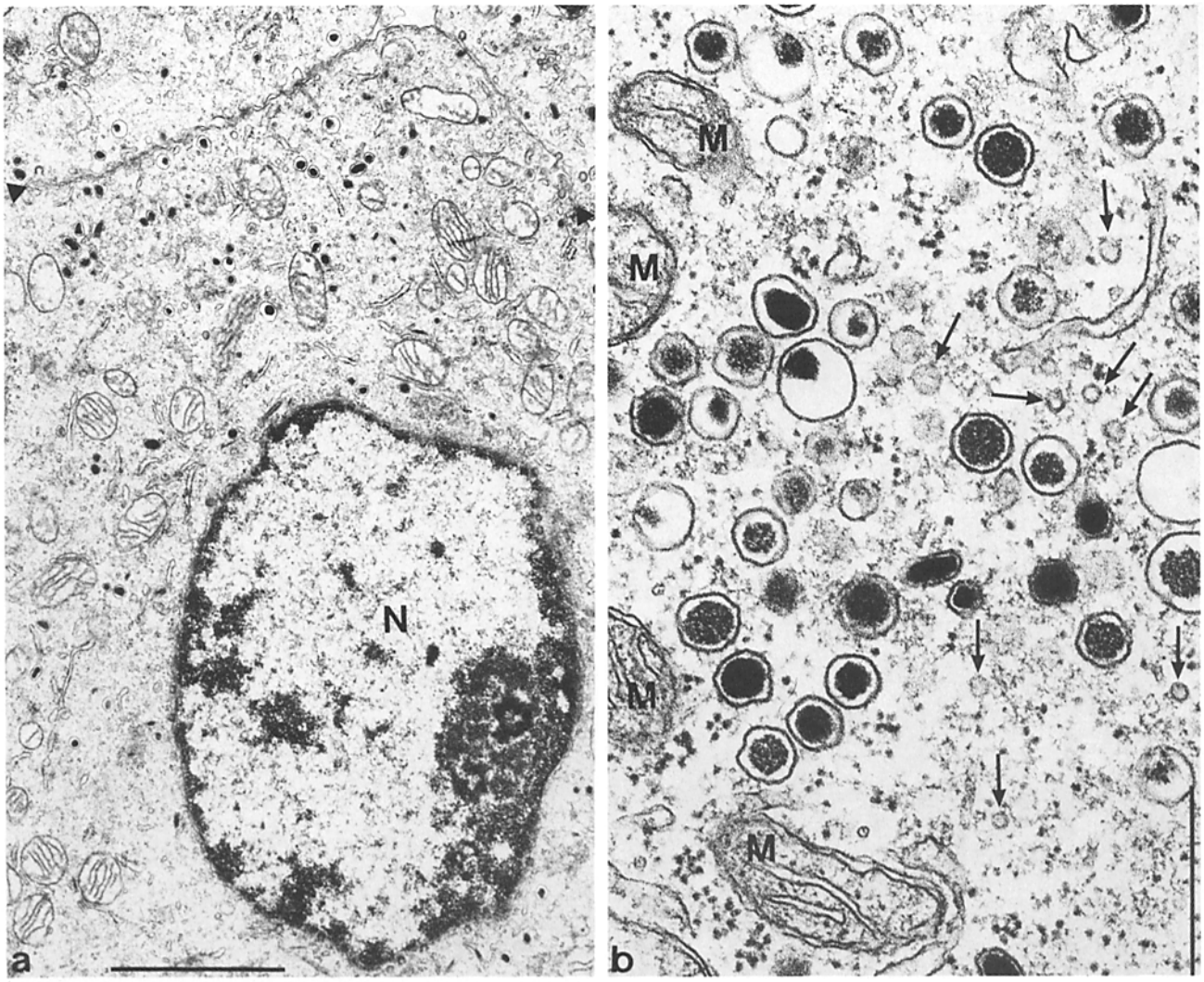

Figure 1. Electron micrographs of the rat PC12 cell colonies used in this study (the main cell bodies of cultures treated with NGF for 5 d are shown). (*a*) Survey micrograph showing the close apposition of the cells (arrowheads denote plasma membranes), the frequency of the mitochondria, and the chromatin appearance of the nucleus (*N*). (*b*) Higher magnification showing the abundance of dense-core vesicles and small (30–80 nm) vesicles with an electron-translucent content (some are denoted by arrows). *M*, mitochondria. The insert in the upper right shows a dense-core vesicle with a bristle-coat (*arrow*) resembling that of typical coated vesicles. Bars: (*a*) 2 μm; (*b*) 0.5 μm.

(Fig. 2 *e*). Such juxtanuclear IF aggregates were rather large (1–5 μm) and often associated with the Golgi apparatus (Fig. 2 *e*; see also Tischler and Greene, 1978; Lee et al., 1982). Moreover, numerous microtubules and microfilaments were seen both in the cell body and in the NGF-induced processes (data not shown; see Greene and Tischler, 1976; Luckenbill-Edds et al., 1979; Drubin et al., 1985; Joshi et al., 1985, 1986).

