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Transformation by Oncogenic Ras-p21 Alters the Processing and Subcellular Localization of the Lysosomal Protease Cathepsin D

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Abstract The expression, processing, and intracellular localization of cathepsin D (CD), an endosomal-lysosomal protease involved in malignancy, were studied in rat embryo fibroblasts transformed with an active mutant of c-Ha-*ras* oncogene. The pattern of the processed molecular forms of CD, comprising two single-chain mature forms of 45 and 43 kDa and two double-chain mature forms of 34 + 9 kDa and 30 + 14 kDa, expressed by the parental cell line was similar to that found in normal rat liver cells. By contrast, in the *ras*-transfected counterpart this pattern was profoundly altered in that the 45 kDa species was much less represented and the 30 + 14 kDa species virtually absent. In both untransformed and *ras*-transformed cells the conversion of proCD into mature forms was not inhibited by ammonium chloride, which is known to increase the intravacuolar pH of post-Golgi compartments. Yet, this drug induced the accumulation of the 43 and 45 kDa molecular forms of mature CD in *ras*-transformed cells and of the 34 kDa molecule in untransformed cells. As compared to controls, in *ras*-transformed fibroblasts vacuolar compartments containing CD were reduced in number and mostly located toward the periphery of the cell. This contrasted with the perinuclear distribution of CD-positive granules in untransformed cells. Serum deprivation did not affect the growth, nor the intra- and extracellular accumulation of CD activity in *ras*-transformed cultures, while it blocked the growth and strongly stimulated the accumulation of CD in the medium in cultures of control fibroblasts. Altogether these data are indicative for a crucial role of *ras* GTPase in the regulation of the transport between post-Golgi organelles. J. Cell. Biochem. 73:370–378, 1999.

Key words: cathepsin D; c-Ha-*ras;* lysosomes

The monomeric GTPase coded by genes of the *ras* family plays crucial roles in the signal transduction pathways coupled to cell proliferation and differentiation [Marshall, 1991; Lowy and Willumsen, 1993]. It is not surprising, therefore, that a deregulated activity of this small GTP binding protein represents a common finding in many different tumors [Tabin et al., 1982; Capon et al., 1983; Graham, 1985; Manhues and Pellicer, 1992]. Accordingly, transfection of constitutively activated mutant forms of c-Ha-*ras* into suitable recipient cells has been shown to cause cellular transformation characterized by loss of cell contacts and adhesivity, uncontrolled proliferation, morphological alterations, and acquisition of invasive and metastatic properties [Spandidos and Wilkie, 1984; Muschel et al., 1985; Ochieng et al., 1991]. Altered expression and trafficking of proteolytic enzymes, including collagenases and lysosomal cathepsins, often parallel the neoplastic progression and several lines of evidence strongly suggest that these alterations actively contribute to the ac-

Abbreviations used: CD, cathepsin D; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HRP, horseradish peroxidase; M6P, mannose-6-phosphate; MPR, M6P-receptor; REF, rat embryo fibroblasts; 4R, c-Ha-*ras*-transformed rat embryo fibroblasts; SDS, sodium dodecyl sulphate.

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quisition of the malignant behavior [Garbisa et al., 1987; Rochefort, 1990; Sloane et al., 1994; Isidoro et al., 1997a]. In the present work we analyze the effects of oncogenic *ras* transformation on the expression and trafficking of cathepsin D (CD), an acid endopeptidase acting within the endosomal and lysosomal compartments and also extracellularly [Dingle et al., 1971; Barrett, 1977; Berg et al., 1995]. Various experimental data assign to CD a key role in tissue homeostasis [Saftig et al., 1995; Deiss et al., 1996] and in various aspects of malignancy [Rochefort, 1990; Isidoro et al., 1995a,b]. These studies have revealed that the presence of enzymically-active mature CD in endosomes is an essential requisite for carrying out many of the phenotypical responses that are crucial in tumor progression. Thus, in transfected tumor cells, overexpression of CD caused an increase of the metastatic potential only if the protein was allowed to reach the endosomal-lysosomal apparatus, while chimeric proCD retained within the endoplasmic reticulum was ineffective [Liaudet et al., 1994]. Also, cytokine-induced apoptosis of HeLa and U937 tumor cells was dependent on the accumulation of mature CD in endosomes [Deiss et al., 1996]. Of interest, differentiation of coloncarcinoma cells was associated with a higher expression of the endosomal form of CD [Isidoro et al., 1997c]. In the present study we show that *ras*-transformation of embryonic fibroblasts is paralleled by (1) altered maturation and cellular location of CD, and (2) loss of serum influence on CD expression and secretion.

