

## Membrane association of cathepsin B can be induced by transfection of human breast epithelial cells with c-Ha-ras oncogene

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### SUMMARY

Alterations in trafficking and increases in expression of the lysosomal proteases cathepsins B, D and L have been observed in transformed cells and malignant tumors, including human breast carcinoma. *ras* and the related *rab* proteins participate in the vesicular transport processes required for normal trafficking of lysosomal enzymes. In addition, transfection of murine fibroblasts with the *ras* oncogene has been shown to increase the expression of cathepsins L and B. As human cancers are primarily epithelial in origin, we have investigated whether there are alterations in the trafficking and expression of cathepsin B in MCF-10 human breast epithelial cells transfected with wild-type and mutated *ras*. In all cells examined, i.e. mortal MCF-10M cells, immortal MCF-10A or MCF-10F cells, and transfected MCF-10A cells (transfected with the neomycin resistance gene (MCF-10Aneo) or cotransfected with wild-type protooncogenic *ras* (MCF-10AneoN) or mutated oncogenic *ras* (MCF-10AneoT)), levels of mRNA transcripts for cathepsin B were similar. However, alterations in trafficking of cathepsin B were observed in the cells transfected with oncogenic *ras*. In these cells there was an increased association of cathepsin B activity and cathepsin B protein with plasma membrane/endosomal

fractions and a more peripheral distribution of immunofluorescent staining for cathepsin B. At the electron microscopic level, immunogold labeling for cathepsin B was localized to the cell membrane as well as to vesicles in the microvilli and adjacent to the cell membrane. In the parental MCF-10A cells, in contrast, cathepsin B was localized to vesicles in the perinuclear region. The cathepsin B associated with plasma membrane/endosomal fractions in the cells transfected with oncogenic *ras* was mature cathepsin B as demonstrated by immunoblot analysis. This was confirmed further by showing an absence of peripheral immunofluorescent staining in these cells using an antibody specific for the propeptide of cathepsin B. Thus, we have demonstrated by multiple techniques that transfection of human breast epithelial cells with oncogenic *ras* results in alterations in the trafficking of cathepsin B similar to those observed previously in human and animal tumors of both epithelial and mesenchymal origin.

Key words: cathepsin, cysteine protease, breast cancer, neoplasia, *ras*

### INTRODUCTION

Increases in expression, alterations in subcellular distribution and increases in secretion of lysosomal proteases have been reported to parallel malignant progression (for review, see Sloane et al., 1994). Increases in expression of the cysteine protease cathepsin L (mRNA and protein) and secretion of cathepsin L precursor (procathepsin L or MEP; major excreted protein) are observed in murine 3T3 fibroblasts transformed by phorbol esters, growth factors, viruses or transfection with the *ras* oncogene (for review, see Kane and Gottesman, 1990). Rochefort and colleagues (for review, see Rochefort, 1990; Rochefort et al., 1990) have demonstrated a link between malignancy of human breast carcinoma and increases in expression of the aspartic protease cathepsin D (mRNA and protein) and secretion of procathepsin D. The lysosomal protease first associated with malignancy of human breast

carcinoma is cathepsin B, a cysteine protease shown to be secreted from explants of human breast carcinoma, by Poole and colleagues (Poole et al., 1980; Recklies et al., 1980, 1982). Secretion of either mature cathepsin B (Poole et al., 1980; Maciewicz et al., 1989) or procathepsin B (Recklies et al., 1980, 1982; Mort et al., 1981; Qian et al., 1989) can parallel malignancy of human and animal tumors. Increased secretion of cathepsin B often correlates with its distribution in a fraction enriched in plasma membrane and endosomal vesicles (M/E), a phenomenon observed by our laboratory in a wide variety of animal and human tumors and transformed cells (for review, see Sloane, 1990; Sloane et al., 1990, 1994). Increases in mRNA transcripts for cathepsin B have been reported in *ras*-transformed murine 3T3 fibroblasts (Chambers et al., 1992; Zhang and Schultz, 1992) and murine and human tumors (Moin et al., 1989; Qian et al., 1989; Murnane et al., 1991). Nevertheless, although alterations in the expression and traf-

ficking of the lysosomal proteases occur during the progression of cells to a malignant phenotype, the stage at which these alterations occur and whether the alterations are causally linked to progression remain in question.

Furthermore, despite the prevalence of carcinoma among human cancers, the studies in which lysosomal proteases have been linked to malignant progression have utilized transformed fibroblasts rather than epithelial cells. Recently, a model system has become available in which to analyze early stages in the progression of human breast epithelial cells, the MCF-10 model system (Soule et al., 1990; Basolo et al., 1991; Ochieng et al., 1991). Because cathepsin B has been linked to malignancy of breast carcinoma, we have investigated whether any of the alterations in expression or trafficking of cathepsin B observed in other human and animal tumors can be observed in MCF-10 cells spanning stages in progression from immortalization to the transition from preneoplasia to neoplasia. The parental MCF-10A line underwent spontaneous immortalization in culture (Soule et al., 1990) and is non-tumorigenic (Basolo et al., 1991; Miller et al., 1993). The MCF-10AneoT, a variant transfected with oncogenic *ras*, exhibits a transformed phenotype in vitro (Basolo et al., 1991; Ochieng et al., 1991) and forms persistent preneoplastic lesions in nude beige mice, which progress to neoplasias in 30% of the mice (Miller et al., 1994). We demonstrate here an altered subcellular distribution of cathepsin B in MCF-10A cells transfected with mutated *ras*, but not with wild-type *ras*. This altered subcellular distribution included an association of cathepsin B with the cell membrane. These findings suggest that alterations in cathepsin B trafficking occur at the point of transition between the pre-neoplastic and neoplastic state.

## MATERIALS AND METHODS

### Materials

Carbobenzyloxy-arginyl-arginyl-7-amino-4-methylcoumarin (Z-Arg-Arg-NHMec) was purchased from Enzyme Systems Products (Livermore, CA); papain, Percoll, Tween and 2[*N*-morpholino]ethanesulfonic acid from Sigma (St Louis, MO); Micro BCA assay kits from Pierce Chemical Co. (Rockford, IL); [<sup>32</sup>P]dCTP from New England Nuclear (Boston, MA); nick-translation kits from Bethesda Research Laboratories (Gaithersburg, MD); GeneScreen Plus nylon membranes from New England Nuclear (Boston, MA); nitrocellulose membranes and DEAE-Affigel Blue columns from Bio-Rad (Richmond, CA); Protein A-Superose and PD-10 columns from Pharmacia-LKB (Piscataway, NJ); Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture from Gibco (Grand Island, NY); and 4',6-diamidin-2-phenylindol-dihydrochlorid from Boehringer-Mannheim (Indianapolis, IN). A monoclonal antibody to  $\beta$ -tubulin was purchased from Sigma (St. Louis, MO). Texas Red-conjugated affinity-purified donkey anti-rabbit or anti-mouse immunoglobulin (IgG), fluorescein-conjugated affinity-purified donkey anti-mouse or anti-rabbit IgG and normal donkey serum were obtained from Jackson ImmunoResearch (West Grove, PA), SlowFade anti-fade reagent from Molecular Probes (Eugene, OR) and gold-conjugated IgG-specific secondary antibodies from Polysciences (Warrington, PA). The enhanced chemiluminescence western blot detection system was purchased from Amersham (Arlington Heights, IL). The cDNAs for human cathepsin B and 18 S rRNA were kind gifts from Dr D. Fong (Rutgers University, Piscataway, NJ), and Dr O. J. Miller (Wayne State University). The antibody specific for the proregion of cathepsin B and the conditioned medium from the human SK-HEP-1 liver adenocarcinoma cells were kind gifts

from Dr J. Zabrecky (Applied bioTechnology, Cambridge, MA). All other chemicals were of reagent grade and were obtained from commercial sources.