The cells displayed extended regions of intercellular contact characterized by junctions of sizes varying from 0.1 to ~0.5 μm that were associated with dense cytoplasmic plaques and loose microfilamentous webs (Fig. 2, *b–d*). The IF bundles occasionally came near to such plaques but did not directly associate with them (Fig. 2, *b–d*). Such plaque-associated junctions of the adhering type were also noted in previous electron microscopic studies of PC12 cells (Tischler and Green, 1978; Luckenbill-Edds et al., 1979).

Immunolocalization Studies

The PC12 cells, grown both with and without NGF, were positive for certain neuronal markers. As an example, we show the reaction (Fig. 3) with antibodies to synaptophysin, a transmembrane, Ca^{2+}-binding glycoprotein of small, translucent vesicles that occur in presynaptic regions of neurons but also throughout the cytoplasm of other neuroendocrine cells, including adrenal medulla and pheochromocytomas (Jahn et al., 1985; Wiedenmann and Franke, 1985; Wiedenmann et al., 1986). In PC12 cells this antigen appeared in a punctate pattern over most of the cytoplasm (Fig. 3 *a*). Immunoelectron microscopy showed that most, if not all, of the immunological reaction with colloidal gold particles was not associated with the dense-core vesicles but with the smaller (30–80 nm), "empty-looking" vesicles (Fig. 3 *b*), i.e., vesicles of a similar size distribution and morphology as the acetylcholine-containing vesicles of presynaptic regions. As-

