

Selective Degradation of Accumulated Secretory Proteins in the Endoplasmic Reticulum

A POSSIBLE CLEARANCE PATHWAY FOR ABNORMAL TROPOELASTIN*

(Received for publication, September 29, 1995, and in revised form, November 29, 1995)

Elaine C. Davis‡ and Robert P. Mecham§

From the Department of Cell Biology and Physiology, Washington University School of Medicine and §Respiratory and Critical Care Division, Department of Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, Missouri 63110

The specific pathway of tropoelastin secretion was investigated in fetal calf ligamentum nuchae (FCL) cells using brefeldin A (BFA) to disrupt the secretory pathway. Electron microscopic studies of BFA-treated FCL cells showed ultrastructural changes consistent with the reported effects of BFA on intracellular organelles. When FCL cells were labeled with [³H]leucine in the presence of BFA, radiolabeled tropoelastin was not secreted, nor was there an intracellular accumulation of the protein. In contrast, fibronectin accumulated within the cells in the presence of BFA. Northern analysis of mRNA levels in FCL cells showed that the message for tropoelastin was unaffected by BFA treatment. Pulse chase experiments conducted in the presence of BFA demonstrated that the tropoelastin retained within the cells was rapidly degraded. Ammonium chloride, nocodazole, and cycloheximide had no effect on the degradation of tropoelastin, indicating that the degradation did not involve the endosome/lysosome pathway, movement via microtubules, or a short-lived protein, respectively. Incubation of FCL cells with BFA in the presence of *N*-acetyl-Leu-Leu-norleucinal, however, allowed tropoelastin to steadily accumulate in the cells. Cells pulsed in the presence of BFA alone showed that tropoelastin initially accumulates within the cells for approximately 1 h prior to being degraded, thus indicating that a critical threshold of tropoelastin must be reached before degradation can occur. Results from this study provide evidence for selective degradation of a soluble secreted protein by a cysteine protease following retention of the protein in the endoplasmic reticulum.

The formation of elastic fibers in the extracellular matrix involves the secretion and subsequent alignment of tropoelastin monomers onto a microfibrillar scaffold where they then become cross-linked to form an insoluble elastin matrix. Ultrastructural studies have suggested that the secretion of tropoelastin occurs at specific sites on the cell surface (1). This targeted secretion is thought to be mediated by an elastin receptor or chaperone complex that not only directs the secre-

tion of tropoelastin but facilitates the assembly of the protein onto the developing elastic fibers at the cell surface (2–4). The definitive function of the elastin chaperone complex, however, has yet to be established. This is due, in large part, to the fact that very little is known concerning the intracellular events required for the trafficking and secretion of the tropoelastin monomer.

Cell-free translation studies using tropoelastin mRNA have shown that the 70-kDa tropoelastin monomer contains a signal sequence of 24–26 residues (5), which is cleaved as the completed polypeptide chain enters the ER¹ (6, 7). From the ER to the cell surface, tropoelastin remains unchanged with no glycosylation and little, if any, other post-translational modifications. Early attempts to explore the synthesis and secretion of tropoelastin, using morphological and cytochemical techniques, showed the presence of small vesicles in the vicinity of the Golgi and cell periphery that contained amorphous material with staining properties identical to that of elastin (8). Similar material was also identified within the ER cisternae and Golgi saccules. Later studies, using immunoelectron microscopy, confirmed the presence of tropoelastin in elastogenic cells (9, 10); however, in many cases, the specific identity of the immunolabeled compartments was unclear.

One approach to study the intracellular pathway of a secreted protein is to use drugs that affect protein trafficking at distinct sites along the secretory pathway. Since tropoelastin does not require any modifications within the Golgi, it was of interest to study the effect of brefeldin A (BFA) on the secretion of tropoelastin. Over the past several years, this fungal metabolite has been used extensively to block protein transport from the ER to the Golgi (11). Morphological studies have shown that in the presence of BFA, the Golgi apparatus disassembles and the ER becomes extensively dilated (12, 13). These morphological attributes reflect a retrograde fusion of the *cis*-, *medial*-, and *trans*-Golgi cisternae into the ER (12, 14–16). Since the effect of BFA on protein synthesis is minimal, secretory proteins tend to accumulate within the mixed ER/Golgi compartment (12, 17). Remarkably, the effects of BFA have been shown to be completely reversible upon removal of the drug, with reassembly of the Golgi apparatus and resumed secretion (18, 19).

In the present study, the secretion of tropoelastin was investigated in fetal calf ligamentum nuchae (FCL) cells. In fetal tissues, cells that are committed to elastogenesis can devote as much as 40% of their total protein synthesis to tropoelastin.

* This work was supported by National Institutes of Health Grants HL-41926 and HL-53325 (to R. P. M.) and by a grant from the National Marfan Foundation (to E. C. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ An American Lung Association Research Fellow. To whom correspondence should be addressed: Dept. of Cell Biology and Physiology, Washington University School of Medicine, Box 8228, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-2254; Fax: 314-362-2252; edavis@cellbio.wustl.edu.

¹ The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; FCL, fetal calf ligamentum nuchae; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; ALLN, *N*-acetyl-Leu-Leu-norleucinal; NH₄Cl, ammonium chloride.

Here we demonstrate that BFA treatment of FCL cells results in complete inhibition of tropoelastin secretion. It was expected that an intracellular accumulation of the protein would therefore occur; however, tropoelastin was found to be rapidly and selectively degraded in the ER. Although not well characterized, there is increasing evidence to support the existence of a pre-Golgi degradation pathway that is independent of lysosomes (20). Such a degradative system is required to dispose of proteins that are retained in the ER due to misfolding or failure to assemble into oligomeric complexes. In addition to abnormal proteins, several normal proteins also undergo ER degradation in a regulated manner, such as the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (21, 22) and apolipoprotein B (23). The results from this study provide the first evidence of a BFA-induced degradation event mediated by a cysteine protease and provide supportive evidence for a selective process of "quality control" in the ER. The implications of these results, with respect to the intracellular events required for tropoelastin secretion and the general process of ER degradation, are discussed.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Bovine ligamentum nuchae were obtained from late gestation fetuses at a local slaughterhouse. FCL fibroblasts were obtained from tissue explants and cultured as described previously (24). All experiments described in this report were with first passage cells from 230–270-day fetal nuchal ligaments. For all experiments, cells were grown to confluency in Dulbecco's modified Eagle's medium supplemented with L-glutamine, nonessential amino acids, antibiotics, and 10% fortified bovine calf serum (Hyclone Laboratories, Inc., Logan, UT) prior to metabolic labeling.

