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Differential targeting of recombinant fibronectins in AtT-20 cells based on their efficiency of aggregation

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SUMMARY

In pituitary-derived AtT-20 cells, recombinant fibronectin containing the N-terminal matrix assembly domain and the C-terminal half of fibronectin does not follow the regulated secretory pathway but instead concentrates in distinct organelles prior to secretion. These organelles are larger than the dense-core granules and localize to the cell body at sites that differ from lysosomes, endosomes and endoplasmic reticulum. Unlike the dense-core granules, their discharge is not stimulated by 8-bromo-cyclic-AMP or phorbol esters. The kinetics of intracellular transport and secretion of the recombinant fibronectin suggest that it is present in a post-Golgi pool that turns over more slowly than constitutive vesicles. Indeed, the fibronectin-containing organelles disappear with a half-time of 3 hours after inhibiting protein synthesis. Presence of the organelles correlates with intracellular aggregation of dimeric fibronectin polypeptides. The organelles are absent in cells expressing monomeric recombinant fibronectin (lacking C-terminal dimerization sites) or the C-terminal half of fibronectin (which dimerizes but lacks the N-terminal matrix assembly domain), both of which aggregate less efficiently than dimeric fibronectin. Instead, the latter polypeptides enter the dense-core granules. Thus while the formation of the fibronectin-containing organelles may require efficient aggregation, it may not require a specific structural signal. Moreover, efficient aggregation is not necessarily a prerequisite for following the regulated pathway.

Key words: fibronectin, secretion, aggregation, AtT-20 cell, targeting

INTRODUCTION

After completion of synthesis and folding, secretory proteins are transported from the endoplasmic reticulum (ER) to the Golgi where post-translational modifications and sorting occur. In many cells secretion occurs constitutively (reviewed by Kelly, 1985); however, a number of cell types regulate the secretion of specific proteins that are stored in intracellular granules until secretion is induced by an external stimulus (Kelly, 1985; Burgess and Kelly, 1987). While extracellular matrix and cell adhesion proteins usually follow constitutive secretory pathways (e.g. see Burgess et al., 1985; Verweij et al., 1987; Hynes, 1990; Weinstock and Leblond, 1974), there are specific circumstances where secretion is controlled. For example, fibronectin (FN) is secreted constitutively by fibroblasts (Hynes, 1990) but is packaged into α-granules in the platelet for release upon platelet activation at wound sites (Wencel-Drake et al., 1985).

FN is a large multifunctional dimeric glycoprotein that plays central roles in a variety of developmental and cellular processes such as cell adhesion and migration, tissue remodeling and wound repair (reviewed by McDonald, 1988; Ruoslahti, 1988; Mosher, 1989; Hynes, 1990). In the extracellular space, FN functions as a major component of a fibrillar matrix assembled via interactions with cell surface integrin receptors, other matrix proteins, and FN itself. The extracellular functions of specific domains have been quite thoroughly studied, but relatively little is known about the sequences required for intracellular transport and secretion. FN is assembled into a disulfide-bonded dimer in the ER and subsequently transported through the Golgi and secreted (Choi and Hynes, 1979). Efficient secretion is dependent on the presence of the alternatively spliced V region in at least one of the subunits of the dimer (Schwarzbauer et al., 1989). A number of different recombinant FNs lacking large domains have been expressed in fibroblasts and appear to be secreted at the same rate as native FN (Schwarzbauer, 1991). However, the determinants required for cell-type-specific transport of FN along regulated versus constitutive pathways have not been identified.

To begin to understand signals involved in sorting, we have used the mouse pituitary cell line AtT-20, which produces and stores adrenocorticotropic hormone (ACTH) and β-endorphin in a population of dense core granules. Exocytosis of these granules is regulated through protein kinases A and C (Thiele and Eipper, 1990). Other endocrine and exocrine secretory proteins expressed in these cells by transfection are routed to granules to varying extents and can be detected in regulated

secretion (e.g. see Burgess et al., 1985; Moore et al., 1983; Moore and Kelly, 1985; Castle et al., 1992). However, not all regulated secretory proteins are targeted to the granules, suggesting specificity in the sorting mechanism (Colomer et al., 1994).