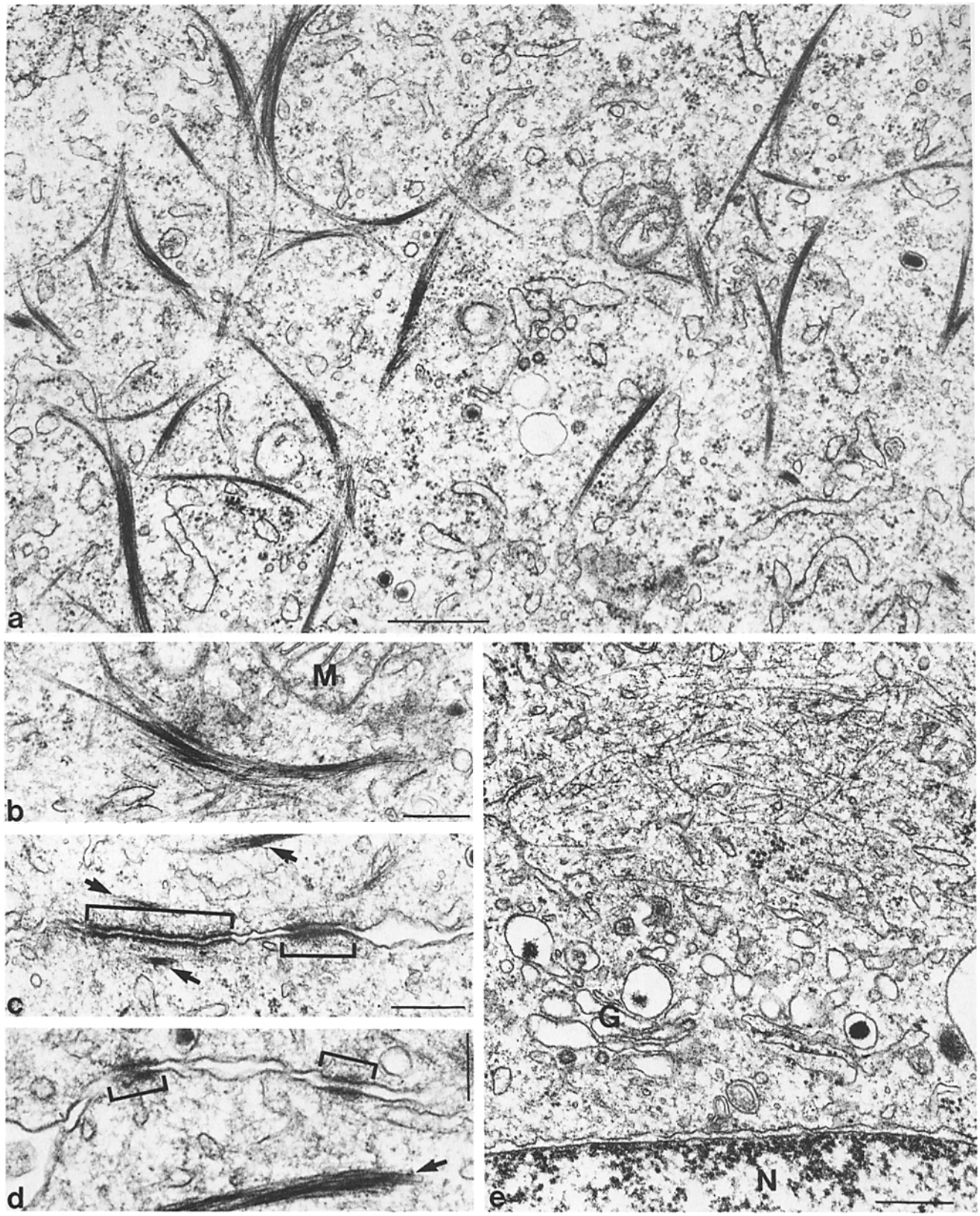

Figure 2. Electron microscopic appearance of IFs and intercellular junctions in PC12 cells. (*a*) Region of cytoplasm in a section oblique to the plane of the substratum, showing abundance of bundles of densely fasciated IF as they are characteristic of cytokeratin IF bundles. (*b*) Higher magnification of such an IF bundle in which the individual IFs are resolved. (*c*) Adherens-type junctions with dense plaques and microfilamentous cells (brackets). Note that the IF bundles do not (*arrows*) attach to these plaques. (*d*) Small-sized (80–200-nm diam) junctions of the adherens category (*puncta adhaerentia*; brackets) with which microfilaments but not IF bundles (*arrow*) attach. (*e*) Perinuclear cytoplasm displaying a spheroidal aggregate of loosely and haphazardly arranged IFs. *G*, Golgi apparatus; *N*, nucleus. Bars: (*a*) 2 μm; and (*b–e*) 0.2 μm.

suming that synaptophysin is specific for acetylcholine vesicles also in PC12 cells, this localization would be in agreement with the interpretation that vesicles of this type store acetylcholine (Greene and Tischler, 1976; Tischler and Greene, 1978; see, however, Schubert and Klier, 1977).

When the antibodies to the various kinds of IF proteins (see Materials and Methods) were used, PC12 cells were positive only for both neurofilaments and, unexpectedly, cytokeratin IFs. Positive cytokeratin staining was seen with all the cytokeratin antibodies, both monoclonal ones (PKK1, LE61, LE65) and the two different guinea pig antisera mentioned under Materials and Methods. While the cytokeratin IFs usually apeared in fibrillar meshworks extending throughout most of the cytoplasm, often even into the thin cell processes formed in the presence of NGF (Figs. 4, *a* and *b* and 5 *a*), the neurofilaments were usually more concentrated in the perinuclear region, often forming large, cap-like or spheroidal aggregates (Figs. 4 *c* and 5 *b*). Only in cells exposed for several days to NGF did we notice neurofilament staining in the cytoplasmic processes, in agreement with observations reported by Lee et al. (1982). The differential location of the two types of IF proteins in the same cells is best demonstrable by double label immunofluorescence microscopy as shown in Fig. 5, *a–c*. When antibodies specific for NF-L, NF-M, or NF-H were compared, strong positive reactivity was consistently observed for all three antigens, although staining for NF-H was often relatively weaker (not shown). We did not see consistent differences in the distribution of these three antigens as reported with certain antibodies by Lee et al. (1982; see also Lee, 1985).

Results obtained with vimentin antibodies were not consistent and the significance of the results difficult to assess. While some cultures, notably those derived from passages 30–40, showed weak vimentin staining in the juxtanuclear "balls" that were also positive for neurofilament protein, similar to the report of Lee and Page (1984), other cultures, notably those of subline PC-51, were completely negative for vimentin (data not shown).

We also attempted to identify the nature of the plaque-bearing intercellular junctions by immunolocalization. While antibodies to desmoplakin did not result in significant staining, cell boundaries were positive for vinculin and plakoglobin (not shown), indicating that these plaque-containing junctions are not desmosomes as thought by other authors (Tischler and Greene, 1978) but belong to the group of non-desmosomal junctions of the adherens category ("fasciae adhaerentes" and "puncta adhaerentia"; cf. Geiger et al., 1983; Cowin et al., 1986).

Biochemical Analyses

When preparations from PC12 cytoskeletons enriched in IFs were examined by one- (not shown) and two-dimensional gel electrophoresis (Fig. 6, *a–c*), two major polypeptides of M_r 55,000 and 49,000 were seen which were identical in positions to cytokeratins A and D as originally described in rat hepatocytes (Franke et al., 1981*a*) and intestinal cells (Franke et al., 1981*d*) as well as in various rat hepatoma cell cultures (Franke et al., 1981*b,c*; Schmidt et al., 1982; Venetianer et al., 1983). These two polypeptides, which occur in equimolar amounts in heterotypic tetrameric complexes (Quinlan et al., 1984), are the rat polypeptides equivalent to human cytokeratins Nos. 8 and 18 (Moll et al., 1982; Schiller et al., 1982) as demonstrable by antibody reactions and peptide map comparisons (Fig. 6, *h* and *i* and Schiller et al., 1982; Hubbard and Ma, 1983) and immunoblot reaction with guinea pig antibodies to cytokeratins (data not shown).