For metabolic labeling, L-[4,5-³H]leucine (1 mCi/ml) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA), and dialyzed fetal bovine serum was purchased from Hyclone Laboratories, Inc. Protease inhibitors, ϵ -amino-*n*-caproic acid, phenylmethylsulfonyl fluoride, and *N*-ethylmaleimide were purchased from Sigma and used in the lysis buffer at final concentrations of 10, 5, and 5 mM, respectively. For immunoprecipitation experiments, a monoclonal tropoelastin antibody BA-4, raised to bovine α -elastin (25), and a polyclonal fibronectin antibody (Chemicon, Temecula, CA) were used. Immune complexes were precipitated using heat-killed *Staphylococcus aureus* (Pansorbin cells, Calbiochem) or protein A immobilized on Trisacryl (Pierce).

Reagents used during metabolic labeling included ammonium chloride (NH₄Cl), *N*-acetyl-Leu-Leu-norleucinal (ALLN), BFA, cycloheximide, leupeptin, nocodazole, and pepstatin A (Sigma). NH₄Cl was prepared fresh as a 2 M stock in distilled water and used at a final concentration of 20 mM. ALLN was stored at –20 °C as a 10 mg/ml stock in ethanol and used at final concentration of 10 μ M. BFA and nocodazole were stored at –20 °C as 10 mg/ml stocks in Me₂SO and used at final concentrations of 10 and 20 μ M, respectively. Cycloheximide and leupeptin were prepared fresh in distilled water as a 0.1 M stock, used at a final concentration of 0.5 mM, and as a 10 mM stock, used at a final concentration of 200 μ M, respectively. Pepstatin A was prepared fresh as a 1 mg/ml stock in methanol and used at a final concentration of 10 μ M.

Metabolic Labeling and Immunoprecipitation—Confluent monolayers of FCL cells, grown in six-well tissue culture plates (Falcon number 3046, Baxter, McGaw Park, IL), were washed with leucine-free medium containing 5% dialyzed fetal bovine serum and incubated in this medium for 1 h. Any pretreatment of cells with ALLN or nocodazole is as indicated in the text and figure legends. Each well of cells was metabolically labeled with 50 μ Ci of [³H]leucine in 1 ml of leucine-free medium containing 5% dialyzed fetal bovine serum for various lengths of time as indicated. For pulse chase experiments, cells were rinsed twice with complete medium and chased in 1 ml of complete medium containing 5% dialyzed fetal bovine serum. Following metabolic labeling, medium was collected, and cell layers were washed twice with cold phosphate-buffered saline. To each well, 1 ml of cold lysis buffer (25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 250 mM NaCl, 0.1% Triton X-100) with protease inhibitors was added. The culture dishes were placed on a platform shaker at 4 °C, and cell lysates together with the detached cells were collected after 30 min. Cellular debris was pelleted by centrifugation, and the cell lysates were transferred to clean microfuge tubes.

To determine total protein synthesis and secretion, trichloroacetic acid-precipitable radioactivity was determined from lysates and medium of cells metabolically labeled as indicated in the text. For each sample, 10 μ l of lysate or medium was mixed with an equal volume of 10 mg/ml bovine serum albumin, and 15 μ l of this solution was spotted on dry glass microfiber filters (Whatman, Hillsboro, OR). After 30 min at 4 °C in dishes flooded with cold 10% trichloroacetic acid, the filters were washed 3 \times 5 min with room temperature 10% trichloroacetic acid and 1 \times 5 min with 95% ethanol and left to dry prior to counting.

For immunoprecipitation, medium and lysates were precleared by incubation with 10 μ g/ml normal mouse IgG for 2 h and an additional 1 h with 25 μ l of *Staphylococcus aureus* added to each tube. The *Staphylococcus aureus* was pelleted by centrifugation, and the supernatants were transferred to clean tubes containing 100 μ l of 50 mg/ml bovine serum albumin in lysis buffer and 10 μ g of BA-4 antibody. Medium and lysates were incubated overnight at 4 °C with gentle agitation. The following day, 40 μ l of *Staphylococcus aureus* was added to each tube and incubated for 1 h at 4 °C with gentle agitation. The immune complexes were pelleted, and the pellets were washed two times with lysis buffer and one time with nondetergent buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5)). After the final wash, each pellet was resuspended in 35 μ l of Laemmli sample buffer containing dithiothreitol and incubated at 100 °C for 6 min. The samples were electrophoresed on SDS-polyacrylamide gels, fixed for 20 min, and treated with EN³HANCE (DuPont NEN) for 1 h. Gels were then dried and exposed to XAR-5 x-ray film (Eastman Kodak Co.).

All immunoprecipitation experiments were conducted a minimum of three times to ensure reproducibility of results. Furthermore, in each experiment, one well of cells was always a control, with no treatment, in order to access the quality of labeling for that particular experiment and provide a direct comparison for the treated cells.

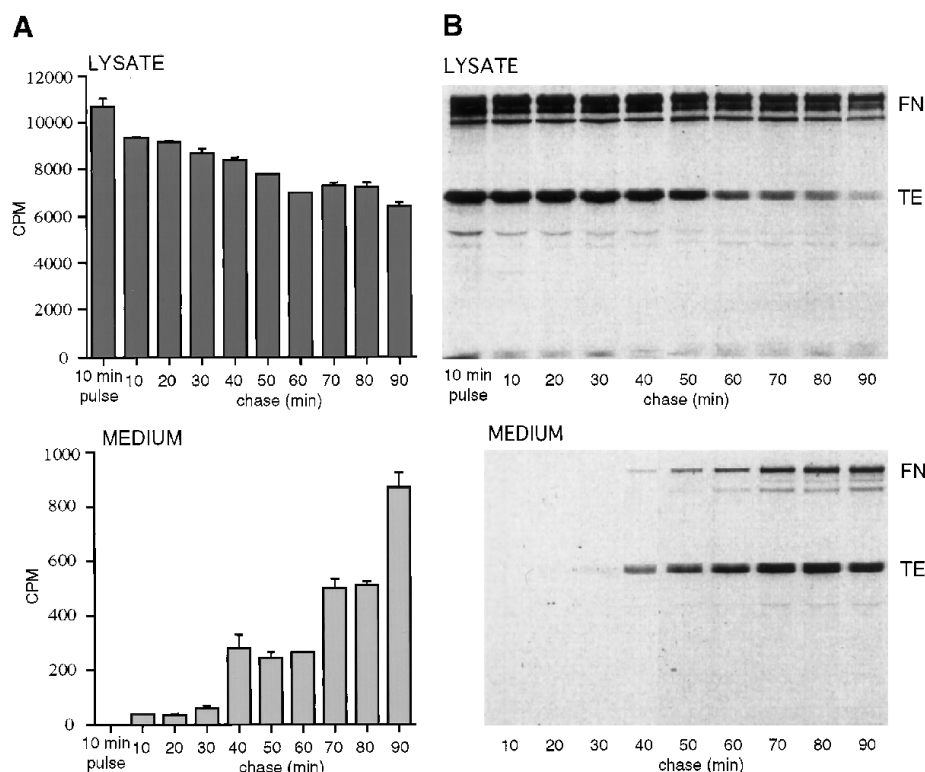
Electron Microscopy—Confluent cultures of FCL cells, plated in four-well Lab-Tek tissue culture chamber slides (Thomas Scientific, Swedesboro, NJ), were incubated with 10 μ g/ml BFA or 1 μ M Me₂SO in complete medium for 4 h. Cell layers were then washed in phosphate-buffered saline and fixed *in situ* with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min. After being washed several times with cacodylate buffer, the cell layers were treated sequentially with 1% osmium tetroxide in buffer, 2% tannic acid in buffer, and 2% uranyl acetate in distilled water. Cell layers were then dehydrated in a graded series of methanol to propylene oxide, infiltrated and embedded with Epon (SPI Supplies, West Chester, PA). *En face* thin tissue sections were cut on a Reichert ultracut ultramicrotome and counterstained with methanolic uranyl acetate (26) followed by lead citrate (27). Sections were examined in a Zeiss 902 transmission electron microscope at an accelerating voltage of 80 kV.