### Cells and culture conditions

MCF-10 is a diploid human breast epithelial cell line derived from a subcutaneous mastectomy in a patient with fibrocystic breast disease. This line underwent spontaneous immortalization in culture and grows attached in the presence of calcium or floating in the absence of calcium (Soule et al., 1990). Transfection and cotransfections were performed using the calcium phosphate method with a plasmid containing the neomycin resistance gene as a transfection vector either alone (MCF-10Aneo) or with constructs containing wild-type (MCF-10AneoN) or mutated (MCF-10AneoT) c-Ha-*ras* (Basolo et al., 1991). All MCF-10 cell lines were screened and shown to be free of *Mycoplasma* with 4',6-diamidin-2-phenylindol-dihydrochlorid. The MCF-10 lines were grown in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, containing 5% equine serum, supplemented with antibiotics and epidermal growth factor (Soule et al., 1990; Basolo et al., 1991; Ochieng et al., 1991), but without amphotericin and cholera toxin.

### Northern blot hybridization

Total cellular RNA from MCF-10 cell lines (either isolated in our laboratory by our published protocols (Moin et al., 1989) or received as a kind gift from Dr R. Pauley (Michigan Cancer Foundation, Detroit, MI)) was analyzed by RNA blot hybridization as described previously (Moin et al., 1989). Total cellular RNAs were electrophoresed on 1% agarose formaldehyde denaturing gels (10  $\mu$ g/lane) according to the procedure of Lehrach et al. (1977) and transferred to nylon membranes by electroblotting (Hoefer Scientific, San Francisco, CA). The membranes were then analyzed by hybridization as follows: one microgram of purified human cathepsin B cDNA or plasmid pUC19 containing cDNA to human 18 S rRNA were <sup>32</sup>P-labeled by nick translation to a specific activity of 2.6 $\times$ 10<sup>7</sup> to 5.8 $\times$ 10<sup>7</sup> cpm/ $\mu$ g depending on the probe. Nylon membranes were prehybridized and then hybridized at 42°C to radioactive probes according to the procedure of Maniatis et al. (1982). Washing was done twice in 2 $\times$  standard saline citrate (SSC; 1 $\times$  SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7) at room temperature for 5 minutes, twice in 2 $\times$  SSC, 1% sodium dodecyl sulfate at 60°C for 30 minutes, and twice in 0.1 $\times$  SSC at room temperature for 30 minutes. Autoradiography was performed at -70°C for 30 minutes to 24 hours depending on the intensity of the signal. In order to analyze the same membrane with different probes, the existing probe was removed by washing the membranes in 0.5 $\times$  Denhardt's solution, 25 mM Tris-HCl, pH 7.5, and 0.1% sodium dodecyl sulfate for 1 hour at 90-95°C followed by a wash in distilled water for 15 minutes at 65°C.

### Preparation of conditioned medium and M/E fractions

MCF-10 cells (4 $\times$ 10<sup>7</sup> to 7 $\times$ 10<sup>7</sup>) grown to ~80% confluency in four T-150 flasks were harvested after an overnight incubation in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture without equine serum. The conditioned medium was concentrated by centrifugation through Millipore UltraFree 10 K concentrators. MCF-10 cells were homogenized in isotonic sucrose (250 mM sucrose, 25 mM 2[*N*-morpholino]ethanesulfonic acid, 1 mM EDTA, pH 6.5) and an M/E fraction enriched in plasma membrane and endosomes isolated by sequential differential and 30% Percoll density gradient centrifugation (Rozhin et al., 1987). SK-HEP-1 conditioned medium was prepared from cells incubated for 4 days in serum-free Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine and containing 10 mM NH<sub>4</sub>Cl to stimulate the secretion of lysosomal enzymes.

### Biochemical assays

Activity of cathepsin B in homogenates, in M/E fractions and in con-

centrated, conditioned media was determined as  $V_{max}$  against the substrate Z-Arg-Arg-NHMeC following our published protocols (Rozhin et al., 1987). The presence of latent activities was assessed by activation with pepsin (0.5 mg/ml) at pH 3.5 for 60 minutes. Latent activity represents the difference between native activity measured in the absence of activation and the total activity measured subsequent to activation with pepsin. Protein was determined using the bicinchoninic acid method of Pierce with bovine serum albumin as standard. DNA in cell homogenates was assayed according to the fluorometric procedure of Downs and Wilfinger (1983).

### Preparation of monospecific anti-cathepsin B IgGs

Antisera were raised in rabbits (New Zealand white male) against the native double-chain form of human liver cathepsin B, as we have described previously (Moin et al., 1992). An IgG fraction was purified and stored at  $-20^{\circ}\text{C}$ . The specificity of the IgG for cathepsin B has been confirmed by slotblotting using purified cathepsin B and cathepsin L, and by immunoblotting using purified cathepsin B (Moin et al., 1992) and extracts (acetone fractions) of normal human liver and human sarcoma (Moin et al., 1992), human prostate tumors (Sinha et al., unpublished data) and matched pairs of normal human colonic mucosa and human colon tumors (Campo et al., unpublished data). The monospecific anti-cathepsin B IgG recognizes procathepsin B and single-chain and double-chain forms of the mature enzyme in immunoblots (Moin et al., 1992; see also Fig. 6a) and immunoprecipitates mature and pro forms of the enzyme (Sloane et al., 1993). This antibody has been used for staining of cathepsin B in human normal and tumor tissues (Visscher et al., 1994; Campo et al., unpublished data; Sinha et al., unpublished data).

### Immunoblotting

Samples electrophoresed in 12% polyacrylamide gel slabs were transferred to nitrocellulose membranes as we have described (Moin et al., 1992). Membranes were developed with an enhanced chemiluminescence western blot detection system using dry milk (10%) and Tween as blocking agents. The samples analyzed were human liver cathepsin B purified according to our published protocol (Moin et al., 1992), and homogenates and M/E fractions prepared from parental 10A cells and their *ras*-transfected neoT counterpart using for each cell line three T-150 flasks harvested at  $\sim 80\%$  confluency.

### Immunofluorescence

Intracellular cathepsin B was localized using a modification of the general immunocytochemical methodologies described by Willingham (1990). Cells grown on glass coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.4. After washing with PBS, cells were blocked with PBS-2 mg/ml bovine serum albumin. All subsequent antibody and wash solutions contained 0.1% saponin. Cells were incubated with primary antibodies (rabbit anti-human liver cathepsin B IgG and mouse anti- $\beta$  tubulin IgG1 or rabbit anti-human cathepsin B propeptide IgG) for 2 hours and washed. In controls, either preimmune serum (rabbit or mouse) was substituted for the primary antibody or no primary antibody was used. After blocking with normal donkey serum (5% in PBS-0.1% saponin), cells were incubated for one hour with either Texas Red-conjugated or fluorescein-conjugated affinity-purified donkey anti-rabbit IgG and fluorescein-conjugated or Texas Red-conjugated affinity-purified donkey anti-mouse IgG at 20  $\mu\text{g}/\text{ml}$ . Cells were then washed, mounted with SlowFade anti-fade reagent and observed with a Zeiss LSM 310.

### Transmission electron microscopy

Cells grown to 60%-100% confluency in 35 mm Petri dishes were fixed in 1.0% glutaraldehyde in phosphate buffer, pH 7.2, at  $4^{\circ}\text{C}$  for 2 hours. After washing in phosphate buffer at  $4^{\circ}\text{C}$ , the cells were postfixed in osmium tetroxide for one hour, dehydrated with graded ethanols and propylene oxide, and embedded in Araldite-Epon. Thin sections were cut on a Sorvall MT-2 ultramicrotome, counterstained

with uranyl acetate and lead citrate, and examined in a Zeiss 10C electron microscope.

### Immunogold staining

Cells grown as for transmission electron microscopy were preserved in 0.5% glutaraldehyde in phosphate buffer, pH 7.2, at  $4^{\circ}\text{C}$  for 1 hour. After washing in phosphate buffer and dehydration in an ethanol gradient, the cells were embedded in LR White medium grade acrylic. Semi-thin sections were collected and mounted on mesh nickel grids. The grids were incubated with primary antibody (rabbit anti-human liver cathepsin B IgG) overnight at  $4^{\circ}\text{C}$ . The grids were then incubated for 4 hours at  $25^{\circ}\text{C}$  with a 1:10 dilution of gold-conjugated (mean particle size of 20 nm) IgG-specific secondary antibody (goat anti-rabbit). The grids were counterstained as described above for transmission electron microscopy.