MATERIALS AND METHODS

Cell Types and Culture Conditions

Rat embryo fibroblasts (REF) and their mutated c-Ha-*ras* 1 (4R) transfected derivatives [Pozzati et al., 1986] were kindly provided by Dr. Garbisa (University of Padova). Cells were maintained under standard culture conditions in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) and 4 mM glutamine. Culture reagents were purchased from Biochrom (Berlin, Germany). Cell-cycle analysis of cells fixed in ice-cold 70% ethanol and stained with propidium iodide (0.18 mg/ml in PBS) in the presence of RNase was performed with a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer.

Cathepsin D Activity and Protein Determination

CD activity was assayed on cell homogenates and medium using a radioactive substrate as previously described [Isidoro et al., 1995b]. In our assay conditions, this proteolytic activity was inhibited in the presence of 1 μM pepstatin. Cell protein was determined by the method of Lowry et al. [1951] using bovine serum albumin as the standard.

Analysis of Cathepsin D Molecular Forms

Rat liver CD was purified by affinity chromatography on pepstatin-Sepharose as previously described [Bonelli et al., 1987] and separated by SDS-polyacrylamide gel electrophoresis. Cell-associated CD molecular forms were identified by the method of western blotting using specific anti-rat CD antiserum. Briefly, 30–40 μg of cell protein were denatured and separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) and then electroblotted onto nitrocellulose N^+ sheet (Hybond N, Amersham, Buckinghamshire, UK). Albumin saturated blots were probed with rabbit anti-rat CD antiserum [Isidoro et al., 1995b] followed by a HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA). Immunocomplexes were revealed by chemiluminescence (Dupont-NEN, Boston, MA) and autoradiography (Amersham). Control loading of homogenates proteins was performed on a parallel gel stained with Coomassie Brilliant Blue or by staining the filter with Ponceau Red. Data shown are representative of at least three independent experiments.

Morphological Studies

For this purpose cells were grown on sterile glass coverslips. Fixed (20 min with 3.7% formaldehyde in phosphate buffered saline, pH 7.4) and permeabilized (7 min with 0.1% Triton X-100) cells were used for immunofluorescent detection of CD by incubating with rabbit anti-rat CD antiserum [Isidoro et al., 1995b] (1:50 in phosphate buffered saline containing 0.1% Triton X-100 and 1:25 fetal calf serum) followed by a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Biorad; 1:100 as above).

RESULTS

Effects of *ras*-Transformation on the Expression of CD

First, we compared the expression and maturation of cathepsin D in cultures of rat embryo