RNA Isolation and Northern Analysis—Confluent cultures of FCL cells in 100-mm dishes were incubated for 15 min, 1 h, 2 h, or 4 h with 10 μ g/ml BFA in complete medium. Control cultures were incubated for 1 h with an equal volume of Me₂SO alone (1 μ l/ml). Following incubation, total RNA was isolated from the cell layers by guanidinium isothiocyanate/phenol extraction (28). RNA concentration and purity was determined by spectrophotometric analysis. Total RNA (5 μ g) was separated by electrophoresis through a 1% agarose gel containing 1 M formaldehyde, transferred to Hybond-N⁺ nylon membrane (Amersham Corp.), and hybridized and washed under stringent conditions as described previously (29). Probes for this study were labeled with [α -³²P]CTP (ICN, Irvine, CA) by random primed labeling. The tropoelastin probe was prepared from clone 12.1, a 2.2-kilobase bovine tropoelastin cDNA (30). The membrane was hybridized concurrently with the tropoelastin probe and a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase. Hybridized complexes were detected by exposure of the membrane to X-OMAT AR film (Kodak).

RESULTS

Time Course of Tropoelastin Secretion—To characterize the normal secretion rate of tropoelastin in FCL cells, cells were pulsed for 10 min and the fate of that pulse was followed over the course of a 90-min chase. Counts of total trichloroacetic acid-precipitated proteins from the cell lysate and medium indicated that approximately 40 min passed before proteins began to be secreted (Fig. 1A). Consistent with this time course, a very faint band of immunoprecipitated tropoelastin appeared in the medium after 30 min of chase with a steady increase in band density thereafter (Fig. 1B). Since fibronectin binds directly to *Staphylococcus aureus* independent of any primary

FIG. 1. Time course of secretion of (A) total trichloroacetic acid-precipitable proteins and (B) immunoprecipitated tropoelastin (TE) in FCL cell lysates and medium following a 10-min pulse with [3 H]leucine and a 90-min chase. Trichloroacetic acid-precipitable radioactivity was from 7.5 μ l of cell lysate or medium. Values are shown in cpm \pm S.E. of triplicate samples. In panel B, radiolabeled fibronectin (FN) is seen at the top of the gel due to direct binding of fibronectin to the *Staphylococcus aureus* used to immunoprecipitate tropoelastin (see Fig. 2).



antibody (31), the use of *Staphylococcus aureus* to immunoprecipitate tropoelastin resulted in the presence of radiolabeled fibronectin on the autoradiographs as well as tropoelastin. The time course of fibronectin secretion was similar to that observed for tropoelastin. To verify that the bands at the top of the autoradiograph were indeed fibronectin, the cell lysate and medium from cells labeled for 4 h with [3 H]leucine were divided into three equal aliquots and immunoprecipitated for fibronectin or tropoelastin using their respective primary antibodies followed by protein A to precipitate the immune complex or by using the tropoelastin antibody followed by *Staphylococcus aureus*. Fig. 2 shows that the mobility and relative intensity of the radiolabeled bands on autoradiographs from the samples immunoprecipitated with the specific antibodies and recovered with protein A were identical to those observed using the tropoelastin antibody and *Staphylococcus aureus*.

BFA Prevents Tropoelastin Secretion, but No Intracellular Accumulation Occurs—To investigate the intracellular pathway of tropoelastin secretion, FCL cells were metabolically labeled for 4 h with [3 H]leucine in the presence of 10 μ g/ml BFA, and the cell lysates and medium were immunoprecipitated for tropoelastin (Fig. 3). In the absence of BFA, radiolabeled tropoelastin was observed in both the cell lysate and medium. As expected with this length of pulse, the proportion of tropoelastin in the medium was greater than that present in the cell. Treatment of the cells with BFA during the pulse resulted in a complete inhibition of tropoelastin secretion. Although the tropoelastin was not secreted, no accumulation of tropoelastin was observed within the cells. In contrast, radiolabeled fibronectin accumulated in the cells following the inhibition of its secretion by BFA treatment.

Characterization of the Effect of BFA on FCL Cell Ultrastructure and Total Protein Synthesis—Since the BFA treatment did not result in an intracellular accumulation of tropoelastin, it was important to verify that the BFA treatment of FCL cells induced morphological changes that were characteristic of the reported effects of BFA on cell ultrastructure. FCL cells treated for 4 h with 1 μ l/ml Me₂SO alone showed no aberrant morphol-

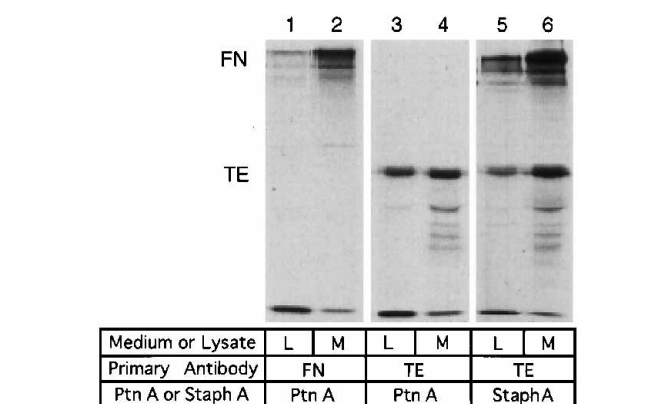


FIG. 2. Fibronectin is immunoprecipitated with *Staphylococcus aureus* independent of fibronectin primary antibody. FCL cells were radiolabeled for 2 h with [3 H]leucine, and the cell lysate and medium were divided into three equal aliquots. From one aliquot, fibronectin was immunoprecipitated with a specific primary antibody followed by protein A (lanes 1 and 2). Tropoelastin was immunoprecipitated from the other two aliquots with a tropoelastin antibody followed by either protein A (lanes 3 and 4) or *Staphylococcus aureus* (Staph A) (lanes 5 and 6).

ogy. At one pole of the nucleus, numerous stacks of Golgi cisternae were observed surrounding the microtubule-organizing center (Fig. 4A). In contrast, cells treated with 10 μ g/ml BFA in Me₂SO for 4 h showed no recognizable Golgi structures (Fig. 4B). Instead, the Golgi region of these cells was occupied by an extensive array of tubulovesicular elements situated in the vicinity of the microtubule-organizing center. In addition to the Golgi apparatus, the cisternae of ER in FCL cells treated with BFA were also affected. In normal FCL cells, the ER is situated in the periphery of the cell and consists of small, slightly dilated cisternae (Fig. 5A). In BFA-treated cells, the ER is still peripherally located; however, the cisternae are grossly expanded and highly irregular in shape (Fig. 5B). This morphology is consistent with the reported accumulation of secreted proteins and retrograde fusion of Golgi elements into

the ER that occur in BFA-treated cells (13, 15, 19).