We have used AtT-20 cells to analyze the sorting of recombinant FNs (recFNs) differing in the presence and absence of specific functional domains. We find that the recombinant polypeptides exhibit a striking difference in localization and in sorting between different secretory pathways. Interestingly, this difference relates to the ability to aggregate intracellularly and to self-associate in the extracellular matrix.

MATERIALS AND METHODS

Materials

Restriction enzymes and other cloning enzymes were from Boehringer/Mannheim (Indianapollis, IN). Growth media for tissue culture were from Gibco/BRL (Gaithersburg, MD). Gel electrophoresis reagents and Tran^{35}S-label were from ICN Biomedicals (Irvine, CA). The nonionic detergent Nikkol was from Nikko chemicals (Tokyo, Japan). Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], MES (2[*N*-morpholino]ethanesulfonic acid), cycloheximide, chloroquine, 8-bromo-cyclic AMP (8-Br-cAMP), phorbol 12-myristate 13-acetate, xyloside, protease inhibitors trasylol, leupeptin, antipain, PMSF (phenylmethylsulfonyl fluoride) and all other chemicals used in this study were purchased from Sigma Chemical (St Louis, MO). Expre^{35}S^{35}S label and $^{35}SO_4$ were from New England Nuclear (Wilmington, DE). Anti-ACTH antiserum was characterized previously (Castle et al., 1992). Anti-FN polyclonal antibody was described previously (Schwarzbauer et al., 1989) and affinity purified for immunofluorescence (Schwarzbauer, 1991). Anti-FN monoclonal antibody IC3 is rat-specific and recognizes an epitope in the cell binding domain, within repeats III$_{8\text{-}11}$. Polyclonal antibody against mannose 6-phosphate receptor was a gift from Dr Bill Brown. Polyclonal antibody against mouse TGN38 was a gift from Dr Sharon Milgram, Soochen Tricia Kho and Dr Betty Eipper. Monoclonal antibody against BiP was a gift from Dr David Bole. Monoclonal antibody against lysosomal membrane glycoproteins LAMP-1 and LAMP-2 was obtained from the developmental hybridoma bank. Antibody against human cathepsin D was a gift from Dr Stuart Kornfeld. Other immunoreagents were from Jackson (West Grove, PA).

Plasmid construction

The retroviral vector pLJ (Schwarzbauer et al., 1989) was used for expression of all the constructs. Construction and expression in fibroblasts of FNΔIII$_{1\text{-}7}$ and FNMΔIII$_{1\text{-}7}$ have been described previously (Schwarzbauer, 1991). FN-C110 contains the carboxy-terminal 1,060 amino acids of rat FN beginning at a *Bam*HI site at position 3,920 within the alternatively spiced EIIIB exon. At the amino terminus, it is fused to the FN signal sequence (Patel et al., 1987). The two sequences are joined with a *Bam*HI site and the amino acid sequence across the junction is CLGTSGIPIFE.

Cell culture and transfection

Mouse pituitary AtT-20 D16v cells were cultured as described previously (Castle et al., 1992). Retroviral plasmids were introduced into AtT-20 cells by liposome-mediated transfection using Lipofectin Reagent (Life Technologies Inc.) and following the manufacturer's directions. Transfected cells were grown in medium plus 0.25 mg/ml G418 and resistant colonies were screened for expression of recombinant FNs as previously described (Castle et al., 1992).

Metabolic labeling and immunoprecipitations

Cells were plated at a density of 3×10^4 cells/cm^2. At 48 to 72 hours later the cells were labeled for 16 hours with 200 μCi/ml Tran^{35}S-label or Expre^{35}S^{35}S label in methionine- and cysteine-free minimal essential medium supplemented with 15% dialyzed Nuserum, 20 mM Hepes, 4 mM glutamine. For labeling periods longer than 1 hour, 4 mg/l cystine and 2.5 mg/l methionine were also included in medium. Chases were carried out in the same medium containing excess cold methionine and cystine, and trasylol as proteinase inhibitor (50 kallikrein inactivating units/ml). To stimulate release of granule contents, 5 mM 8-Br-cAMP was added to the chase medium. The chase media were collected and a proteinase inhibitor cocktail (2 mM PMSF, 2 mM iodoacetamide and 10 mM ethylenediaminetetraacetic acid (EDTA)) was added. Cells were lysed in 1% NP-40, 0.4% deoxycholic acid, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA containing inhibitor cocktail by incubating on ice for 10 minutes, and the lysates were cleared by centrifugation for 1 minute at 12,000 ***g***.