fibroblasts (REF) and of their Ha-*ras*-transformed derivatives (4R) [Pozzati et al., 1988]. The hyperexpression of the oncogenic Ras-p21 in the transformed cell line was checked by Western blot analysis (not shown). Cells were plated at an initial density of 50,000/cm^2, allowed to attach and grown for 2 days in renewed medium. Then, after culturing for further 24 h in fresh medium, cells were collected, homogenized, and analyzed for CD activity and processing. In these experimental conditions final densities were approximately 190,000 cells/cm^2 and 380,000 cells/cm^2 for REF and 4R, respectively (see also below). Intracellular specific activity of CD was reduced by some 20% in *ras*-transformed cells with respect to the values measured in parental untransformed cells (see below). We analyzed and compared the molecular forms of CD synthesized by normal and *ras*-transformed fibroblasts by Western blotting (Fig. 1B). As a control, mature CD isolated from rat liver by chromatography was used (Fig. 1A). As revealed by SDS-polyacrylamide gel electrophoresis mature rat liver CD consisted of a mixture of three molecular species with an approximate mass of 43–45 kDa, 32 kDa, and 10 kDa, respectively. The largest molecular species was by far the most represented (accounting for more than 95% of total), the smallest ones being detectable only if samples were overloaded in the gel. Beside these molecular forms, in homogenates of both cell types a higher molecular weight polypeptide of approximate mass of 50 kDa was also detected (Fig. 1B). According to the predicted sizes based on cDNA sequence [Fujita et al., 1991], data shown in Figure 1B can be interpreted as follows: the 50 kDa species represents the proenzyme form, the 45 and 43 kDa polypeptides represent the two single-chain forms and the 34 kDa and 30 kDa bands represent the large chains of the two double-chain forms made up, respectively, of 34 kDa plus 9 kDa and of 30 kDa plus 14 kDa polypeptides. The latter species was by far the most represented double-chain form in REF. In *ras*-transformed cells the 45 kDa species was little represented and the 30 + 14 kDa species was virtually absent (Fig. 1B). As mature, enzymically active, molecular forms of CD are generated in the endosomal-lysosomal apparatus by limited proteolysis of the precursor [Hasilik, 1992], the above results suggest that in REF and 4R cells proCD was differently transported,

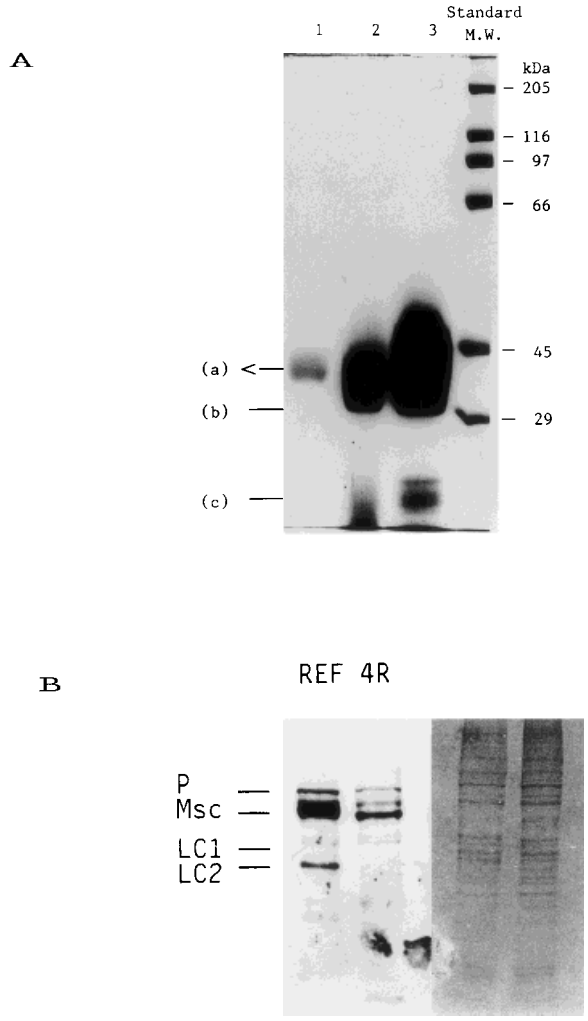

Fig. 1. Molecular forms of cathepsin D in normal and *ras*-transformed rat cells. **A:** Molecular composition of purified rat CD: increasing amounts (5 µg, 40 µg, and 120 µg, **lanes 1, 2, and 3,** respectively) of CD purified from rat liver were separated on SDS-containing 10% polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. The position of the various molecular forms (a, single-chains; b and c, heavy and light chains of the double-chain, respectively) are indicated. **B:** Molecular forms of CD revealed by immunoblotting in homogenates of REF and 4R cells: P, precursor, Msc, mature single-chain, LC1 and LC2, large-chains of the two double-chain forms (see text). The small chains of these molecular species probably run out the gel. The right side of the picture shows that equal amounts of homogenates proteins were loaded.

matured, and accumulated within post-Golgi organelles.