In addition to FCL cell ultrastructure, the general condition of the cells with respect to protein synthesis and secretion was investigated by measuring total trichloroacetic acid-precipitable counts from lysates and medium of cells treated with 1 μ l/ml Me_2SO alone (control) or 10 μ g/ml BFA over a 4-h time course. As shown in Fig. 6, the synthesis of total Triton X-100-extractable proteins, radiolabeled with [^3H]leucine, was found to continue in both control and BFA-treated cells during the 4-h pulse. BFA treatment does, however, lead to a 19% reduction over control cells in the total trichloroacetic acid-precipitable counts after 4 h of labeling. The treatment of FCL cells with 10 μ g/ml BFA was sufficient to block total protein secretion for at least 4 h, while in control cells secretion of most radiolabeled proteins occurred after approximately 1 h of pulse and steadily continued for the 4-h time course.

Tropoelastin mRNA Levels Are Unaffected by BFA—It has previously been reported that a time- and dose-dependent decrease in tropoelastin gene expression occurs in smooth muscle cells treated with monensin due to the repression of tropoelas-

tin secretion (32). In the present study, even though tropoelastin secretion was inhibited, the amount of radiolabeled tropoelastin in the lysate of BFA-treated FCL cells was actually less than that normally present in the secretory pathway of untreated cells after 4 h. This raised the possibility that a rapid down-regulation of tropoelastin gene expression might result as a consequence of an accumulation of tropoelastin in the ER during the initial stages of BFA treatment. To investigate this possibility, the effect of BFA treatment on mRNA levels of tropoelastin was investigated by Northern analysis. Fig. 7 shows that treatment of FCL cells for up to 4 h with 10 μ g/ml BFA has no effect on mRNA levels for tropoelastin.

Selective Degradation of Tropoelastin Is Induced by BFA Treatment—Since the synthesis of tropoelastin appears to continue during BFA treatment, the lack of any accumulation of the protein in the cell was hypothesized to be a result of intracellular degradation. To study this possibility, FCL cells were pulsed for 1 h in the presence of BFA and then chased in complete medium still containing BFA in order to follow the fate of the radiolabeled tropoelastin trapped within the cell. After 1.5 h of chase, the amount of radiolabeled tropoelastin immunoprecipitated from the cells was found to be less than half of the original amount, and by 3 h of chase, little or no radiolabeled tropoelastin could be detected in the cell lysate (Fig. 8). In contrast, the amount of fibronectin that was initially radiolabeled during the 1 h pulse remained unchanged during the 3-h chase. These results indicate that the tropoelastin trapped in the fused ER/Golgi compartment is rapidly and selectively degraded.

Degradation of Tropoelastin Is Inhibited by ALLN but Unaffected by Leupeptin, Ammonium Chloride, Pepstatin A, Cycloheximide, and Nocodazole—To characterize the nature of the protease responsible for the degradation of tropoelastin, several protease inhibitors were tested for their ability to inhibit the BFA-induced degradation. In a degradation assay similar to that shown in Fig. 8, the cysteine protease inhibitor, ALLN, was found to completely inhibit the degradation of tropoelastin when included in the chase together with BFA following the 1-h pulse (Fig. 9A). Since it has been demonstrated that ALLN inhibits the activity of the lysosomal proteases, cathepsin L and cathepsin B (33), the ability of lysosomotropic agents to inhibit degradation was also tested. NH_4Cl , which raises intracellular pH and thus inhibits acid proteases in endosome/lysosome degradative pathway, did not inhibit the rapid degradation of

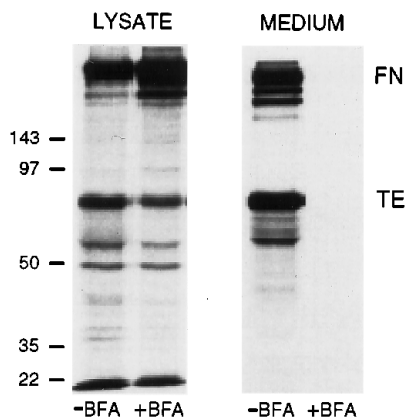


FIG. 3. BFA treatment of FCL cells results in blocked secretion of tropoelastin with no concurrent intracellular accumulation. FCL cells were pulsed with [^3H]leucine for 4 h in the absence or presence of 10 μ g/ml BFA. Tropoelastin (TE) was immunoprecipitated from the cell lysates and medium using *Staphylococcus aureus* to collect the immune complexes. Samples were run on an 8.75% SDS-polyacrylamide gel, fixed, treated with EN 3 HANCE, and exposed to x-ray film. Fibronectin (FN) binds directly to *Staphylococcus aureus* as shown in Fig. 2. Molecular mass markers (kDa) are as indicated on the left.