^{35}S-labeled recFNs were immunoprecipitated in radioimmune precipitation buffer (1% NP40, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM EDTA, 1.7×10^{-6} M PMSF) with an anti-FN polyclonal for 12-24 hours at 4°C. Immune complexes were adsorbed to Protein A-Sepharose, washed with radioimmune precipitation buffer, eluted in SDS sample buffer (Laemmli, 1970) and resolved on 6% or 7.5% gels. Gels were treated and proteins were visualized and quantitated as described (Castle et al., 1992). Endoglycosidase H (Endo H) sensitivity/resistance was determined as described (Castle et al., 1992).

Labeling with $^{35}SO_4$ and quantitation of labeled GAG chains

AtT-20 cells were plated at 2×10^5 per well in 12-well dishes and used 48-72 hours later. The cells were starved for sulfate in sulfate-free minimal essential medium containing 1 mM xyloside for 30 minutes and then pulse-labeled for 5 minutes with the same medium containing 0.2 mCi/ml of $^{35}SO_4$. Labeled cells were chased in minimal essential medium containing 5 mM sodium sulfate. At the end of the chase, the medium was removed and the cells were extracted with 0.1 ml of 1% TX-100, 0.15 M sodium chloride, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$. Wells were washed with 0.4 ml of phosphate-buffered saline and the wash was pooled with the original lysate. $^{35}SO_4$-labeled GAG chains were quantitated exactly as described by Miller and Moore (1991).

Immunofluorescence

Cells were plated on coverslips as described (Castle and Castle, 1993), fixed in 2-4% paraformaldehyde in 0.12 M sodium phosphate, pH 7.4, for 30 minutes, washed in PBS and permeabilized and quenched in 0.2% TX-100, 1 M glycine in PBS for 20 minutes. The coverslips were blocked in 2% ovalbumin in PBS (FN blocking buffer) and incubated with primary antibodies in FN blocking buffer for 1-2 hours at room temperature. The following dilutions were used: anti-FN monoclonal antibody IC3, 1:10 of culture supernatant; anti-FN affinity purified polyclonal antibody, 1 μg/ml; anti-mannose 6-phosphate receptor, 1:500; anti-TGN38, 1:200; anti-BiP, 1:10 of culture supernatant; anti-ACTH, 1:2,000; anti-LAMP-1 and LAMP-2, 1:1 of culture supernatant, anti-human cathepsin, 1:100. The coverslips were washed with PBS and incubated with fluorescein- or Texas Red-conjugated secondary antibodies in FN blocking solution for 1 hour at room temperature. The coverslips were washed with PBS, mounted using Vectashield and viewed under epifluorescence using a Leitz microscope or by confocal microscopy using a Zeiss LSM 410 confocal microscope.

For studies involving uptake of Texas Red-conjugated ovalbumin into lysosomes, cells were first incubated with 3 mg/ml of Texas Red-conjugated ovalbumin in normal growth medium for 3 hours. They were then incubated for an additional 3 hours in normal growth medium and fixed and processed as described above.

In experiments involving the quantitation of the number of FNΔIII$_{1\text{-}7}$-containing organelles per cell, groups of cells (60-150 per

photograph) were photographed under fluorescence and differential interference contrast. The number of fluorescently labeled structures was counted in each photograph and normalized to the total number of cells found in the DIC image.