Role of Vacuolar Acidification in CD Maturation

Maturation of proCD occurs in acidified post-Golgi compartments, namely endosomes and lysosomes [Hasilik, 1992]. We asked whether disruption of the pH gradient in these compart-

ments would differentially interfere with the rate of synthesis and accumulation of the various mature forms of CD in normal and *ras*-transformed cells. Since transport of rat proCD from the site of synthesis to endosomal/lysosomal compartments is largely completed within 2 h [Ludwig et al., 1991; Isidoro et al., 1995a] a prolonged alkalinization would result in the accumulation of immature forms of the enzyme that could be easily revealed by Western blotting. We compared the effects of 10 mM ammonium chloride treatment on the trafficking and processing of proCD in both REF and 4R cell lines. As revealed by Western blotting the pattern of CD molecules was unchanged in treated REF cells as compared to controls, except for a slight increase in the accumulation of the 30 + 14 kDa species (Fig. 2). In 4R cells ammonium chloride treatment resulted in a prominent accumulation of the two single-chain molecular forms of CD, while no change in the pattern of the double-chain forms was observed (Fig. 2). This experiment also (1) revealed that prolonged alkalinization of vacuolar compartments did not prevent maturation of proCD in both untransformed and transformed REF, and (2) confirmed the inability of *ras*-transformed fibroblasts to generate the mature 30 + 14 kDa species.

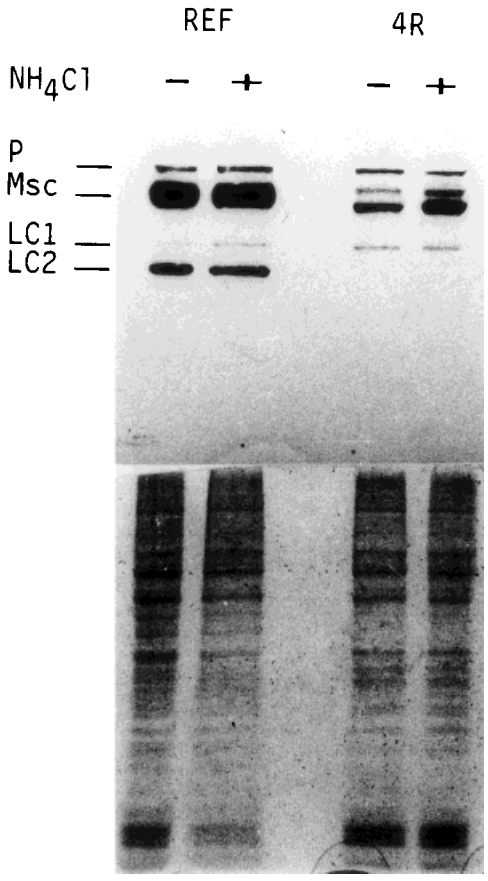

Fig. 2. Effects of ammonium chloride on the processing of CD in REF and 4R cells. Adherent cells were incubated 24 h in the absence or the presence of 10 mM ammonium chloride and homogenates analyzed by Western blotting. For CD symbols refer to Figure 1. A Coomassie-stained gel run in parallel is included (bottom) to show equal loading of cellular proteins.