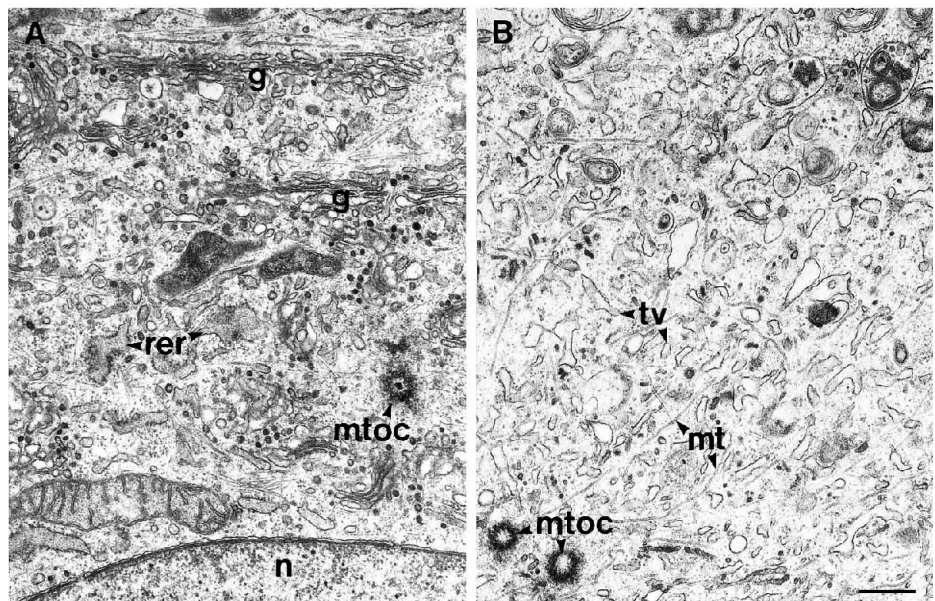


FIG. 4. Electron micrographs of cultured FCL cells showing the Golgi region of control cells treated with 1 μ l/ml Me_2SO alone (A) and cells treated for 4 h with 10 μ g/ml BFA (B). A, in control cells, numerous stacks of Golgi (g) are observed at one pole of the nucleus (n) centered around the microtubule-organizing center (mtoc). This region also contains a multitude of small vesicles and a few small cisternae of rough endoplasmic reticulum (rer). B, no visible stacks of Golgi are evident in BFA-treated cells. Instead, a multitude of tubulovesicular structures (tv) are observed surrounding the microtubule-organizing center. mt, microtubule. Bar, 0.5 μ m.

FIG. 5. Electron micrographs of cultured FCL cells showing the peripheral region of control cells treated with 1 μ l/ml Me₂SO alone (A) and cells treated for 4 h with 10 μ g/ml BFA (B). A, the cisternae of endoplasmic reticulum (*rer*) in control cells show normal ER morphology and are interspersed with cables of actin filaments (*a*). B, in contrast, the cisternae of rough endoplasmic reticulum (*rer*) in cells treated with BFA appear swollen, diffuse, and irregular in shape. Bar, 1.0 μ m.

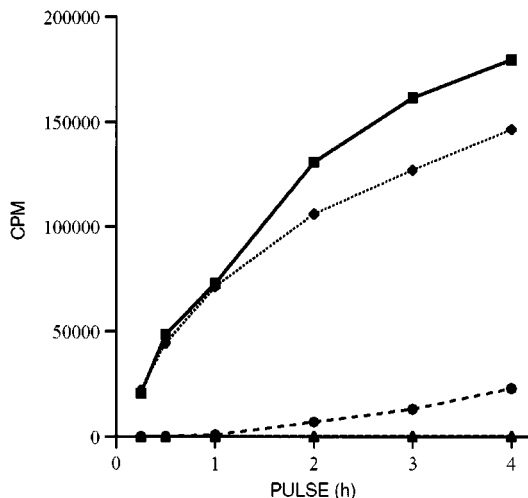
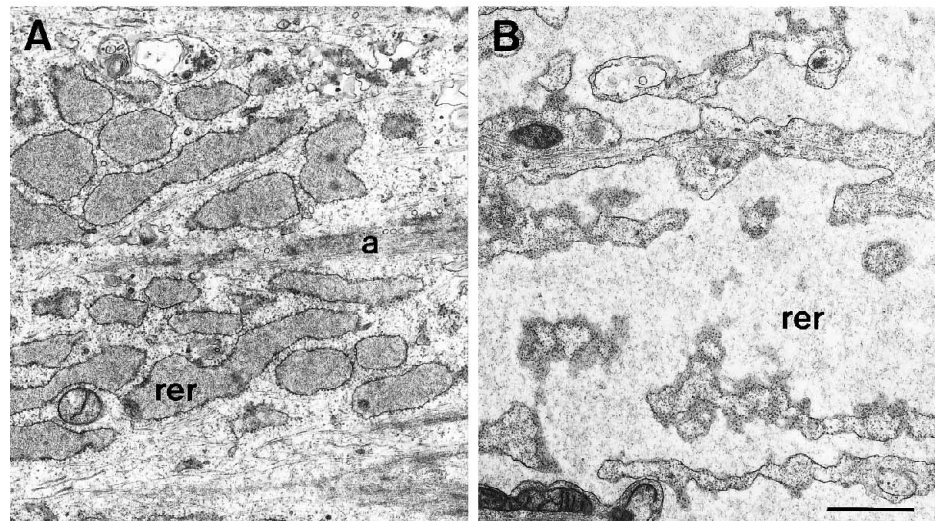


FIG. 6. Effect of BFA treatment on total protein synthesis and secretion. FCL cells were metabolically labeled in complete medium with [³H]leucine for up to 4 h in the presence of 1 μ l/ml Me₂SO alone (control) or 10 μ g/ml BFA. Cell lysates and medium were collected and trichloroacetic acid-precipitable radioactivity was determined from 7.5 μ l of each sample. ■, control, lysate; ●, control, medium; ◆, BFA, lysate; ▲, BFA, medium.

tropoelastin in BFA-treated cells (Fig. 9A). Similarly, leupeptin, a reversible inhibitor of trypsin-like serine proteases and most cysteine proteases, and pepstatin A, an aspartic protease inhibitor, had no effect on tropoelastin degradation (not shown).

Studies on the ER degradation of HMG-CoA reductase and T-cell receptor α subunit have shown that the degradation of both proteins is inhibited by ALLN, but only HMG-CoA reductase degradation is prevented by cycloheximide (34). Thus, the effect of inhibiting protein synthesis by cycloheximide on tropoelastin stability was tested. Fig. 9A shows that the presence of cycloheximide in the chase following the 1-h pulse had no effect on the rate of tropoelastin degradation. This result suggests that *de novo* protein synthesis is not needed for the tropoelastin to be degraded, and thus a short-lived protein is not required for the degradation event.

Although it is well documented that BFA treatment results in the retention of secreted proteins in the fused ER/Golgi compartment, the possibility that tropoelastin is transported to some degradation compartment following BFA treatment could not be ruled out. However, nocodazole had no effect on tro-

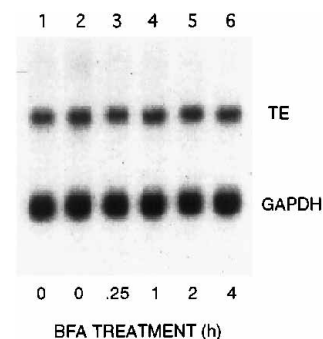


FIG. 7. Effect of BFA treatment on mRNA levels of tropoelastin in FCL cells. FCL cells were incubated in the presence of 10 μ g/ml BFA for 15 min, 1 h, 2 h, and 4 h in complete medium (lanes 3–6). As a control, one plate of cells was incubated in leucine-free medium alone for 1.25 h (lane 1), and a second plate was incubated with 1 μ l/ml Me₂SO only for 1 h (lane 2). Ten μ g of total RNA from each experimental group was fractionated on a 1% agarose gel, transferred to nylon membrane, and probed for tropoelastin (*TE*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