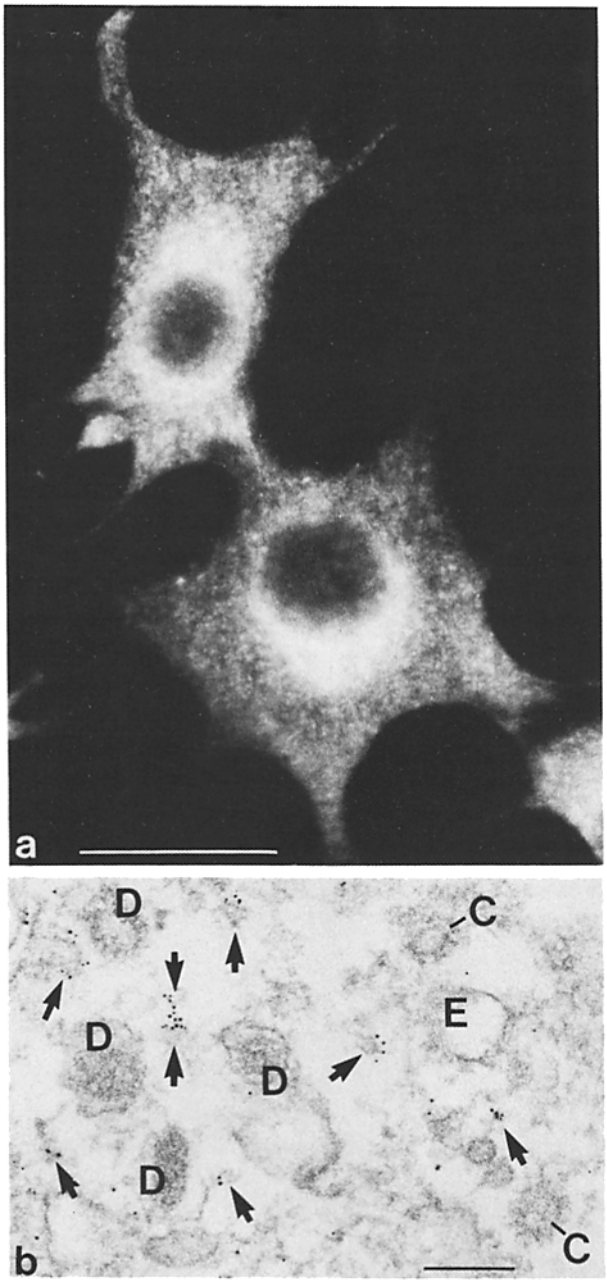

Figure 3. Immunolocalization of synaptophysin as a neuroendocrine marker protein in PC12 cells treated for 24 h with NGF. (*a*) Immunofluorescence microscopy with monoclonal antibody SY38 against synaptophysin, showing a punctate staining throughout the cytoplasm with some accumulation in the perinuclear region. (*b*) Immunoelectron microscopy using the same antibody in combination with secondary antibodies coupled to colloidal gold particles of ~5-nm diam. Note that the gold particles decorate a category of the small (30–80 nm) vesicles (*arrows*) but not the large dense-core vesicles (*D*), large "empty-looking" vesicles (*E*), and coated vesicles (*C*). Bars: (*a*) 20 μm; and (*b*) 0.2 μm.

PC12 cytoskeletons also revealed two polypeptides of M_r ~68,000 and 160,000 with coordinates identical to those of