Ras Expression Alters the Cellular Location of Cathepsin D

Further, we analyzed the subcellular localization of CD-containing organelles in REF and 4R cells. As compared to the parental untransformed cells, *ras*-transformed embryo fibroblasts appear smaller in size, show a reduced cytoplasm around the nucleus and also show long and thin cellular processes (Fig. 3). In REF immunofluorescent staining of CD revealed a perinuclear distribution of the positive organelles, which is consistent with the usual localization of the endosomal-lysosomal apparatus in normal cells (Fig. 3). By contrast, in 4R cells CD-positive organelles were mostly peripheral and accumulated close to the plasmamembrane along the cytoplasmic extensions (Fig. 3). Of interest, the number of CD-positive granules per cell was strongly reduced in 4R cells, as compared to controls (Fig. 3).

Expression and Trafficking of CD as Affected by Serum Growth Factors

In normal, but not in transformed, 3T3 mouse fibroblasts the expression of CD was shown to be regulated depending on their proliferative state, being upregulated in density-arrested cultures [Isidoro et al., 1995b]. In these cells, serum-deprivation caused an increase of the expression and secretion of CD [Isidoro et al., 1995b]. The relationship between cell growth and expression and trafficking of CD was examined in REF and 4R cultured in the absence of serum factors. Cells were plated at an initial density of 50,000 cells/cm^2 and allowed to adhere for 24 h, then the medium was changed daily to avoid side effects due to nutrient consumption. For each cell type two parallel cultures were set, one of which was fed with medium not supplemented with serum. In serum-fed exponentially growing cultures the doubling time was approximately 26 h for REF and 13 h for 4R cells. While the growth rate of 4R cells

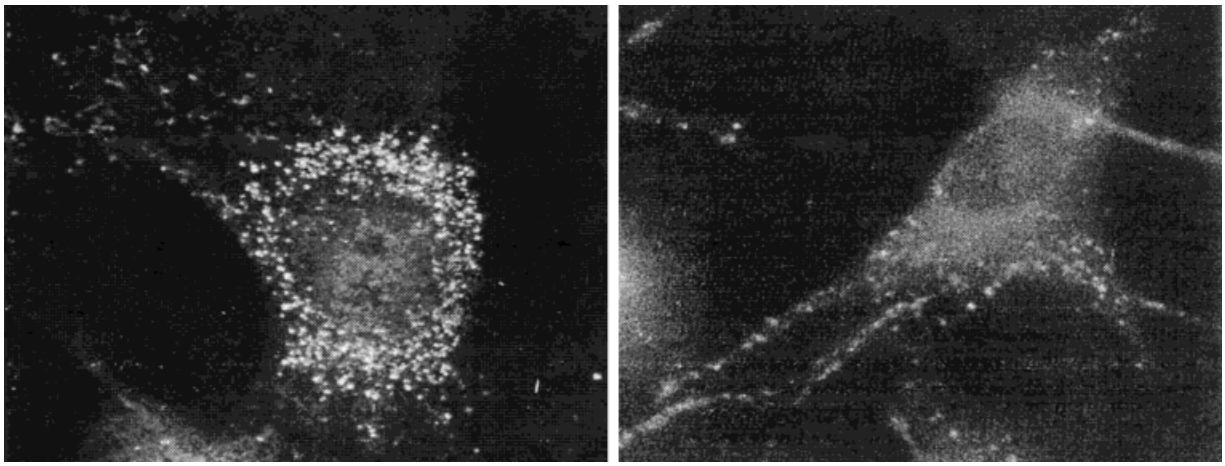

Fig. 3. Immunolocalization of CD in REF (left) and 4R (right) cells. Both images were taken at the same magnification (1,000×). With respect to untransfected cells, 4R appears much smaller in size. In the latter, CD-positive organelles are scattered throughout the cytoplasm and more close to the periphery of the cell.