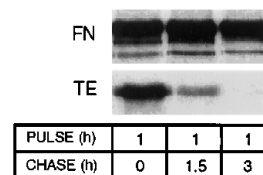


FIG. 8. FCL cells pulsed and chased in the presence of BFA result in rapid degradation of the tropoelastin trapped within the cells, while fibronectin remains unchanged. FCL cells were pulsed for 1 h with [³H]leucine in medium containing 10 μ g/ml BFA. Cell lysates and media were then either collected immediately or collected following a chase for a further 1.5 or 3 h in complete medium containing 10 μ g/ml BFA. This protocol allows the fate of the radiolabel tropoelastin to be studied in the presence of BFA. Tropoelastin (*TE*) was immunoprecipitated from the cell lysates as described in the legend to Fig. 3. Fibronectin (*FN*) binds directly to *Staphylococcus aureus* as seen in Fig. 2.

poelastin degradation when the drug was added into the chase medium (Fig. 9A). This observation suggests that movement of tropoelastin via microtubules is not a prerequisite for the degradation to occur. The inclusion of nocodazole in the chase, however, does not preclude a degradative compartment from fusing with the ER during BFA treatment. Thus, FCL cells were pretreated with nocodazole before being metabolically labeled and chased, with both BFA and nocodazole being present in the pulse and chase. Since the retrograde transport of

Golgi elements into the ER is a microtubule-dependent event (35), the preincubation of the cells with nocodazole prior to being pulsed in the presence of BFA would block, or at least severely reduce, the redistribution of Golgi and other vesicular compartments into the ER. As shown in Fig. 9B, the addition of nocodazole prior to BFA treatment did not increase the stability of tropoelastin during the chase. In contrast, the degradation of tropoelastin in these cells appeared to occur even more rapidly. These results confirm that the degradation of tropoelastin takes place in the ER as a result of its retention in that compartment.

Tropoelastin Accumulation in the ER Reaches a Threshold Level before Degradation Occurs—A consistent observation made during the course of these experiments was that the amount of tropoelastin immunoprecipitated from cell lysates of FCL cells pulsed for 1 h in the presence of BFA was always greater than that obtained from cells pulsed for 4 h under similar conditions. To investigate the kinetics of the BFA-induced degradation of tropoelastin, the effect of BFA on intracellular accumulation and secretion of tropoelastin was studied by metabolically labeling cells for increasing lengths of time in the presence of BFA alone or together with the inhibitor of degradation, ALLN. In control cells, with no drug added during

the pulse, or in cells with ALLN only, the amount of tropoelastin immunoprecipitated from the cell lysates appeared to steadily increase until approximately 1 h (Fig. 10). At this time point, tropoelastin could also be immunoprecipitated from the medium. As expected, BFA alone, or together with ALLN, completely inhibited tropoelastin secretion. In the cell lysate of BFA-treated cells, radiolabeled tropoelastin was observed to accumulate within the cells for up to 1 h (Fig. 10). However, with increasing pulse times, the amount of radiolabeled tropoelastin in the cell lysate steadily decreased to a level lower than that observed for the 15-min pulse. Although this appears to suggest that the synthesis of tropoelastin is affected by the longer incubation times in BFA, the presence of ALLN in the labeling medium together with BFA allowed a steady accumulation of intracellular radiolabeled tropoelastin to occur (Fig. 10). This observation provides conclusive evidence that tropoelastin continues to be synthesized in the presence of BFA throughout the 4-h time period.

DISCUSSION

Over the past several years, a number of studies have provided good evidence for the existence of a proteolytic system contained within the ER that functions as a "quality control" mechanism. Although not well characterized, this system is thought to be responsible for the degradation of unassembled or misfolded proteins as well as excess subunits of proteins that undergo oligomerization in the ER, such as the T-cell antigen receptor (36). In addition to unassembled and misfolded proteins, normal proteins are also degraded in the ER, indicating that this system may play a role in regulated proteolytic degradation. Examples of such proteins include HMG-CoA reductase (21) and apolipoprotein B-100 (23, 37, 38).

In many of the studies of proteins that undergo intracellular degradation, BFA has been used to characterize the nature of the degradative compartment. It has been shown, for example, that the regulated degradation of HMG-CoA reductase and apolipoprotein B-100 is unaffected by BFA treatment, indicating that the proteolytic event must take place in a pre-Golgi compartment. In contrast, the degradation of secretory immunoglobulin M in B lymphocytes has been reported to occur in a post-ER compartment, since the degradation of this protein is strongly inhibited by BFA treatment (39). In the present study, treatment of FCL cells with BFA induced in the degradation of tropoelastin as a consequence of the protein being retained in the fused ER/Golgi compartment. Retention alone, however, is not sufficient to cause the degradation of proteins, since fibronectin, another protein normally secreted by these cells, readily accumulated in the ER of BFA-treated FCL cells without any apparent degradation.

The selective degradation of tropoelastin raises important questions concerning the specificity and regulation of the ER proteolytic system. Of particular interest from the present study was the observation that tropoelastin initially accumulated in the fused ER/Golgi compartment prior to its rapid

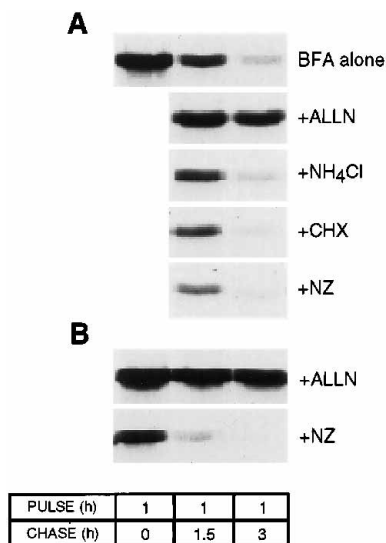
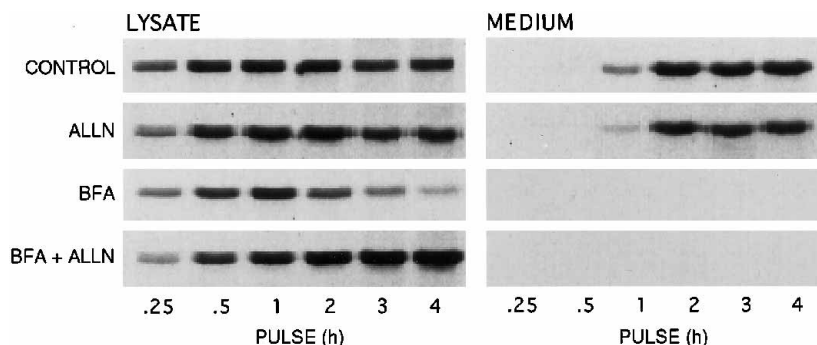


FIG. 9. The cysteine protease inhibitor, ALLN, inhibits the BFA-induced degradation of tropoelastin. A, FCL cells were pulsed for 1 h with [3 H]leucine in complete medium containing 10 μ g/ml BFA. Following the pulse, the cell lysates were collected immediately or after a 1.5 or 3 h chase in complete medium containing BFA alone or together with either ALLN, NH_4Cl , cycloheximide (CHX) or nocodazole (NZ). B, FCL cells were pretreated for 2 h with either ALLN or NZ and then pulsed for 1 h with [3 H]leucine in the presence of BFA + ALLN or BFA + NZ. Following the pulse, the cell lysates were collected immediately or after a 1.5- or 3-h chase in complete medium containing BFA + ALLN or BFA + NZ. Cell lysates were immunoprecipitated for tropoelastin as described in the legend to Fig. 3.