Sedimentation analysis of aggregation

Transfected AtT-20 cells were pulse-labeled for 15 minutes and chased for 30 minutes in studies of FN-C110 and for 75 minutes in studies of $FN\Delta III_{1\text{-}7}$ and $FN_M\Delta III_{1\text{-}7}$. The dishes were placed on ice to stop intracellular transport, washed twice with PBS and solubilized in 0.4% Nikkol, 150 mM NaCl, 5 mM EDTA, 10 mM MES, pH 6.3, or 10 mM Tris-HCl, pH 7.5, containing the proteinase inhibitor cocktail. After 30 minutes incubation at 0°C, solubilized cells were homogenized in a Dounce homogenizer with 10 strokes and cleared by centrifugation at 6,600 ***g*** for 40 seconds. Supernatants were examined for aggregation by sedimentation at 200,000 ***g*** for 30 minutes in TLX100 tabletop ultracentrifuge. Some samples were treated with 0.5 M NaCl for 30 minutes before centrifugation. recFNs were immunoprecipitated from supernatants and pellets, and analyzed by SDS-PAGE and fluorogaphy as above.

RESULTS

Structure of recombinant FN polypeptides

Three well-characterized recFNs that differ in their functional abilities were used to analyze FN transport (Fig. 1) (Schwarzbauer et al., 1989; Schwarzbauer, 1991; Aguirre et al., 1994). $FN\Delta III_{1\text{-}7}$ contains the amino-terminal matrix assembly and collagen binding domains directly connected to the carboxy-terminal half of FN, consisting of cell, heparin and fibrin domains. This recFN is secreted as a disulfide-bonded dimer and, when expressed in fibroblasts, it is assembled into a fibrillar extracellular matrix. $FN\Delta III_{1\text{-}7}$ behaved similarly to full-length FN in a number of assays (Aguirre et al., 1994; Schwarzbauer, 1991; Wilson and Schwarzbauer, 1992) and because of the difficulty with expressing full-length FN in AtT-20 cells and other cell lines (J.E. Schwarzbauer, unpublished observations), we elected to use this recombinant protein in our experiments. $FN_M\Delta III_{1\text{-}7}$, a monomeric version of $FN\Delta III_{1\text{-}7}$, lacks a short segment near the carboxy terminus containing the pair of cysteines that form the interchain disulfide bonds in the FN dimer. Although this protein contains the amino-terminal assembly domain, its monomeric structure prevents it from being assembled into fibrils. FN-C110 consists of the carboxy-terminal 110 kDa of FN but lacks the amino-terminal domains. The FN signal sequence is fused to the amino terminus to provide for transport into the ER and all recFNs have the alternatively spliced V120 segment. Retroviral vectors encoding these recFNs were transfected into mouse pituitary AtT-20 cells and stable cell lines secreting each polypeptide were created. We have established that the cell lines synthesized polypeptides of appropriate M_r (i.e. 180,000 for $FN\Delta III_{1\text{-}7}$ and $FN_M\Delta III_{1\text{-}7}$; 110,000 for FN-C110) by immunoprecipitation from media and cell lysates of metabolically labeled cells followed by SDS-PAGE and fluorography (data not shown).

Interestingly, no labeled full-length FN (M_r 250,000) was observed in the immunoprecipitates from metabolically labeled cells, suggesting that AtT-20 cells do not express it. This finding is consistent with the lack of immunofluorescent staining of nontransfected AtT-20 cells (data not shown). The absence of synthesis of endogenous FN in AtT-20 cells allows us to study the secretion of different recFNs without the potentially complicating formation of recFN-FN heterodimers (Schwarzbauer et al., 1989).

Intracellular transport of recFNs and their storage in dense-core granules of AtT-20 cells

To evaluate the efficiency of intracellular transport of recFNs in AtT-20 cells, we estimated the rates of their transport from the ER by following the time course of acquisition of resistance to digestion with Endo H (Fig. 2). AtT-20 cells expressing recFNs were pulse-labeled for 5 minutes and chased for different lengths of time. At each time point, secreted and cell-associated recFNs were immunoprecipitated, subjected to Endo H digestion and the fraction of Endo H-resistant recFN was quantitated. Transport of all recFNs from ER-related compartments (judged by the acquisiton of Endo H resistance) occurred without an appreciable lag and was efficient with half-times of 40 minutes for $FN\Delta III_{1\text{-}7}$, 30 minutes for $FN_M\Delta III_{1\text{-}7}$ and 15 minutes for FN-C110. These half-times are shorter than that of full-length FN in fibroblasts, which exits the ER with a half-time of 1 hour (A. Castle, unpublished observations). No loss of total ^{35}S-labeled recFN was observed over an 8 hour period (data not shown), indicating intracellular transport without degradation.