## RESULTS

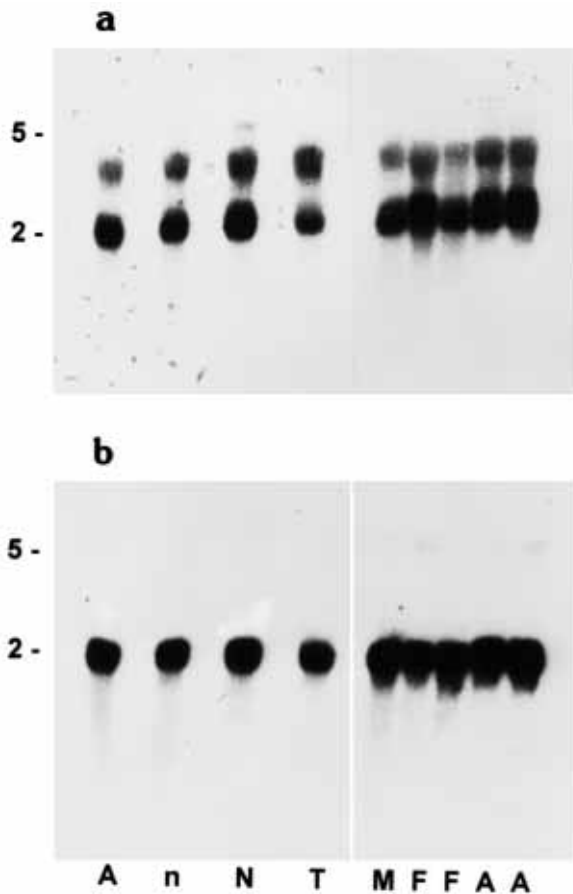
### Levels of mRNA transcripts

Because increases in mRNA transcripts for cathepsin B have been reported in murine 3T3 fibroblasts transformed with the *ras* oncogene (Chambers et al., 1992; Zhang and Schultz, 1992), we investigated whether transfection of MCF-10 human breast epithelial cells with either wild-type *ras* or oncogenic *ras* would affect the levels of mRNA transcripts for cathepsin B. Total RNAs from the following MCF-10 lines were analyzed: mortal MCF-10 cells, spontaneously immortalized MCF-10 cells that grow attached (10A) or floating (10F), and MCF-10A cells transfected with the neomycin resistance gene (10Aneo) or cotransfected with the neomycin resistance gene plus protooncogenic *ras* (10AneoN) or plus oncogenic *ras* (10AneoT). In all MCF-10 cell lines, including the mortal cell line, two mRNA transcripts of 2.2 and 4.1 kb hybridized to the human cathepsin B cDNA (Fig. 1) as has been reported by ourselves (Moin et al., 1989) and others (Murnane et al., 1991; Gong et al., 1993). Furthermore, the levels of cathepsin B-specific transcripts were similar in all MCF-10 cell lines (Fig. 1). Thus, transfection of the MCF-10 human breast epithelial cells with oncogenic *ras* did not result in an increase in the levels of mRNA transcripts for cathepsin B.

### Membrane association

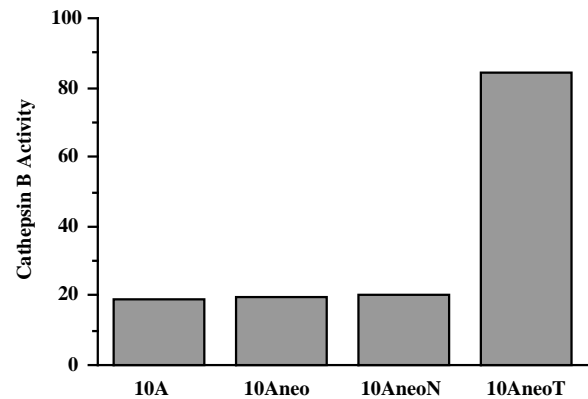
Increased association of cathepsin B with M/E fractions often parallels malignancy (for review, see Sloane, 1990; Sloane et al., 1990, 1994). Therefore, we investigated whether transfection of MCF-10A cells with wild-type or oncogenic *ras* would result in M/E association of cathepsin B. Cathepsin B activities in M/E fractions of immortal 10A cells and 10A cells transfected with neo or protooncogenic *ras* were similar. However, in M/E fractions of 10A cells transfected with mutated *ras*, the cathepsin B activity was  $>4$ -fold more than in the other MCF-10A cell lines analyzed (Fig. 2). Cathepsin B activity in the M/E fractions did not require activation with pepsin at acidic pH and thus did not appear to be in a latent precursor form.

Because the presence of native activity in the M/E fraction suggested that the cathepsin B had undergone proteolytic processing to its mature form, we determined the molecular size(s) of the cathepsin B in the M/E fraction of the *ras*-transfected neoT cells by immunoblotting (Fig. 3) using antibody produced to the purified double-chain form of human liver cathepsin B (Moin et al., 1992). We compared the molecular

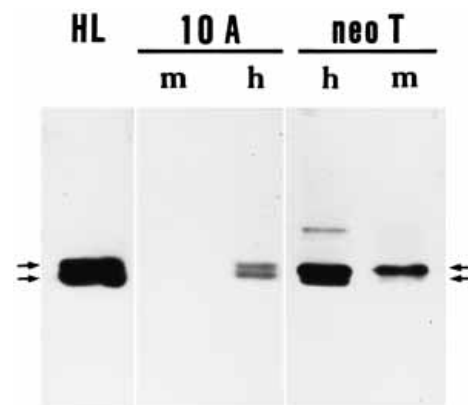


**Fig. 1.** Blot hybridization of total RNAs from MCF-10 cell lines to human cathepsin B (a) cDNA. Filters used for analysis of cathepsin B-specific transcripts were washed and reprobbed for 18 S human ribosomal RNA (b) to ensure equivalent loading of lanes (10  $\mu$ g/lane). In addition, the amount of RNA loaded per lane was judged to be equivalent by ethidium bromide staining of the ribosomal bands (not shown). Lanes represent: A, MCF-10A; n, MCF-10Aneo; N, MCF-10AneoN; T, MCF-10AneoT; M, MCF-10M; F, MCF-10F (see text for further details). Multiple lanes with the same letter designation represent different passage numbers of the same cell type. Blot hybridizations have been repeated with comparable results for each cell type on at least three separate occasions. Ribosomal RNA sizes are indicated in kb.

sizes of cathepsin B in homogenates and M/E fractions of the parental 10A cells and *ras*-transfected neoT cells to those of cathepsin B purified from human liver (Moin et al., 1992). The double-chain form or fully processed form of human liver cathepsin B runs as a heavy-chain doublet of 26 and 25 kDa plus a 5 kDa light chain not visible on this gel (Fig. 3, HL). The heavy-chain doublet of cathepsin B was detected in homogenates of both the parental 10A and *ras*-transfected neoT cells (Fig. 3, h). A band of 31 kDa representing mature single-chain cathepsin B (Moin et al., 1992) also was present in homogenates of the neoT cells (Fig. 3, h), suggesting that proteolytic processing of cathepsin B may be somewhat compromised in these cells. Cathepsin B protein was not detected in M/E fractions of the parental 10A cells (Fig. 3, m). In the *ras*-transfected neoT cells, the primary form of cathepsin B in M/E fractions was the 26 kDa upper band of the heavy-chain doublet (Fig. 3, m). We have shown previously that this band

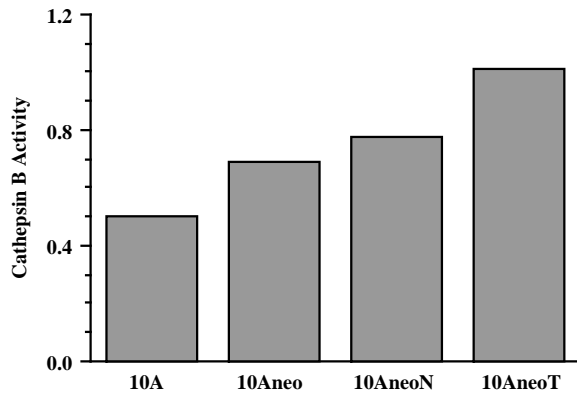


**Fig. 2.** Cathepsin B activity associated with plasma membrane/endosomal fractions of parental MCF-10A cells and their counterparts transfected with neo alone (10Aneo) or cotransfected with neo + wild-type *ras* (10AneoN) or neo + oncogenic *ras* (10AneoT). Only native activities could be measured in the plasma membrane/endosomal fractions. Activity is expressed as nmol NHMec/min  $\times$  mg protein and was determined as  $V_{max}$ , as described in Materials and Methods, in at least three separate experiments.