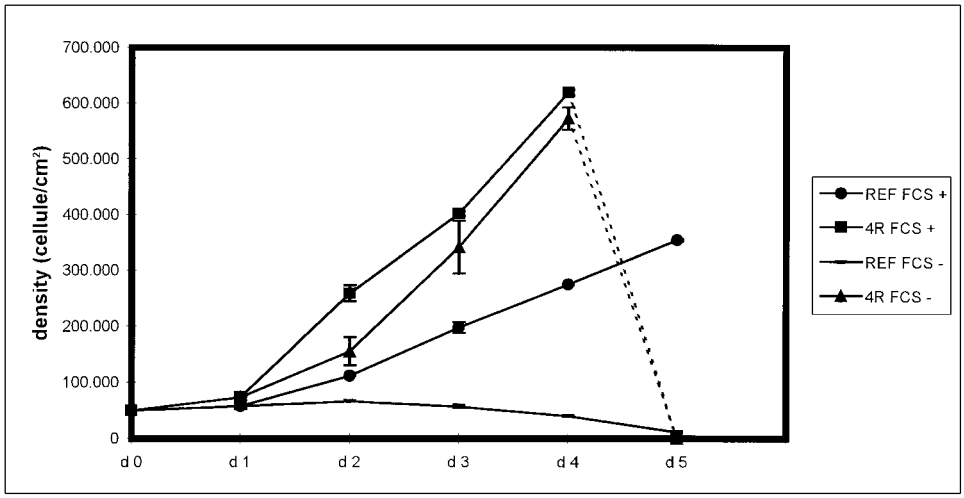

Fig. 4. Effect of serum on the growth of REF and 4R cells. Cells were seeded in Petri dishes at a density of 50,000 cells/cm^2, allowed to attach for 24 h and then cultured in daily-renewed medium containing or not 10% fetal calf serum. Cells were trypsinized and counted every 24 h. Data represent the mean ± SD of three separate experiments.

was not affected by serum deprivation, this treatment strongly impaired the growth of REF (Fig. 4). 4R cells, cultured either in the absence or the presence of serum reached a density of about 600,000 cells/cm^2, which was limit for the surviving of the cultures. By cytofluorimetry the percentage of actively dividing cells was similar in both serum-fed and serum-deprived 4R cultures (Table I). In contrast, after 48 h of serum starvation REF cultures started to die. Flow cytometric analysis confirmed that in this condition surviving rat embryo fibroblasts accumulated in the G0/G1 phase of the cell cycle (Table I). In the absence of serum the level of intracellular CD activity was not affected both in REF and 4R cells (Fig. 5A). However, serum deprivation induced an increase of the CD activity recovered in the medium, this effect being much more prominent in the case of REF cultures (Fig. 5A). This experiment also revealed that in both REF and 4R control cultures only a small fraction (less than 8% of total) of the enzyme was mistargeted extracellularly during the 24 h of observation (Fig. 5A). By Western blotting it was shown that only the proenzyme form was secreted (not shown). That serum differently influenced the trafficking of proCD in REF and 4R cells is also suggested by a

TABLE I. Effect of Serum on the Cell-Cycle Distribution of REF and 4R Cells[a]

Cell type	FCS	Cell cycle phase G_0/G_1	S	G_2/M
REF	+	42 ± 3	36 ± 2	22 ± 1
	−	78 ± 5	21 ± 4	1 ± 1
4R	+	38 ± 2	46 ± 3	16 ± 1
	−	33 ± 2	56 ± 3	11 ± 1

[a]Cells seeded at a density of 50,000/cm^2, allowed to adhere 48 h and then cultured the subsequent 24 h in renewed medium containing or not 10% fetal calf serum. At the end of the incubation the cells were subjected to cell cycle analysis as described in the Materials and Methods section. Data are given in percentage and represent the means ± S.D. of three separate determinations.

comparison of the molecular forms accumulated within the cells. In fact, in serum-deprived REF the presence of the 30 + 14 kDa species was reduced, while in serum-deprived 4R cells the 45 kDa and the 34 + 9 kDa species were more prominent (Fig. 5B). Again, the 30 + 14 kDa molecular form of lysosomal CD was not apparent in 4R cells cultured in the presence or the absence of serum (Fig. 5B).