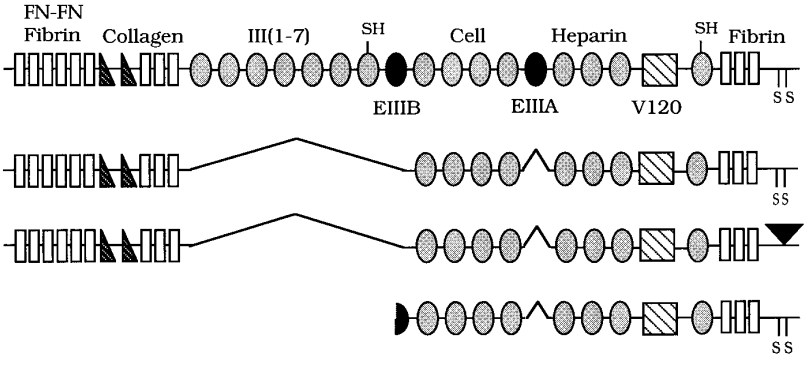

Fig. 1. Domain structure of FN and recFNs. A schematic representation of full-length FN (top) shows domains for binding to FN (FN-FN), fibrin, collagen, cells and heparin. Two cysteine residues at the C terminus (SS) form interchain disulfide bonds in the FN dimer. The three recFNs used in this study are shown. $FN\Delta III_{1\text{-}7}$ and $FN_M\Delta III_{1\text{-}7}$ contain an N-terminal domain consisting of six type I repeats ($I_{1\text{-}6}$), two type II repeats ($II_{1,2}$), and three type I repeats ($I_{7\text{-}9}$). The N-terminal domain is connected to the FN-C110 half of the molecule beginning with repeat III_8, thus excluding repeats $III_{1\text{-}7}$. The deletion of the carboxy-terminal cysteines is indicated by a filled triangle. The FN-C110 construct consists of the C-terminal domain of FN beginning in the middle of repeat EIIIB. All constructs lack the EIIIA repeat but contain the alternatively spliced segment V120. The M_r values of the constructs are 180,000 for $FN\Delta III_{1\text{-}7}$ and $FN_M\Delta III_{1\text{-}7}$, and 110,000 for FN-C110.

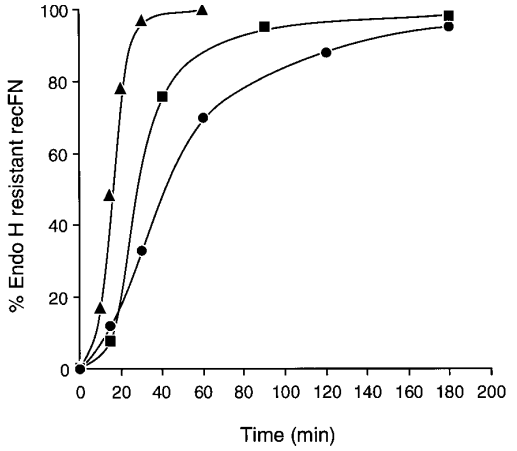

Fig. 2. Kinetics of transport from the ER to the Golgi. AtT-20 cells expressing FNΔIII$_{1-7}$ (●), FN$_M$ΔIII$_{1-7}$ (■) and FN-C110 (▲) were labeled for 5 minutes with Tran^{35}S-label and chased for various lengths of time. At each timepoint labeled recFNs from the medium and cell lysate were immunoprecipitated and subjected to Endo H digestion, SDS-PAGE and fluorography as described in Materials and Methods. Labeled recFNs were quantitated by fluorography and Endo H resistant recFN was expressed as a fraction (%) of total labeled recFN (secreted and cell-associated).