**Fig. 3.** Immunoblot analysis of homogenates (h) and plasma membrane/endosomal (m) fractions of parental MCF-10A and *ras*-transfected neoT cells utilizing a monospecific cathepsin B antibody. In the lane labeled HL is cathepsin B purified from human liver by our published methodologies (Moin et al., 1992). The two arrows indicate the heavy-chain doublet of double-chain cathepsin B, i.e. the fully processed mature form of the enzyme. This heavy-chain doublet of cathepsin B was detected in homogenates of both parental and *ras*-transfected MCF-10 cell lines. In contrast, single-chain cathepsin B was detected only in the homogenate of the *ras*-transfected cell line. Cathepsin B could not be detected in the plasma membrane/endosomal fraction of the MCF-10A cell line, whereas the primary form of cathepsin B detected in the membrane fraction of the neoT cell line was the upper band of the heavy-chain doublet of cathepsin B (compare with lane labeled HL). The amount of protein loaded in each lane was: HL, 100 ng; h, 10 mg each; and m, 0.2 mg each. Immunoblotting analyses have been repeated ten times with comparable results.

represents the glycosylated heavy chain of mature double-chain cathepsin B (Moin et al., 1992). The presence in the immunoblot of a mature form of cathepsin B in the M/E fraction of neoT cells verified our measurement of native cathepsin B activity in this fraction of neoT cells.



**Fig. 4.** Cathepsin B activity in concentrated, conditioned media of parental MCF-10A cells and their counterparts transfected with neo alone (10Aneo) or cotransfected with neo + wild-type *ras* (10AneoN) or neo + oncogenic *ras* (10AneoT). Only latent activities could be measured in the media. Activity is expressed as nmol NHMec/min × mg DNA in cell homogenates from the four T-150 culture flasks and was determined as  $V_{max}$  as described in Materials and Methods in at least three separate experiments.

The immunoblotting analyses indicated that the amount of mature cathepsin B protein in both the homogenate and M/E fraction of the *ras*-transfected neoT cells was greater than in the homogenate and M/E fraction of parental 10A cells (Fig. 3). The cathepsin B activities in M/E fractions of the two cell lines (Fig. 2) paralleled the amounts of mature cathepsin B protein in those fractions (Fig. 4). In contrast, although the amount of mature cathepsin B protein in neoT homogenates was greater than that in 10A homogenates (Fig. 4), cathepsin B activities in the homogenates of the two MCF-10 lines did not differ. The  $V_{max}$  values for cathepsin B activities in nmol NHMec/min-mg protein were 36 in the parental 10A line, 38 in the line transfected with neo alone (10Aneo), 32 in the line cotransfected with neo + protooncogenic *ras* (10AneoN), and 32 in the line cotransfected with neo + oncogenic *ras* (10AneoT). Our inability to measure differences in cathepsin B activities in cell homogenates may reflect the presence of alternative substrates for the enzyme and/or of endogenous cysteine protease inhibitors in cell homogenates. However, the activities of heat-stable cysteine protease inhibitors in homogenates of the parental 10A and the *ras*-transfected neoT cells were identical (i.e. a mean ± s.d. of 100±18 and 98±12 munits per mg protein, respectively; see Sloane et al., 1992, for details of the assay procedure). Therefore, our inability to measure increased cathepsin B activities in the homogenates of the *ras*-transfected neoT cells did not appear to be due to differences in activities of heat-stable cysteine protease inhibitors in the parental 10A and neoT cell lines.

**Secretion**

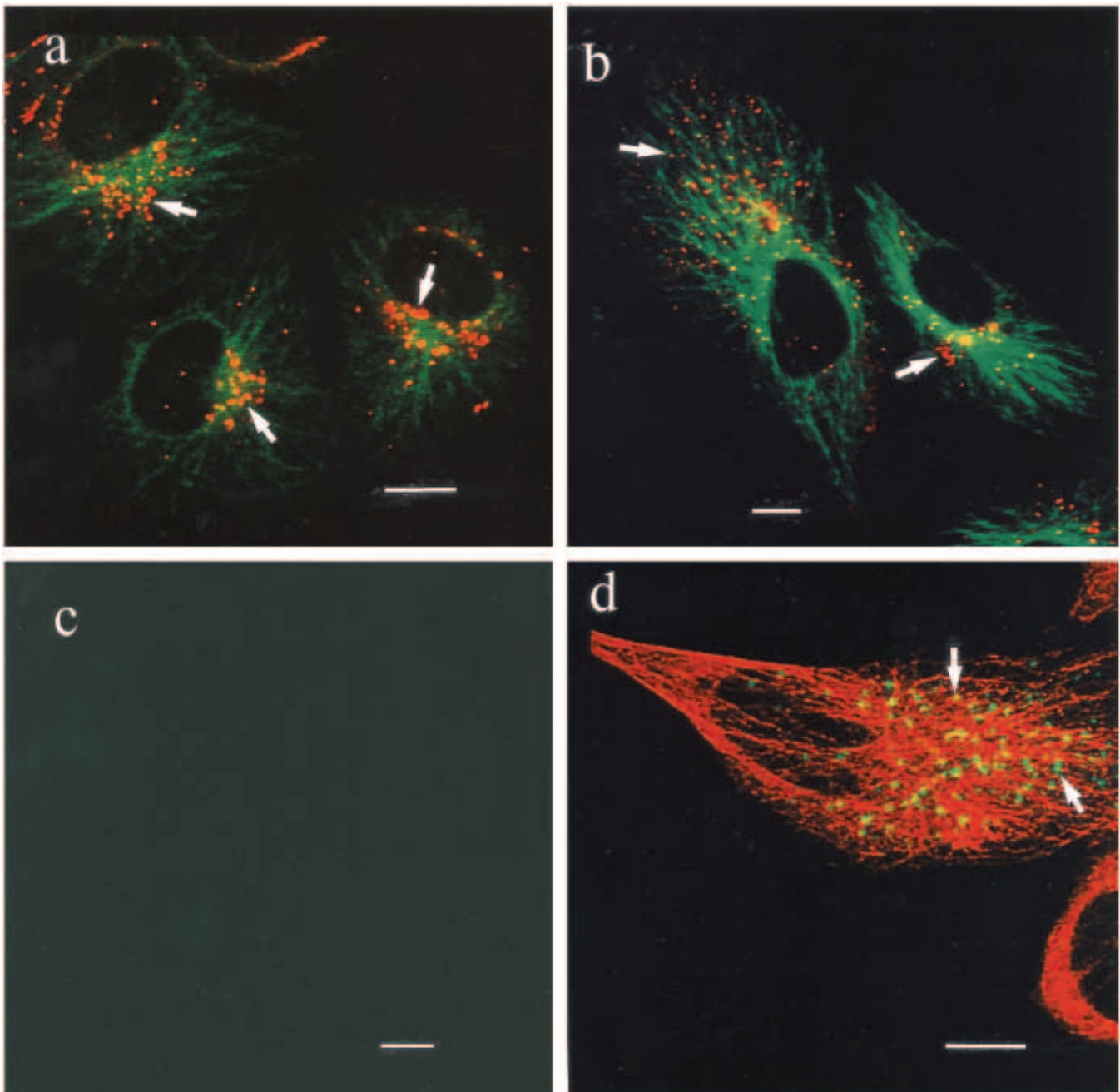
Transfection of murine 3T3 fibroblasts with the *ras* oncogene dramatically elevates secretion of procathepsin L (for review, see Kane and Gottesman, 1990), whereas lesser increases in the secretion of either mature cathepsin B or procathepsin B have been shown to correlate with malignancy of human and murine tumors, respectively (Maciewicz et al., 1989; Qian et al., 1989). Therefore, we determined the effect of transfection of MCF-10A cells with wild-type and oncogenic *ras* on the