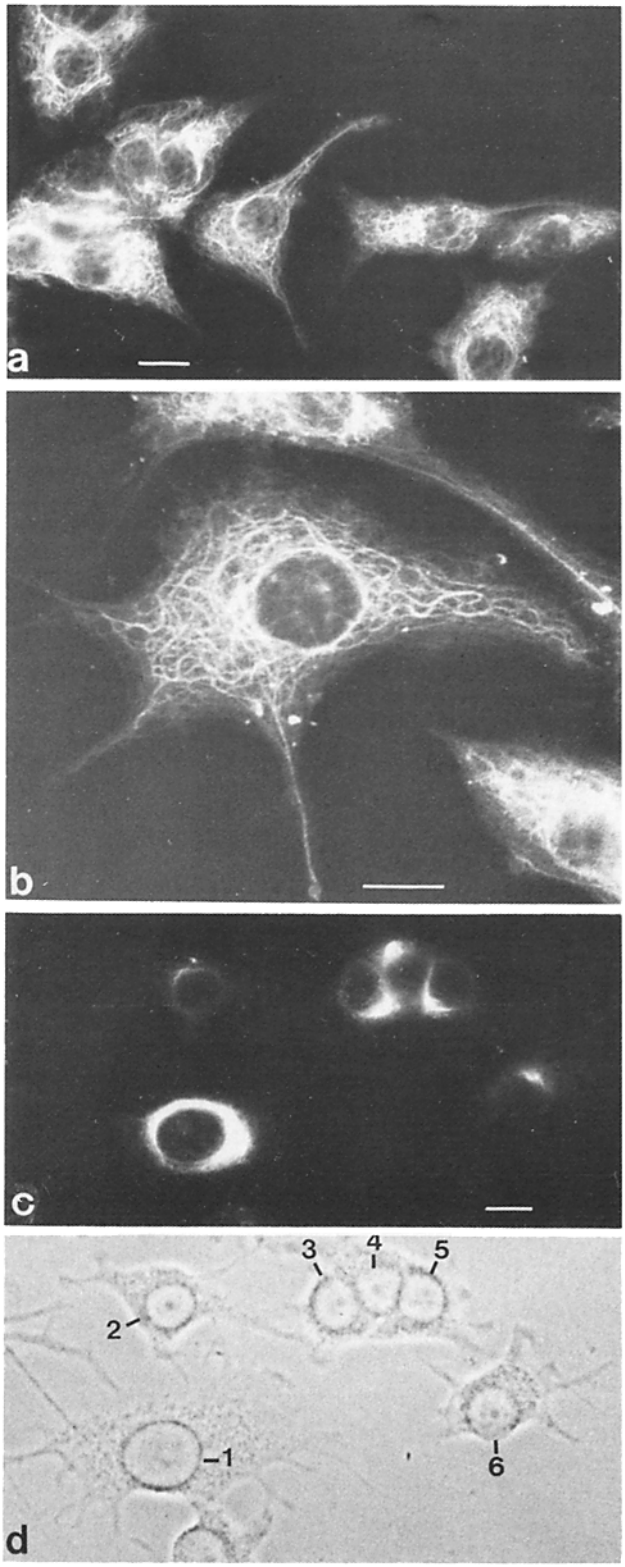

Figure 4. Immunofluorescence microscopy using antibodies to intermediate filament proteins on PC12 cells that had been treated with NGF for 24 h. (*a*) Survey micrograph of culture showing staining with monoclonal cytokeratin antibody (PKK1) throughout the cytoplasm of all cells. (*b*) Higher magnification, showing that the cytokeratin IF also occur in the processes formed at this time of NGF treatment. (*c*) Staining of the same culture with monoclonal antibody to neurofilament polypeptide NF-L (M_r 68,000), showing juxtanuclear staining in a perinuclear ring- or cap-like fashion, although at different intensities in the different cells. (*d*) Phase-contrast picture corresponding to *c*, showing that the cell designated *1* is very intensely labelled, cell *2* is very weakly stained, and cells designated *3–6* are moderately stained. Bars, 10 μm.

neurofilament polypeptides NF-L and NF-M from rat spinal cord (Fig. 6, *a–c*). The relative amounts of these neurofilament proteins were always lower than those of cytokeratins A and D (SDS PAGE densitometer tracing indicated an >15-fold excess) but was clearly enhanced in NGF-treated cells. The amounts of the NF-H component were too low to be detected in the gel system used, probably also due to some degradative losses during preparation.

The identity of these IF proteins was established by co-electrophoresis of [^{35}S]methionine-labeled PC12 cytoskeletal proteins with unlabeled cytoskeletal proteins from rat liver tissue and spinal cord, respectively (not shown), and by peptide map comparisons of the individual polypeptide spots excised after two-dimensional gel electrophoresis as those shown in Fig. 6, *a–c*. Fig. 6, *d* and *e* demonstrates an essentially identical peptide map for NF-L from PC12 cells and from rat spinal cord, and Fig. 6, *e* and *f* shows the same for the NF-M polypeptide from these two sources. The peptide maps of both NF-L and NF-M differed grossly from those of cytokeratins A and D analyzed in parallel (Fig. 6, *h* and *i*).

We have not been able to detect, by Coomassie Blue staining, vimentin in two-dimensional gel electrophoresis of cytoskeletal proteins from the various PC12 passages and sublines examined. This suggests that vimentin, if present, occurs in amounts far below those of cytokeratins and neurofilament proteins. As Lee and Page (1984; see also Lee, 1985) have reported that the concentration of vimentin filaments in their PC12 cultures increases during the NGF treatment we cannot exclude that the NGF effect on our cultures might have been insufficient to produce detectable amounts of vimentin.

In addition, two-dimensional gel electrophoresis of these cytoskeletons revealed the presence of an unidentified polypeptide of M_r ~59,000 (Fig. 6, *a–c*) and the nuclear lamina proteins (lamin B is shown in Fig. 6, *a–c*) which are frequent contaminations copurifying with IF proteins (e.g., Staufenbiel and Deppert, 1983; Venetianer et al., 1983; Blobel et al., 1984; for a contrasting view see Zackroff et al., 1984; for discussion see also Osborn, 1985).