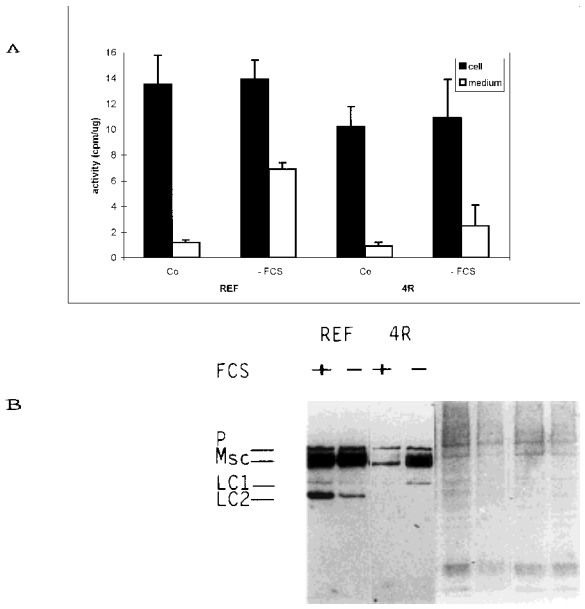

Fig. 5. Effects of serum on the expression and trafficking of CD in REF and 4R cells. Cells were seeded at a density of 50,000/cm^2, allowed to adhere 48 h and then cultured the subsequent 24 h in renewed medium containing or not 10% fetal calf serum. **A:** At the end of the incubation CD activity was assayed in media and cell homogenates using a radioactive substrate. Data are expressed as cpm of TCA-soluble radioactivity per µg cell protein and represent the means ± S.D. of three separate experiments. **B:** Western blotting analysis of cell homogenates. For CD symbols refer to Figure 1. A Coomassie-stained gel run in parallel is included (right side) to show equal loading of cellular proteins.

DISCUSSION

Ras-p21 is a small GTP binding protein that plays crucial roles in the signal transduction cascade coupled to cell proliferation and that is capable of causing cellular transformation when constitutively activated by point mutation. Altered expression of Ha-*ras* oncogene in transformed cells is associated with independence from serum growth factors for growing and the ability to invade the extracellular matrix and to form metastatic colonies when injected in nude mice [Muschell et al., 1985; Pozzati et al., 1986; Hill et al., 1988]. The mechanism through which the expression of oncogenic Ras-p21 leads to this malignant behavior is still obscure [Marshall, 1991]. At least some of the above features have been linked to altered expression and trafficking of proteolytic enzymes that often accompany *ras*-induced cellular transformation [Garbisa et al., 1987; Chambers et al., 1992; Sloane et al., 1994]. In the present work we asked whether oncogenic *ras* transformation would affect the expression and intracellular transport and maturation of cathepsin D, a lysosomal protease involved in many aspects of tumor development and progression [Rochefort, 1990; Isidoro et al., 1997b,c]. We have previously observed that the expression, maturation, and secretion of this protease are profoundly influenced by the state of proliferation or transformation or differentiation of the cell [Isidoro et al., 1995a,b; Isidoro et al., 1997a,b,c; De Stefanis et al., 1997]. Here we show that the expression of the oncogenic form of c-Ha-*ras* profoundly alters the maturation of proCD resulting in the inability to generate the 30 + 14 kDa double-chain form. This contrasts with the pattern of mature CD molecules found in the normal counterpart which resembles that of rat hepatocytes. Also, the proportion between the two single-chain molecular forms was altered in *ras*-transformed fibroblasts in that the 45 kDa species was much less represented. Defective maturation of proCD was already reported in rat hepatoma cells [Isidoro et al., 1995a] and in SV-40-virus- or Benzopyrene-transformed mouse fibroblasts [Isidoro et al., 1995b, 1997b]. Cathepsin D is synthesized in the rough endoplasmic reticulum as a glycosylated precursor enzymically inactive. Its conversion into the mature enzymically active form occurs upon its