FIG. 10. In the presence of BFA, tropoelastin accumulates in the ER to a threshold level before degradation occurs. FCL cells were labeled with [3 H]leucine in the absence of BFA, with ALLN or BFA alone, or with BFA and ALLN together. At time points up to 4 h of pulse, cell lysates and medium were collected and immunoprecipitated for tropoelastin as described in the legend to Fig. 3.



degradation. This observation appears to be a relatively consistent feature of ER degradation, in that a lag period with little degradation often precedes the rapid phase of degradation (20, 40). Although it is unclear as to why a lag period exists, it has been suggested that the site of degradation may be in a specific subcompartment of the ER, and thus a certain degree of time is required to allow for sorting and delivery to this area (20). Another reason for the lag period may be that, within the ER, tropoelastin normally associates with a chaperone protein that protects the protein from degradation. Thus, with BFA treatment, the continued synthesis of tropoelastin into the fused ER/Golgi compartment exhausts the available chaperone and leads to either misfolded or simply unfolded protein that is susceptible to proteolytic degradation. This hypothesis is supported by the fact that half-lives of chaperone proteins are often quite long, for example greater than 30 h for calnexin (41), while the secretion rate of tropoelastin is on the order of 40 min. During a 4-h pulse in the presence of BFA, therefore, the production of a chaperone protein could be relatively minimal as compared with the amount of tropoelastin synthesized and retained in the ER. Furthermore, studies of procollagen I and its association with a collagen-binding glycoprotein localized to the ER, termed colligin or hsp47 (42, 43), have set a precedence for such a protective function of an ER chaperone. It has been demonstrated that procollagen I can bind to colligin and that this interaction can protect the procollagen I from degradation by a serine protease that is present in microsomal preparations (44). Since the expression of colligin has only been detected in collagen-secreting cells (45), the possibility exists that a specific tropoelastin-binding chaperone is present in elastogenic cells.

One interesting property of tropoelastin is its ability to undergo a phase transition and form a coacervate under physiological conditions (46). Because this process is concentration-dependent, it is also possible that retention of tropoelastin in the fused ER/Golgi compartment during BFA treatment leads to coacervation of the protein and thus its subsequent recognition and targeting for degradation as a misfolded protein or aggregate. Support for a concentration-dependent requirement for tropoelastin degradation is provided by the observation that, in the presence of BFA, tropoelastin degradation was more rapid in FCL cells when the cells were pretreated with nocodazole. Since this pretreatment restricts the retrograde fusion of the Golgi saccules with the ER, the concentration of tropoelastin in the ER would increase faster due to the smaller size of the compartment. The tendency of tropoelastin to coacervate provides further evidence that the transport of tropoelastin through the cell may be mediated by a chaperone protein, since coacervation is clearly an undesirable feature of a protein that must traverse the secretory pathway.

The accumulation and lag period observed prior to the BFA-induced degradation of tropoelastin may also represent the time required for the protein to reach a threshold level critical for activation of the enzyme responsible for its degradation. Since the nature of the proteolytic enzymes contained within the ER remains largely unknown, the control of the active states of these enzymes has yet to be determined. In the present study, the degradation of tropoelastin was found to be independent of lysosomal function and strongly inhibited by the cysteine protease inhibitor, ALLN. Similarly, a cysteine protease that is inhibited by ALLN has been implicated in the degradation of HMG-CoA reductase (47), apolipoprotein B-100 (48) and T-cell receptor chains (49). The degradation of these proteins in the ER, however, appears to be considerably more complex than the involvement of a single cysteine protease. For example, while HMG-CoA reductase degradation is sensitive to

depletion of intracellular Ca^{2+} , the degradation of T-cell receptor α is unaffected by Ca^{2+} homeostasis (34). On the contrary, T-cell receptor α degradation is significantly inhibited by *N*-tosyl-L-phenylalanine chloromethyl ketone, whereas *N*-tosyl-L-phenylalanine chloromethyl ketone has no effect on the degradation of HMG-CoA reductase. Furthermore, increasing evidence suggests that resident ER chaperone proteins, such as immunoglobulin heavy chain binding protein (BiP/GRP78) and protein disulfide isomerase, associate with abnormal or excess proteins prior to their degradation in a pre-Golgi compartment (50–52). Overall, these results suggest that the proteolytic system of the ER is extremely intricate and likely involves multiple enzyme systems with cooperation from several different resident ER chaperones.

Recently, ER-60 protease, an ER-resident protein with cysteine protease activity (53, 54), has been characterized and related to ER degradation *in vivo* for the first time by its association with misfolded human lysozyme prior to its degradation (52). In addition to ER-60, a second protease, termed ER-72 protease, has also been identified in the ER and is inhibited by cysteine protease inhibitors (55). Although both of these proteases localize to the ER and can be inhibited by ALLN, their specific role in ER degradation of proteins *in vivo* remains to be determined.

It is important to note that FCL cells treated with ALLN alone during the pulse did not result in an obvious increase in tropoelastin secretion. This observation suggests that the intracellular degradation and turnover of tropoelastin observed in the present study is not a significant event during normal secretion of the protein. The ability of tropoelastin to be selectively degraded in the ER, however, may be of extreme importance in the event of an aberrant accumulation of tropoelastin in the ER. One situation where this may occur is in disease states where the elastin gene is disrupted, such as supravalvular aortic stenosis (56, 57). The production of the abnormal gene product from the defective allele could result in a tropoelastin protein that is incompetent for transport from the ER and thus be lethal for the cell if not disposed of early in the secretory pathway. It remains to be determined if the degradative pathway identified in the present study plays a role in "quality control" of elastin gene products in diseases such as supravalvular aortic stenosis.

Acknowledgments—We thank Lisa Mecham and David Schettler for cell culture assistance and Dr. R. Pierce for assistance with probe preparation and Northern analysis.