secretion of latent and native cathepsin B. Only latent cathepsin B activity could be detected in conditioned media of the MCF-10A cell lines. Native cathepsin B activity could not be detected even in media concentrated by ultrafiltration. Secretion of latent cathepsin B activity was ~100% greater in the cells transfected with mutated *ras* than in the parental 10A cells (Fig. 4), yet secretion of latent cathepsin B activity also was increased in cells transfected with neo alone or neo plus wild-type *ras* (40% and 60%, respectively). Thus, increased secretion of procathepsin B by the MCF-10A human breast epithelial cell lines was not a response to their transfection with oncogenic *ras*.

**Immunofluorescence localization**

The analyses described above demonstrating cathepsin B activity and protein in M/E fractions of neoT cells suggest that trafficking of cathepsin B may have been altered in these cells transfected with mutated *ras*. We, therefore, determined the subcellular localization of cathepsin B in the parental 10A cells and in their neoT counterpart using antibody produced to the purified double-chain form of human liver cathepsin B (Moin et al., 1992). To delineate the intracellular distribution of cathepsin B staining in the MCF-10A and neoT cells, we double-stained for tubulin and cathepsin B using secondary antibodies conjugated to either Texas Red (Fig. 5a-c) or fluorescein (Fig. 5d). In the parental 10A cells, the staining for cathepsin B was localized in the perinuclear region (Fig. 5a). In contrast, in the cells transfected with oncogenic *ras*, staining for cathepsin B (Fig. 5b, arrows) was distributed throughout the cells in both peripheral and perinuclear regions. The peripheral staining for cathepsin B in the neoT cells included staining in cell processes. Only a weak background fluorescence was observed in the presence of preimmune IgG and secondary antibodies (Fig. 5c). In order to better visualize the peripheral distribution of cathepsin B staining in the neoT cells, confocal images of several optical sections were obtained (Fig. 5d). In the confocal images, the staining for cathepsin B was readily seen to be present peripherally. Thus, the staining pattern in the *ras*-transfected cells confirmed our biochemical observations of an altered subcellular distribution of cathepsin B in these cells.

We observed an increase in the secretion of procathepsin B in the neoT cells as compared to the 10A cells, although this increase was not due solely to transfection with the *ras* oncogene (Fig. 4). Nevertheless, given the secretion of procathepsin B by the neoT cells, one might expect to see secretory vesicles containing procathepsin B adjacent to the cell surface in these cells. In order to distinguish the intracellular staining pattern for mature cathepsin B from that for procathepsin B, we used an antibody raised to a synthetic peptide derived from the propeptide of cathepsin B. Initially, we confirmed the specificity of this antibody for procathepsin B by its reactivity with the 43 kDa precursor form of cathepsin B (Sloane et al., 1993) in conditioned medium from human SK-HEP-1 liver adenocarcinoma cells (Fig. 6a, lanes 2 and 3) and its lack of reactivity (Fig. 6a, lane 1) with the purified double-chain form (25/26 kDa heavy chain + 5 kDa light chain) of human liver cathepsin B (Moin et al., 1992). Only a few large perinuclear vesicles exhibited staining with the propeptide antibody in both the 10A (not illustrated) and neoT cells (Fig. 6b, arrows). No staining for procathepsin B was



**Fig. 5.** Immunocytochemical colocalization of intracellular cathepsin B and  $\beta$ -tubulin in parental 10A cells and their neoT counterpart transfected with oncogenic *ras* cell lines. The primary antibodies were rabbit anti-human cathepsin B IgG and mouse anti- $\beta$ -tubulin IgG1. In the 10A cells (a), the cathepsin B staining (arrows) was concentrated in the perinuclear region. In the *ras*-transfected cells (b), the distribution of cathepsin B staining (arrows) was more peripheral with staining observed at the tips of microtubules adjacent to the cell surface. No fluorescent staining was observed in neoT cells in controls in which rabbit and mouse pre-immune IgG replaced the primary antibodies (c). When several optical sections of the neoT cells were superimposed (d), the surface localization of cathepsin B staining (arrows) was more apparent. In (a-c) the secondary antibodies were Texas Red-conjugated anti-rabbit and fluorescein-conjugated anti-mouse IgG and in (d), fluorescein-conjugated anti-rabbit and Texas Red-conjugated anti-mouse IgG. The staining for cathepsin B has been repeated >100 times to date with comparable results. Bars, 10  $\mu$ m.

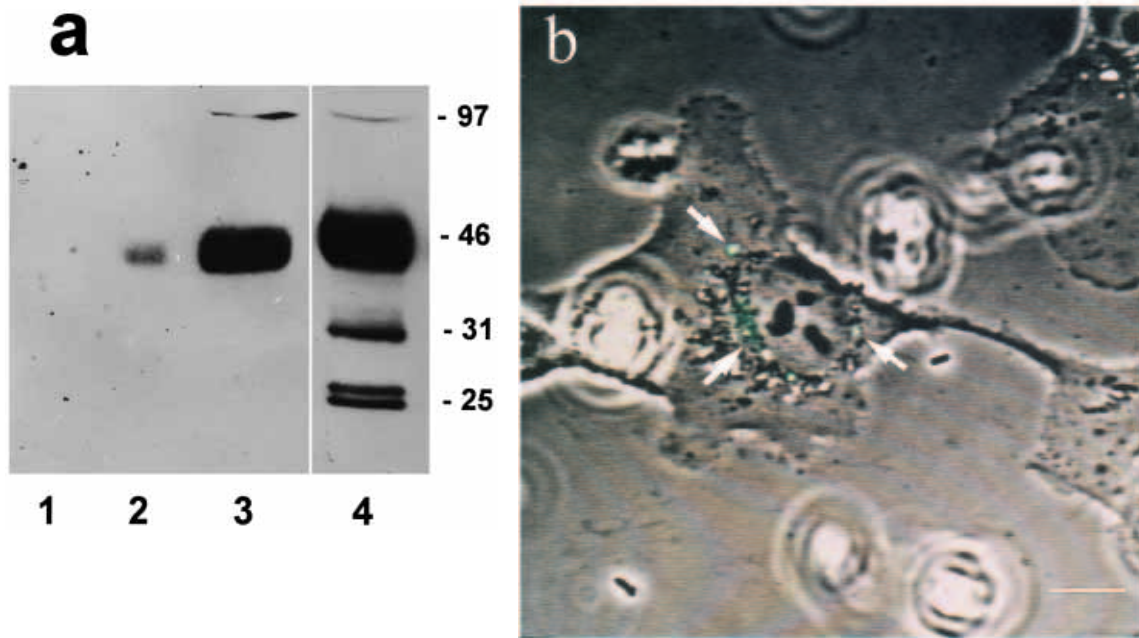
observed in the peripheral vesicles of the neoT cells, i.e. in those vesicles that exhibited staining with the monospecific polyclonal antibody that recognizes both pro and mature forms of cathepsin B (Fig. 5b and d). Thus, the cathepsin B in peripheral vesicles of the neoT cells appeared to be mature cathepsin

B, suggesting that the M/E fraction of the neoT cells may comprise these peripheral vesicles.