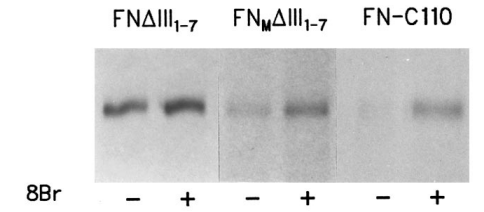

Fig. 3. Effect of 8-Br-cAMP on the secretion of recFNs. Duplicate dishes of AtT-20 cells expressing FNΔIII$_{1-7}$, FN$_M$ΔIII$_{1-7}$ and FN-C110 were labeled for 16 hours with Tran^{35}S-label and chased for three consecutive 3 hour intervals. During the last interval, shown here, the medium of one dish was supplemented with 5 mM 8-Br-cAMP to stimulate granule release. The chase media were immunoprecipitated, and subjected to SDS-PAGE and fluorography.

Table 1. 8-Br-cAMP-dependent stimulation of recFNs and ACTH

	FNΔIII$_{1-7}$	FN$_M$ΔIII$_{1-7}$	FN-C110
Stimulated secretion of recFNs	2±1	11±1	18±1
Stimulated secretion of ACTH	36	36	41

Stimulated secretion of recFNs and ACTH were quantitated from experiments as those shown in Fig. 3. Stimulated secretion of recFNs represents the difference between the percentage of total labeled recFN secreted in the presence and that secreted in the absence of 8-Br-cAMP. Data are expressed as mean ± s.e.m. At least three experiments were performed. Stimulated secretion of ACTH was calculated in the same manner. Labeled ACTH represented the sum of labeled proopiomelanocortin, the ACTH biosynthetic intermediate, and ACTH after correcting for methionine content.

We used 8-Br-cAMP-dependent stimulation of secretion to assess whether recFNs are present in endogenous storage granules. Duplicate dishes of cells were labeled for 15 hours and chased for two 3-hour periods without stimulation to allow secretion of proteins via constitutive pathway(s). One of the dishes was then treated with 5 mM 8-Br-cAMP to stimulate exocytosis. Fig. 3 shows that all three recFNs were secreted but the secretion of FN$_M$ΔIII$_{1-7}$ and FN-C110 was stimulated three- to sixfold, respectively, whereas only a slight stimulation (1.1-fold) of secretion of FNΔIII$_{1-7}$ was observed. The fractions of recFNs and ACTH that were released upon stimulation are shown in Table 1. The stimulus-dependent secretion of FN$_M$ΔIII$_{1-7}$ and FN-C110 suggests that these polypeptides are present in the dense-core granules of AtT-20 cells while FNΔIII$_{1-7}$ is absent or is present at a much reduced level.

recFN FNΔIII$_{1-7}$ localizes to distinct organelles in AtT-20 cells

Immunofluorescent localization of recFNs and ACTH in transfected cells revealed that the distribution of FN$_M$ΔIII$_{1-7}$ and FN-C110 is similar to that of ACTH, with greatest concentration in the tips of cellular processes where dense core granules accumulate (Fig. 4). This distribution is consistent with the presence of these recFNs in the secretory granules, as inferred above. In contrast, cells expressing FNΔIII$_{1-7}$ lack fluorescent staining in the processes and instead contain very brightly stained, relatively large granule-like structures within the cell body, with an average number of 10-20 structures per cell. Although less intense, Golgi staining in the perinuclear region of the cells is also visible.

To further characterize the FNΔIII$_{1-7}$-containing organelles we analyzed their localization in relation to markers of other compartments and their structures by indirect immunofluorescence. The distribution of FNΔIII$_{1-7}$ is distinct from dense-core granules marked by ACTH (Fig. 5A,B); lysosomes marked by internalized Texas Red-conjugated ovalbumin (Fig. 5C,D), cathepsin D (not shown) or lysosomal membrane glycoproteins LAMP-1 and LAMP-2 (Fig. 5E,F); late endosomes/prelysosomes marked by mannose 6-phosphate receptor (Fig. 5G,H); *trans*-Golgi network marked by TGN38 (Fig. 5I,J) and ER marked by BiP (Fig. 5K,L). When sections through different planes in the cell obtained by confocal fluorescence microscopy were aligned, we found no instances of colocalization of the FNΔIII$_{1-7}$ structures with any of the markers. Interestingly, optical sectioning also showed that most of the FNΔIII$_{1-7}$-containing structures were located toward the bottom (attached) surface of the cell whereas organelles involved in uptake were located toward the top of the cell (Fig. 6).