Discussion

Our results show that, in addition to neurofilaments, rat PC12 cells consistently contain large amounts of cytokeratin filaments formed by cytokeratin polypeptides of the simple-epithelium type, i.e., cytokeratins A (No. 8 of humans) and D (human No. 18). The occurrence of neurofilaments in these cells (Lee et al., 1982; Osborn et al., 1982*b*) is as expected for a cell line presumed to be derived from a pheochromocytoma, i.e., a tumor containing only neurofilaments but no other IFs (Osborn et al., 1982*a*,*b*; Lehto et al., 1983; Trojanowski et al., 1984; Miettinen et al., 1985*b*; Wiedenmann et al., 1986). However, the co-existence of cytokeratin IF, i.e., the epithelial type of IFs, in these cells is surprising, all the more as only neurofilaments but not cytokeratins are found in the adrenal medulla of rat and other mammals (not shown; for similar data see also Miettinen et al., 1985*c*). Cytokeratin IFs have escaped detection in previ-

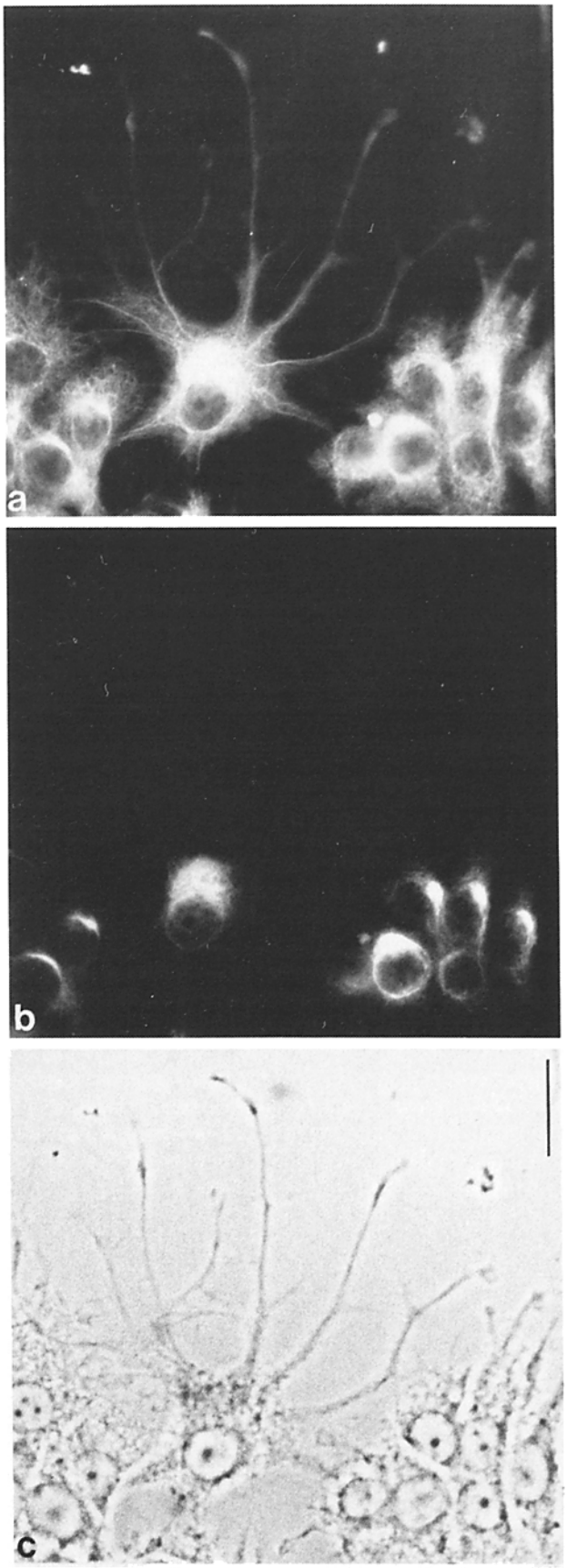

Figure 5. Double-label immunofluorescence microscopy showing the same PC12 cells (similar culture as that shown in Fig. 4) in epifluorescence (*a* and *b*) and phase-contrast (*c*) optics, showing staining with murine monoclonal antibodies to cytokeratins (*a*, same as in Fig. 4, *a* and *b*) and guinea pig antibodies to neurofilament protein (*b*). Note that at this stage of process formation the cytokeratin IF extend throughout the whole cytoplasm, including the basal portions of the processes (*a*), whereas the neurofilaments are still concentrated in the juxtanuclear aggregates which considerably vary in size and/or intensity of reaction (*b*). Bar, 20 μm.

ous studies on the cytoskeleton of PC12 cells (Lee et al., 1982; Osborn et al., 1982*b*; Parysek and Goldman, 1985). This may be because often the simple epithelial type cytokeratins 8 and 18 do not react with antibodies raised against epidermal cytokeratins (cf. Sun et al., 1979) or these authors may have looked at cells kept for very long times under the influence of NGF, which may result in relative reduction of cytokeratin contents.

We do not think that the expression of cytokeratin IFs is a feature selectively acquired by only those PC12 sublines and culture forms examined that happened to be included in this study, as we have noticed in electron micrographs published by other authors (e.g., Tischler and Greene, 1978) the same densely packed IF bundles which are characteristic of cytokeratin IF bundles (compare their Figs. 4 and 10 with Fig. 2 of this study) but are not formed by neurofilaments. Thus we think that co-expression of cytokeratin IFs and neurofilaments is a rather general feature of this cell line. Co-expression of cytokeratins, vimentin and, at least in some cells, neurofilaments has also been suggested for a human cell line (U-1810) assumed to be derived from a large cell carcinoma of the lung (Bergh et al., 1984), but in this case the proteins have not been fully characterized.