#### Immunogold localization

In an effort to find an ultrastructural explanation for the sedi-





**Fig. 6.** Immunoblot analysis of cathepsin B antibodies (a) and immunocytochemical localization of procathepsin B in neoT cells transfected with oncogenic *ras* (b). (a) The primary antibody used in lanes 1-3 was a rabbit IgG produced against a synthetic peptide derived from the propeptide of human cathepsin B and in lane 4 was a rabbit IgG produced against the purified double-chain form of human liver cathepsin B (Moin et al., 1992). Lane 1 contains the purified double-chain form of human liver cathepsin B; lane 2 contains conditioned medium from  $5 \times 10^4$  SK-HEP-1 cells; lanes 3 and 4 contain a 10-fold concentration of the conditioned medium in lane 2. Procathepsin B present in the conditioned medium crossreacted with the anti-propeptide antibody (lanes 2 and 3), but the mature double-chain form of cathepsin B did not (lane 1). The antibody produced to mature double-chain cathepsin B was used to reprobe lane 3 and found to recognize three forms of cathepsin B (procathepsin B, mature single-chain cathepsin B and the heavy-chain doublet of mature double-chain cathepsin B) present in the conditioned medium (lane 4).  $M_r$  size markers ( $\times 10^{-3}$ ) are indicated on the right. (b) The immunocytochemical analysis revealed that procathepsin B was localized exclusively in large perinuclear vesicles (arrows) in both neoT cells (b) and 10A cells (not illustrated). Staining for procathepsin B has been repeated 3 times to date with comparable results. Bar, 10  $\mu$ m.

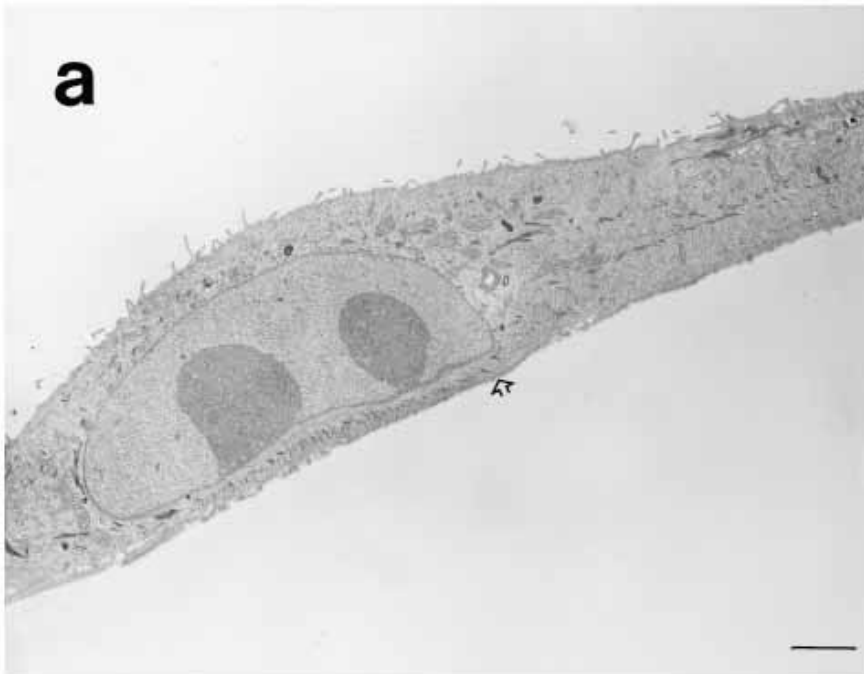
mentation in the neoT cells of cathepsin B activity with the M/E fraction (Fig. 2), we compared the morphology of the parental 10A cells (Fig. 7a) and the *ras*-transfected neoT cells (Fig. 7b). The 10A cells grow as a monolayer (Tait et al., 1990), whereas the neoT cells form multicellular foci as has been described previously (Basolo et al., 1991). The interface of each cell line with the culture dish is indicated in Fig. 7 by an open arrowhead. In terms of vesicular populations in the two cell lines, there were two notable differences. The first was that many of the lysosomes in the neoT cell line contained membrane whorls (Fig. 7b, arrowheads). Similar structures have been observed within the lysosomes of cells from patients with lysosomal storage diseases (van Hoof, 1973). Therefore, the presence of membrane whorls within the lysosomes of neoT cells might indicate that these lysosomes do not contain their normal complement of enzymes as would be expected if *ras*-transfection altered trafficking of lysosomal enzymes. The second notable difference was that the neoT cell line had a large number of small vesicles throughout the cytoplasm. Because endosomes containing cathepsins D and/or B have been observed in macrophages (Rodman et al., 1990), T-cells (Guagliardi et al., 1990) and hepatocytes (Casciola-Rosen et al., 1992), the small vesicles in the neoT cells might represent an endosomal compartment that contains cathepsin B. On the other hand, one of us had demonstrated that the small vesicles in the cytoplasm of the neoT cells contained mucins (L. Tait,

unpublished observation) and, thus, were unlikely to contain cathepsin B.

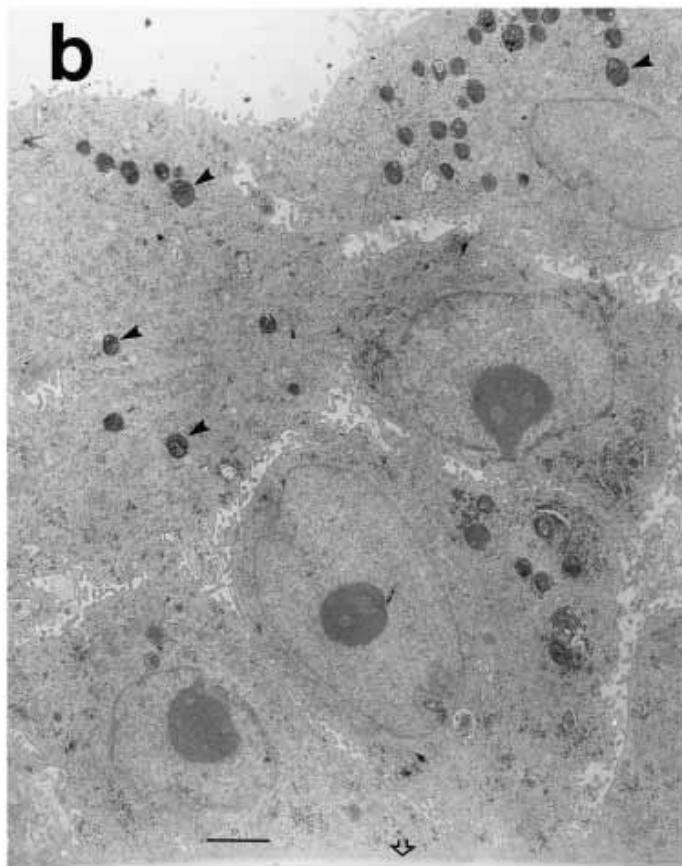
We determined the localization of cathepsin B in the parental 10A cells and their *ras*-transfected neoT counterpart by immunogold labeling using antibody produced to the purified double-chain form of human liver cathepsin B (Moin et al., 1992). In the parental 10A cells, immunogold labeling for cathepsin B was localized to vesicles in the perinuclear region of the cells (Fig. 8a). In the neoT cells transfected with oncogenic *ras*, immunogold labeling for cathepsin B was localized at and adjacent to the surface membrane, primarily to microvilli (Fig. 8b). Cathepsin B-positive vesicles also could be observed in the interior of microvilli (Fig. 9). Such images suggest that vesicles containing cathepsin B might be undergoing exocytosis from the *ras*-transfected neoT cells.