Secretion and turnover of FNΔIII$_{1-7}$-containing organelles

As the FNΔIII$_{1-7}$-containing organelles had a granule-like appearance, we wished to determine whether they represent a second granule population. The distinguishing features of secretory storage granules are a prolonged lifetime in the cytoplasm and regulation of their exocytosis by external stimuli. In AtT-20 cells the endogenous ACTH-containing granules turn over with a halftime of 7-12 hours (Moore and Kelly, 1985; A. Castle, unpublished observations), and their formation but not their release is dependent on continuous protein synthesis (Brion et al., 1992).

If the FNΔIII$_{1-7}$ structures are secreted yet have a longer lifetime than the constitutive pathway, then this should be reflected in the kinetics of secretion of the FNΔIII$_{1-7}$ polypep-

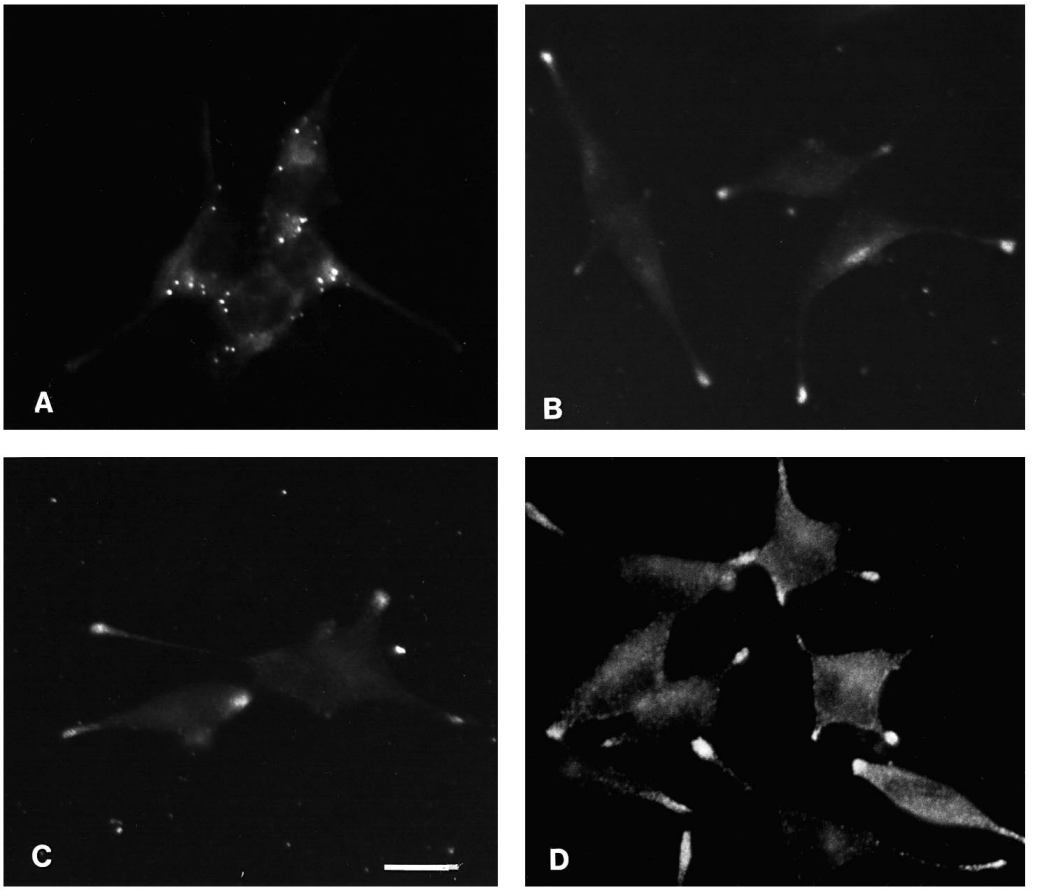

Fig. 4. Immunocytochemical localization of recFNs in AtT-20 cells. Transfected AtT-20 cells were fixed and processed for immunofluorescence as described in Materials and Methods. AtT-20 cells expressing $FN\Delta III_{1-7}$ (A,D), $FN_M\Delta III_{1-7}$ (B) and FN-C110 (C) were stained with an affinity-purified antibody against rat FN (A-C) or an antibody against ACTH (D). Similar distributions of ACTH were obtained in cells expressing $FN_M\Delta III_{1-7}$ and FN-C110. Bar, 10 μm.

tides. Cells expressing $FN\Delta III_{1-7}$ were labeled overnight with Expre^{35}S^{35}S to approach steady state labeling of all intracellular pools and chased for various lengths of time. At each timepoint, we determined the fraction of total $FN\Delta III_{1-7}$ that was secreted (Fig. 7A). We found that $FN\Delta III_{1-7}$ is secreted with a $t_{\frac{1}{2}}$=90 minutes, which is considerably slower than would be expected for a protein with a $t_{\frac{1}{2}}$ of exit from the ER of 40 minutes. Also we have determined that following an extended metabolic labeling, ~35% of the intracellular $FN\Delta III_{1-7}$ is Endo H resistant (data not shown), indicating its presence in a post-Golgi compartment that turns over more slowly than constitutive vesicles. Since $FN\Delta III_{1-7}$ is not present in the dense-core granules, it is likely that the slower kinetics of secretion reflect discharge of the $FN\Delta III_{1-7}$ organelles.