The unusual pattern of co-expression of neurofilaments and cytokeratin IFs shown here for cultured PC12 cells has not been seen in any cell of true neural cell differentiation, including adrenal medulla (see above) and other neural crest derivatives which during embryogenesis develop from cells that have become negative for cytokeratin and usually show a transient period of co-expression of vimentin and neurofilaments, followed by a predominance, if not exclusivity of neurofilaments (Tapscott et al., 1981; Bignami et al., 1982; Jacobs et al., 1982; Houle and Fedoroff, 1983; Ziller et al., 1983; Cochard and Paulin, 1984; for co-expression of vimentin IF and neurofilaments in cells of adult retina see Dräger, 1983). On the other hand, the PC12 IF pattern is similar to that found in tumors derived from neurosecretory epithelial cells such as certain cell types of Merkel cell tumors of the skin as well as carcinoids and neuroendocrine carcinomas of the bronchial and gastrointestinal tracts (Höfler et al., 1984; Van Muijen et al., 1984, 1985; Blobel et al., 1985*a*; Clark et al., 1985; Gould et al., 1985; Lehto et al., 1985; Miettinen et al., 1985*a*,*b*; Moll and Franke, 1985; McNutt et al., 1985; Merot et al., 1986). Moreover, the typical form of juxtanuclear spheroidal aggregates of neurofilaments found in PC12 cells (this study; Lee et al., 1982; Lee, 1985), which has been stated to be absent in normal neurons (Lee and Page, 1984), is also characteristically found in several neuroendocrine epithelial cell tumors (for references see Moll and Franke, 1985; Merot et al., 1986). Co-expression of cytokeratin IFs and neurofilaments, sometimes together with vimentin IF, has also been reported for medullary carcinomas of the thyroid (Wiedenmann et al., 1986).

Therefore, the question arises whether the tumor from which PC12 cells have originated really has been a pheochro-