REFERENCES

1. Mecham, R. P., and Davis, E. C. (1994) in *Extracellular Matrix Assembly and Structure* (Yurchenco, P. D., Birk, D. E., and Mecham, R. P., eds) pp. 281–314, Academic Press, Inc., San Diego
2. Hinek, A., Wrenn, D. S., Mecham, R. P., and Barondes, S. H. (1988) *Science* **239**, 1539–1541
3. Mecham, R. P., Hinek, A., Entwistle, R., Wrenn, D. S., Griffin, G. L., and Senior, R. M. (1989) *Biochemistry* **28**, 3716–3722
4. Mecham, R. P., Hinek, A., Griffin, G. L., Senior, R. M., and Liotta, L. (1989) *J. Biol. Chem.* **264**, 16652–16657
5. Karr, S. R., and Foster, J. A. (1981) *J. Biol. Chem.* **256**, 5946–5949
6. Saunders, N. A., and Grant, M. E. (1984) *Biochem. J.* **221**, 393–400
7. Grosso, L., and Mecham, R. P. (1988) *Biochem. Biophys. Res. Commun.* **151**, 822–826
8. Thyberg, J., Hinek, A., Nilsson, J., and Friberg, U. (1979) *Histochem. J.* **11**, 1–17
9. Damiano, V., Tsang, A., Weinbaum, G., Christner, P., and Rosenbloom, J. (1984) *Collagen Relat. Res.* **4**, 153–164
10. Daga-Gordini, D., Bressan, G. M., Castellani, I., and Volpin, D. (1987) *Histochem. J.* **19**, 623–632
11. Pelham, H. R. B. (1991) *Curr. Opin. Cell Biol.* **3**, 585–591
12. Misumi, Y., Misumi, Y., Miki, A., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) *J. Biol. Chem.* **261**, 11398–11403
13. Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988) *J. Biol. Chem.* **263**, 18545–18552
14. Doms, R. W., Russ, G., and Yewdell, J. W. (1989) *J. Cell Biol.* **109**, 61–72
15. Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S., and Klausner, R. D. (1989) *Cell* **56**, 801–813
16. Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri,

- H. P., Yuan, L. C., and Klausner, R. D. (1990) *Cell* **60**, 821–836
17. Magner, J. A., and Papagiannes, E. (1988) *Endocrinology* **122**, 912–920
18. Alcalde, J., Bonay, P., Roa, A., Vilaro, S., and Sandoval, I. V. (1992) *J. Cell Biol.* **116**, 69–83
19. Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R. D. (1991) *Cell* **67**, 601–616
20. Klausner, R. D., and Sitia, R. (1990) *Cell* **62**, 611–614
21. Chun, K. T., Bar-Nun, S., and Simoni, R. D. (1990) *J. Biol. Chem.* **265**, 22004–22010
22. Lecureux, L. W., and Wittenberg, B. W. (1994) *J. Cell Sci.* **107**, 2635–2642
23. Davis, R. A., Thrift, R. N., Wu, C. C., and Howell, K. E. (1990) *J. Biol. Chem.* **265**, 10005–10011
24. Mecham, R. P. (1987) *Methods Enzymol.* **144**, 232–246
25. Wrenn, D. S., Griffin, G. L., Senior, R. M., and Mecham, R. P. (1986) *Biochemistry* **25**, 5172–5176
26. Franc, S., Garrone, R., Bosch, A., and Franc, J.-M. (1984) *J. Histochem. Cytochem.* **32**, 251–258
27. Reynolds, E. S. (1963) *J. Cell Biol.* **17**, 208–215
28. Chomezynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
29. Parks, W. C., Secrist, H., Wu, L. C., and Mecham, R. P. (1988) *J. Biol. Chem.* **263**, 4416–4423
30. Parks, W. C., Roby, J. D., Wu, L. C., and Grosso, L. E. (1992) *Matrix* **12**, 156–162
31. Kuusela, P., Vartio, T., Vuento, M., and Myhre, E. B. (1984) *Infect. Immun.* **45**, 433–436
32. Frisch, S. M., Davidson, J. M., and Werb, Z. (1985) *Mol. Cell. Biol.* **5**, 253–258
33. Hiwasa, T., Sawada, T., and Sakiyama, S. (1990) *Carcinogenesis* **11**, 75–80
34. Inoue, S., and Simoni, R. D. (1992) *J. Biol. Chem.* **267**, 9080–9086
35. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116**, 1071–1080
36. Klausner, R. D., Lippincott-Schwartz, J., and Bonifacio, J. S. (1990) *Annu. Rev. Cell Biol.* **6**, 403–431
37. Sato, R., Imanaka, T., Takatsuki, A., and Takano, T. (1990) *J. Biol. Chem.* **265**, 11880–11884
38. Dixon, J. L., Furukawa, S., and Ginsberg, H. N. (1991) *J. Biol. Chem.* **266**, 5080–5086
39. Amitay, R., Shachar, I., Rabinovich, E., Haimovich, J., and Bar-Nun, S. (1992) *J. Biol. Chem.* **267**, 20694–20700
40. Hurtley, S. M., and Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5**, 277–307
41. David, V., Hochstenbach, F., Rajagopalan, S., and Brenner, M. B. (1993) *J. Biol. Chem.* **268**, 9585–9592
42. Nakai, A., Satoh, M., Hirayoshi, K., and Nagata, K. (1992) *J. Cell Biol.* **117**, 903–914
43. Jain, N., Brickenden, A., Lorimer, I., Ball, E. H., and Sanwal, B. D. (1994) *Biochem. J.* **304**, 61–68
44. Jain, H., Brickenden, A., Ball, E. H., and Sanwal, B. D. (1994) *Arch. Biochem. Biophys.* **314**, 23–30
45. Saga, S., Nagata, K., Chen, W.-T., and Yamada, K. M. (1987) *J. Cell Biol.* **105**, 517–527
46. Cox, B. A., Starcher, B. C., and Urry, D. W. (1974) *J. Biol. Chem.* **249**, 997–998
47. Inoue, S., Bar-Nun, S., Roitelman, J., and Simoni, R. D. (1991) *J. Biol. Chem.* **266**, 13311–13317
48. Adeli, K. (1994) *J. Biol. Chem.* **269**, 9166–9175
49. Wileman, T., Kane, L. P., and Terhorst, C. (1991) *Cell Regul.* **2**, 753–765
50. Roy, S., Yu, S., Banerjee, D., Overton, O., Mukhopadhyay, G., Oddoux, C., Grieninger, G., and Redman, C. (1992) *J. Biol. Chem.* **267**, 23151–23158
51. Cotner, T., and Pious, D. (1995) *J. Biol. Chem.* **270**, 2379–2386
52. Otsu, M., Urade, R., Kito, M., Omura, F., and Kikuchi, M. (1995) *J. Biol. Chem.* **270**, 14958–14961
53. Urade, R., and Kito, M. (1992) *FEBS Lett.* **312**, 83–86
54. Urade, R., Nasu, M., Moriyama, T., Wada, K., and Kito, M. (1992) *J. Biol. Chem.* **267**, 15152–15159
55. Urade, R., Takenaka, Y., and Kito, M. (1993) *J. Biol. Chem.* **268**, 22004–22009
56. Curran, M. E., Atkinson, D. L., Ewart, A. K., Morris, C. A., Leppert, M. F., and Keating, M. T. (1993) *Cell* **73**, 159–168
57. Ewart, A. K., Morris, C. A., Ensing, G. J., Loker, J., Moore, C., Leppert, M. F., and Keating, M. T. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3226–3230