## DISCUSSION

Increases in expression of lysosomal cathepsins have been associated with malignant transformation of cells. Increases in cathepsin B expression are seen in murine 3T3 fibroblasts transformed with oncogenic *ras* (Chambers et al., 1992; Zhang and Schultz, 1992) and increases in cathepsin L expression in murine 3T3 fibroblasts transformed by phorbol esters, growth factors or viruses, or by transfection with the *ras* oncogene (for review, see Kane and Gottesman, 1990). In contrast, transfec-



**Fig. 7.** Transmission electron micrographs of transverse cross-sections of the parental 10A cells (a) and their neoT counterpart transfected with oncogenic *ras* (b). There are several marked morphological differences between the two cell lines: (1) the 10A cells grow as a monolayer and the neoT cells as multicellular foci; (2) the presence of membrane whorls in the lysosomes in the neoT cells (arrowheads); and (3) the presence of many small vesicles in the cytoplasm of the neoT cells. For both the 10A cells and their neoT counterpart, the site of attachment of the cells to the flask is indicated by an open arrowhead. Bars, 15  $\mu$ m.

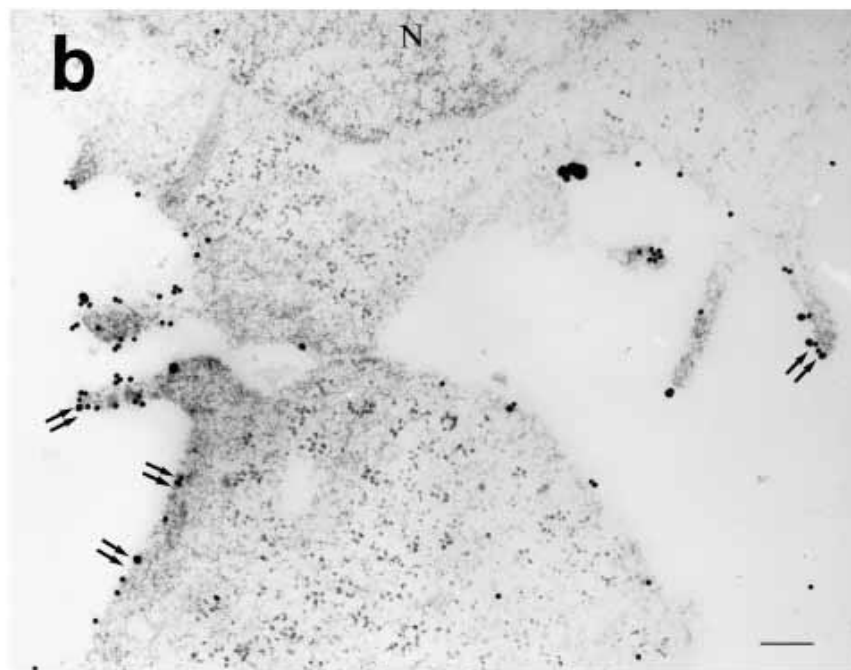
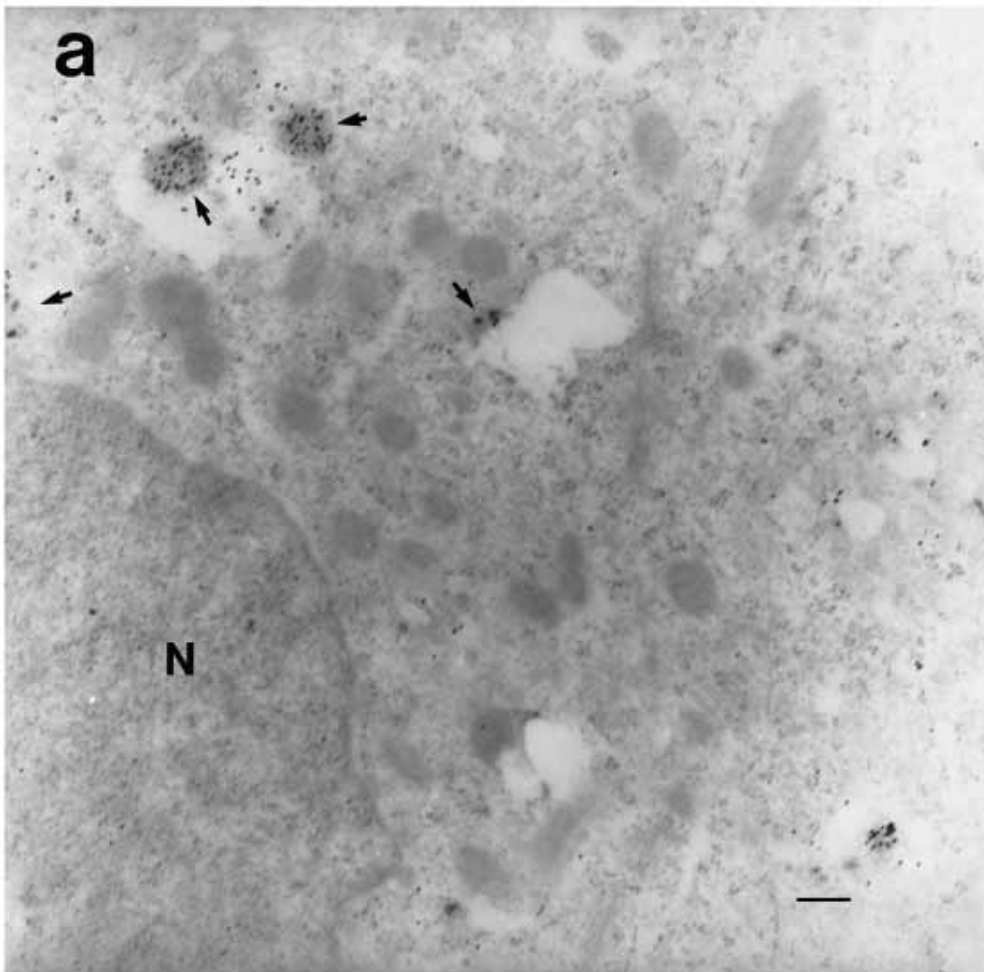


tion with oncogenic *ras* does not result in increased cathepsin B expression in rat embryo fibroblasts (Sloane et al., 1992), nor, in the present study, did transfection with oncogenic *ras* result in increased cathepsin B expression in MCF-10 human breast epithelial cells. Alterations in trafficking that lead to secretion of lysosomal cathepsins have also been associated

with malignant transformation of cells. For example, transfection of murine 3T3 fibroblasts with the *ras* oncogene elevates secretion of procathepsin L (for review, see Kane and Gottesman, 1990) and transformation of murine BALB/3T3 fibroblasts by Moloney murine sarcoma virus elevates secretion of both procathepsin L and procathepsin B (Achkar et al., 1990). In MCF-10 human breast epithelial cells, the process of transfection itself (i.e. transfection with a plasmid containing the neomycin resistance gene) resulted in enhanced secretion of procathepsin B and cotransfection with either wild-type or mutated *ras* moderately increased this secretion. Thus, in the MCF-10 cells increased secretion of procathepsin B was not solely a result of transfection with the *ras* oncogene. Comparing the effects of transfection with oncogenic *ras* on cathepsin B expression and secretion in murine 3T3 fibroblasts, rat embryo fibroblasts and MCF-10 human breast epithelial cells suggests that the ability of oncogenic *ras* to induce increases in cathepsin B expression and secretion is not dependent on cell lineage (epithelial vs fibroblastic) or species (human vs murine), but may be related to the genetic phenotype of the transfected cells (diploid vs aneuploid). In other words, the ability of oncogenic *ras* to induce increases in cathepsin B-specific transcripts and procathepsin B secretion in 3T3 fibroblasts (Chambers et al., 1992; Zhang and Schultz, 1992) may be secondary to other genetic alterations in these aneuploid cells. Perhaps supporting this interpretation is the fact that Balb/3T3 fibroblasts secrete procathepsin B and procathepsin L in the absence of viral transformation (Achkar et al., 1990).