arrival in the endosomal-lysosomal compartment [Hasilik, 1992]. Generation of the various mature molecular forms of CD results from limited proteolysis occurring in the organelle of destination and thus depends on the presence therein of particular pH conditions that allow for autoactivation or proteolysis by specific converting enzymes. Removal of N-terminal propiece leads to the active mature single-chain which may further be processed (likely at the C-terminus) to generate microheterogeneities [cf. Horst and Hasilik, 1991]. Thus, the two single-chain forms may originate in two different compartments or by a two-step limited proteolysis within the same organelle. Accordingly, the present data would imply that *ras* hyperexpression affects either the trafficking between post-Golgi organelles or the rate of maturation of proCD within the same organelle. For instance, altered maturation of proCD in 4R cells could be linked to an increase of the intravacuolar pH in endosomes and lysosomes, which has been shown to occur in fibroblasts hyperexpressing Ha-*ras* [Jang et al., 1990]. However, alkalinization of REF or 4R cells by ammonium chloride did not abrogate the generation of mature CD forms in REF, nor facilitated the synthesis/accumulation of the 30 + 14 kDa molecular species in 4R cells. On the other hand, ammonium chloride treatment resulted in the accumulation of the 34 kDa chain in REF and of both the 45 and 43 kDa single-chain form of CD in REF and 4R cells. This was likely due to an increased stability of the molecules, yet this effect was not apparent for the 34 kDa species in 4R cells. We hypothesize that the inability of *ras*-transformed embryo fibroblasts to generate the 30 + 14 kDa molecular form of CD arises from alterations in the trafficking of the protein between endosomal and lysosomal organelles. Relevant to this hypothesis is the recent finding that functional rab 7, a small GTPases belonging to the *ras*-superfamily, is essential for the transport of CD from early to late endosomes [Press et al., 1998].

Immunofluorescent studies revealed that CD positive organelles per cell were drastically reduced in 4R cells as compared to REF. At present, we have no explanation for such an effect of *ras* transformation. CD-containing organelles also locate differently in REF and 4R cells, being almost perinuclear in the former and peripheral, close to the plasmamembrane, in the latter. Also cathepsin B was found mislocated toward the periphery of the cell in *ras*-transfected breast epithelial cells [Sloane et al., 1994]. Such an atypical localization of cathepsins-containing organelles is reminescent of what is observed in activated macrophagic cells [Baron, 1989] and seems to prelude the secretion of mature enzymically active lysosomal hydrolases. Thus, the mislocation of endosomal- and lysosomal-like organelles from the perinuclear region to the cell periphery may be a common feature of cells engaged in local degradative and invasive processes. Indeed, the cytoplasmic extensions enriched of cathepsins-containing organelles could be regarded as 'invadopodia-like' structures. The mechanism through which the constitutive hyperactivity of Ras-p21 effects this re-routing is not clear, but likely it involves a cascade of small G proteins that includes rho-A and rac-1 components, which in turn regolate the cytoskeletal organization [Denhardt, 1996; Machesky and Hall, 1996].

Another interesting observation made in the present study is the differential response of REF and 4R cells to serum deprivation. In normal fibroblasts this condition is associated with growth arrest, reduced accumulation of the 30 + 14 kDa double-chain form of CD and marked increase of extracellular proCD. By contrast, *ras*-transformed fibroblasts showed insensitivity to serum deprivation with regards to cell growth and secretion of proCD. In addition, serum deprivation induced in 4R cells only the accumulation of the 45 kDa and of the 34 + 9 kDa molecular species of CD, though no apparent effects were observed on the generation of the 30 + 14 kDa form. The differential effect of serum growth factors on the trafficking and maturation of proCD in normal and *ras*-transformed rat embryo fibroblasts strongly suggest a link between extracellular signals, small G-proteins of the *ras* superfamily and trafficking of lysosomal proteases.

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