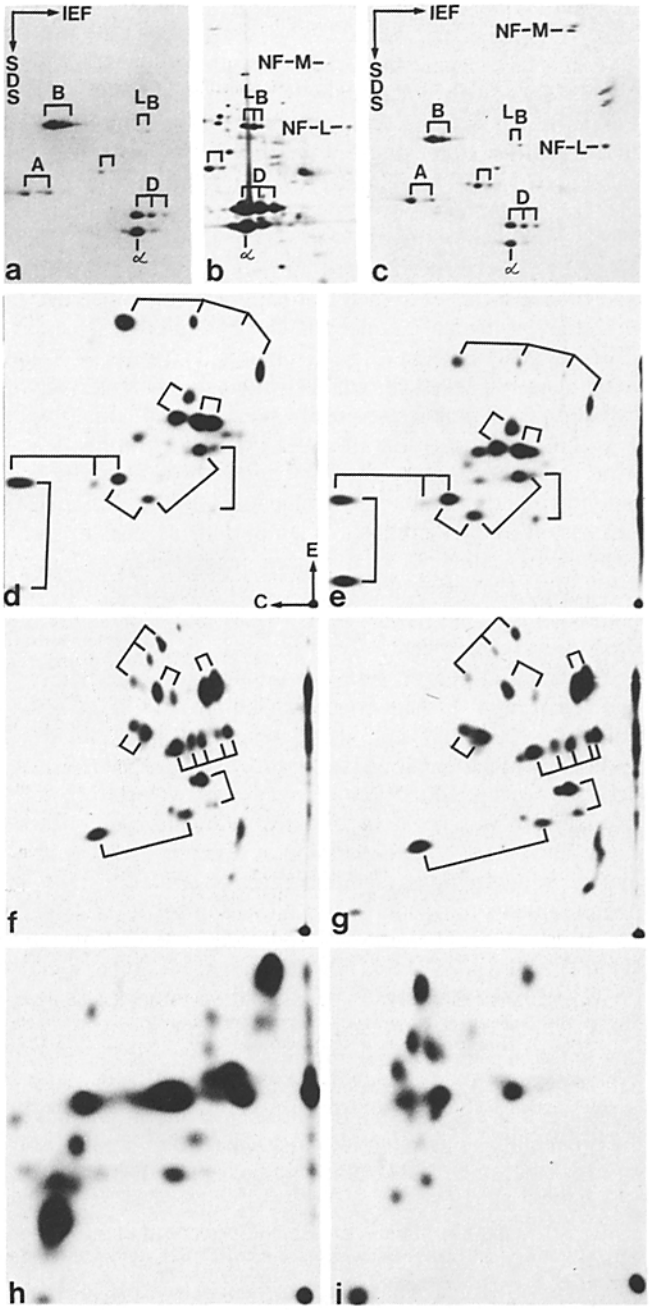

Figure 6. Gel electrophoresis of cytoskeletal proteins from PC12 cells and peptide map analysis of the major components. (*a–c*) Two-dimensional gel electrophoresis, using isoelectric focusing (*IEF*) in the first dimension and SDS PAGE in the second, of PC12 cells not treated with NGF (*a* and *b*) and after treatment with NGF for 36 h (*c*). (*a* and *c*) Coomassie blue stained gels; (*b*) secondary silver staining of the same gel as that shown in *a* to demonstrate the small amounts of neurofilament polypeptides NF-L and NF-M (NF-H has not been detected with this method). Reference proteins used for co-electrophoresis are bovine serum albumin (*B*) and rabbit skeletal muscle α-actin (vertical bar). L_B, nuclear lamin B; *A* and *D*, cytokeratins A and D, equivalent to human cytokeratin polypeptide Nos. 8 and 18. Note that the relative amounts of NF-L and NF-M and of another cytoskeletal protein demarcated by the bracket are increased after NGF treatment (*c*). (*c–i*) Two-dimensional peptide map analyses (*E*, direction of electrophoresis; *C*, that of chromatography) of spots excised from (*c*): (*d*) NF-L, (*f*) NF-M, (*h*) cytokeratin A, and (*i*) cytokeratin D. For comparison, the corresponding maps of NF-L and NF-M from parallel separations of cytoskeletal proteins from rat spinal cord are shown (*e* and *g*). Brackets denote corresponding spots in *d–g*, respectively. The cytokeratin maps of *h* and *i* can be directly compared with those published for these cytokeratins isolated from rat liver (Schiller et al., 1982; Hubbard and Ma, 1983).

mocytoma, as it has been reported (cf. Warren and Chute, 1972) and as they seem to occur frequently as spontaneous tumors in various laboratory strains of rats (Tischler et al., 1985), or whether a neuroendocrine epithelial tumor, i.e., one expressing the IF protein pattern of PC12 cells, could have been the cell of origin of this type of tumor. If the origin of PC12 cells from a pheochromocytoma not expressing cytokeratins is correct then this would represent a case of a neuronally derived cell that has begun to synthesize, in addition to the variable amounts of neurofilaments, large amounts of cytokeratins during growth in vitro, a so far not encountered phenomenon. Alterations of expression of IF proteins during culture in vitro have been reported for epithelial cells that begin to synthesize vimentin (e.g., Franke et al., 1981*b*; Schmid et al., 1983; Venetianer et al., 1983). In these cases, the composition of the culture medium, notably the presence of certain hormones, remarkably influences the pattern of expression of IF proteins (Schmid et al., 1983; Venetianer et al., 1983). It is, therefore, conceivable that changes in the concentration of certain factors influencing the state of differentiation during establishment of the PC12 line such as neurotrophic components (Thoenen et al., 1983) may have contributed to the change in the IF pattern synthesized.

Clearly, the NGF-inducible formation of some neuron-characteristic functions such as production of synapses, electrical excitability, and tetanus toxin receptors demonstrates the value of this cell for studies of the expression of certain neuronal features and functions. On the other hand, the combination of epithelial and neuronal features in the IF cytoskeleton of PC12 cells shows that the state of differentiation of this cell line as now established is not identical to that of a true neuronal or chromaffin cell or a direct precursor in this developmental pathway. As several neurosecretory epithelial cells can also form neurite-like processes, dense-core granules, and various hormones and neurotransmitter substances, specific observations made with PC12 cells in studies of the effects of certain growth factors and ions (e.g., Greene and Tischler, 1976; Mallet et al., 1983; Lander et al., 1983; O'Malley et al., 1983; Nose et al., 1985; Richter-Landsberg et al., 1985; Togari et al., 1985; Friedlander et al., 1986; Green et al., 1986) or oncogene products (Alemà et al., 1985; Bar-Sagi and Feramisco, 1985; Hagag et al., 1986) should not be interpreted a priori in terms of neuronal differentiation. Rather, the PC12 cell system should be considered a neuroendocrine model cell system sui generis with a cytoskeleton that contains epithelial elements.

Clearly, our observations show that the differentiation program involving neurosecretory activity and inducible formation of neurite-like processes, as is characteristic for the PC12 cell line, is well compatible with—and apparently independent from—the simultaneous expression of features of epithelial differentiation. Obviously, the synthesis of neurofilaments does not require the cessation of cytokeratins or vice versa, although in vivo both kinds of IF proteins have been found to co-exist only in a few special tumor cell types. In the studies of the molecular principles of the regulation

of the differential expression of the genes encoding the different IF proteins, the PC12 cell therefore presents a particularly challenging situation.

We are grateful to Drs. H. Betz, H. Rehm and H. Thoenen for providing PC12 cells and valuable discussions as well as Dr. B. Wiedenmann (this institute) for help in the synaptophysin experiments. We thank P. Cowin for reading and correcting the manuscript and I. Purkert for careful typescript.

The work has been supported by the Deutsche Forschungsgemeinschaft and the Research Council "Rauchen und Gesundheit."

Received for publication 27 June 1986, and in revised form 28 July 1986.

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