Association of cathepsin B with the membrane of MCF-10 human breast epithelial cells was induced by transfection with oncogenic *ras*. The ability of *ras* to alter the intracellular trafficking of cathepsin B is of interest, as the most consistent observation that we have made in regard to cathepsin B in malignant cells is its cosedimentation with M/E fractions isolated from tumor cells and transformed cells (for review, see Sloane, 1990; Sloane et al., 1990, 1994). In the present study,

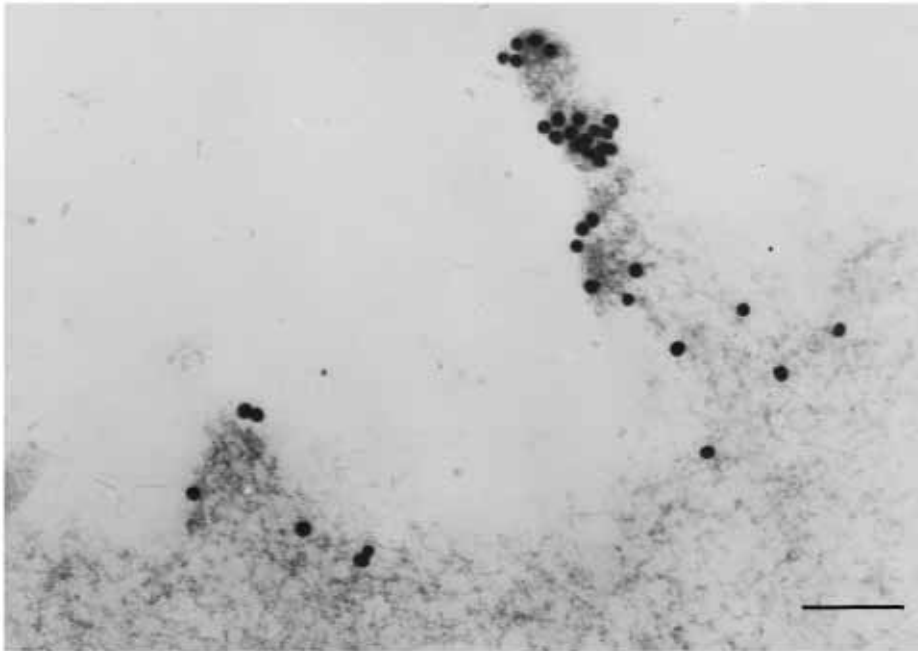




**Fig. 8.** Immunogold labeling of cathepsin B protein in the parental 10A cells and their neoT counterpart transfected with oncogenic *ras*. In the 10A cells (a), the gold particles are localized over large vesicles (arrows) in the perinuclear region. In the neoT cells (b), the gold particles are localized at the cell surface (double arrows), often in association with microvilli. N, nucleus; bars, 0.5  $\mu$ m (a and b).

we confirm the altered trafficking and membrane association of cathepsin B in the *ras*-transfected neoT cells by multiple techniques: the presence of cathepsin B activity and protein in

an M/E fraction isolated by sequential differential and Percoll density gradient centrifugation, immunofluorescent staining for cathepsin B in the cell periphery and cell processes, and



**Fig. 9.** Vesicles exhibiting immunogold labeling for cathepsin B protein appear to be in the process of being secreted from a microvillous. Bar, 0.17  $\mu\text{m}$ .

immunogold labeling for cathepsin B at the cell membrane and in vesicles within microvilli and adjacent to the cell membrane. In the highly malignant murine B16 amelanotic melanoma, we had shown that 80% of the cathepsin B activity in the M/E fraction does not require activation with pepsin at acid pH and, thus, appears to be a native or mature form of cathepsin B rather than a latent precursor form (Sloane et al., 1991). That only mature cathepsin B was present in the M/E fraction of the *ras*-transfected neoT cells and that procathepsin B was not present in peripheral vesicles of the *ras*-transfected neoT cells was verified in the present study by immunoblotting analyses and immunofluorescent staining with an antibody specific for the propeptide of cathepsin B. A peripheral vesicular compartment containing active cathepsin B has been found in murine Swiss 3T3 fibroblasts by Roederer et al. (1987). In this case, the compartment was identified as an endosomal compartment. Either single-chain or double-chain cathepsin B are active (Hanewinkel et al., 1987; Moin et al., 1992) and could account for the activity measured by Murphy and coworkers in the endosomal compartment of 3T3 fibroblasts (Roederer et al., 1987) and for the activity measured by us in M/E fractions of neoT cells.

The cathepsin B that cosedimented with the M/E fraction of the neoT cells was the fully processed double-chain form rather than the partially processed single-chain form. Maturation of the cathepsins to active single-chain forms and subsequently to double-chain forms has been thought to be initiated during their trafficking within the endosomal/prelysosomal vesicles and completed after their delivery to the lysosomes (for discussion of the biosynthesis of lysosomal proteases, see Erickson, 1989a). This has recently been confirmed for the lysosomal aspartic protease cathepsin D (Rijnboutt et al., 1992) where maturation to an active single-chain form occurs within the late endosomes and subsequent cleavage to an active double-chain form is completed after delivery to the lysosomes. A similar paradigm has not yet been confirmed for other lysosomal cathepsins. Nevertheless, one might speculate

that the presence of double-chain cathepsin B in the M/E fraction of the *ras*-transfected neoT cells indicates that: (1) processing of cathepsin B is completed within endosomal/prelysosomal compartments in these cells, or (2) there is organelle mixing, similar to that observed with brefeldin A (for review, see Pelham, 1991), within the vesicular transport system of these cells. That asymmetric cleavage of cathepsin B to its double-chain form occurs in the endosomes of neoT cells seems unlikely as in vitro autocatalytic processing to the double-chain form is dependent on an acidic pH such as that present in the lysosomal compartment (Rowan et al., 1992). Given the association of *ras* and the related *rab* proteins with vesicular transport including transport between the endosomal/lysosomal compartments and the plasma membrane (Ridley and Hall, 1992; Ridley et al., 1992) and with the recycling of mannose 6-phosphate receptors from late endosomes to the *trans*-Golgi network (Lombardi et al., 1993), a disruption of normal vesicular transport in the *ras*-transfected neoT cells would appear to be a more likely explanation for the presence of double-chain cathepsin B in the M/E fraction of these cells.

Lysosomes normally are localized in the perinuclear region of cells (see Fig. 5a). However, redistribution of lysosomes toward the cell periphery as observed in the *ras*-transfected neoT cells (see Fig. 5b) often occurs in cells that participate in degradative or invasive processes. In activated osteoclasts, the lysosomes are transported toward the apical membrane and lysosomal enzymes are secreted into an extracellular resorptive compartment formed between the adherent osteoclast and bone (for review see Baron, 1989). Activated macrophages also secrete lysosomal enzymes (Skudlarek and Swank, 1981; Erickson, 1989b; Tomoda et al., 1989; Tapper and Sundler, 1990) and have been reported to have surface-associated lysosomal enzymes including the lysosomal cysteine protease cathepsin L (Reilly et al., 1989). Movement of lysosomes from the perinuclear region to the cell periphery can be induced in macrophages and fibroblasts (Heuser, 1989) and neuronal and

epithelial cells (Parton et al., 1991) by incubating the cells in an acidic buffer. Osteoclasts and macrophages can acidify the compartments between themselves and bone and collagen films, respectively (Silver et al., 1988), perhaps in turn inducing the further secretion of lysosomal enzymes.

The MCF-10AneoT cells transfected with mutated *ras* have been shown to invade through Matrigel in vitro (Ochieng et al., 1991) and to produce preneoplastic lesions in nude beige mice that progress to neoplasias in 30% of the mice (Miller et al., 1993). These lesions resemble those observed in proliferative breast disease, suggesting that the neoT cells may represent an early step in the malignant progression of human breast epithelium. We hypothesize that having a vesicular compartment containing hydrolytic enzymes poised at the cell surface may be an important component of the preneoplastic phenotype of the neoT cells and a prelude to the induction of invasive processes in neoplastic lesions. The fact that similar patterns of lysosomal redistribution are observed in activated osteoclasts and macrophages indicates that transport of lysosomes to the cell periphery may be a mechanism common to cells that engage in local degradative and invasive processes. The universality of this phenomenon is indicated by the recent observation that the movement of lysosomes to the cell periphery is required for the invasion of epithelial cells by trypanosomes (Tardieux et al., 1992).

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