To confirm that the slow rate of secretion is not due to a clonal variation in the rate of constitutive secretion we followed the kinetics of secretion of free GAG chains, which have been used previously as a marker of the constitutive pathway (e.g. see Miller and Moore, 1991). The data presented in Fig. 7B show that $^{35}SO_4$-labeled GAG chains are secreted with rapid kinetics ($t_{\frac{1}{2}}$=15 minutes) from untransfected AtT-20 cells and from cells expressing $FN\Delta III_{1-7}$, indicating no difference in the rate of constitutive secretion.

To assess the turnover of the $FN\Delta III_{1-7}$ structures more directly, we used indirect immunofluorescence to follow their disappearance after inhibiting protein synthesis. Over the course of 8 hours in the presence of cycloheximide, there was a significant decrease in the number of $FN\Delta III_{1-7}$ organelles (compare Fig. 8A and B). In contrast, there was little change in the staining of ACTH in dense-core granules (at the tips of the processes) (compare Fig. 8E and F), consistent with a very slow basal rate of turnover of the dense-core granules. We quantitated the number of $FN\Delta III_{1-7}$-containing structures per cell at different intervals following the addition of cycloheximide and estimated the half-time of their disappearance to be 3 hours (Fig. 7C). The rate of turnover is unaffected by the presence of 40-100 μM chloroquine (Fig. 7C), which is widely used as an inhibitor of lysosomal degradation. It is interesting to note that chloroquine alone had no effect on the number of $FN\Delta III_{1-7}$ structures present, suggesting that their formation is not dependent on acidic pH, as may be the case for certain neuroendocrine granules (e.g. see Orci et al., 1987; Carnell and Moore, 1994).

Results presented in Fig. 3 indicate that secretion of $FN\Delta III_{1-7}$ is not stimulated by 8-Br-cAMP; however, if the $FN\Delta III_{1-7}$ organelles carry only a fraction of the $FN\Delta III_{1-7}$ polypeptides, stimulation may be difficult to detect due to secretion via the constitutive pathway. Therefore, we evaluated possible stimulation of $FN\Delta III_{1-7}$ organelles by indirect immunofluorescence after treatment with either 5 mM 8-Br-cAMP or 100 nM phorbol 12-myristate 13-acetate. No change in the intensity of staining or in the number of organelles was observed (data not shown), confirming the apparent lack of regulated exocytosis of this compartment.

Sedimentation analysis of intracellular recFNs

The results presented so far show that dimeric recFN differs from the other recFNs in that it is not stored in ACTH-containing

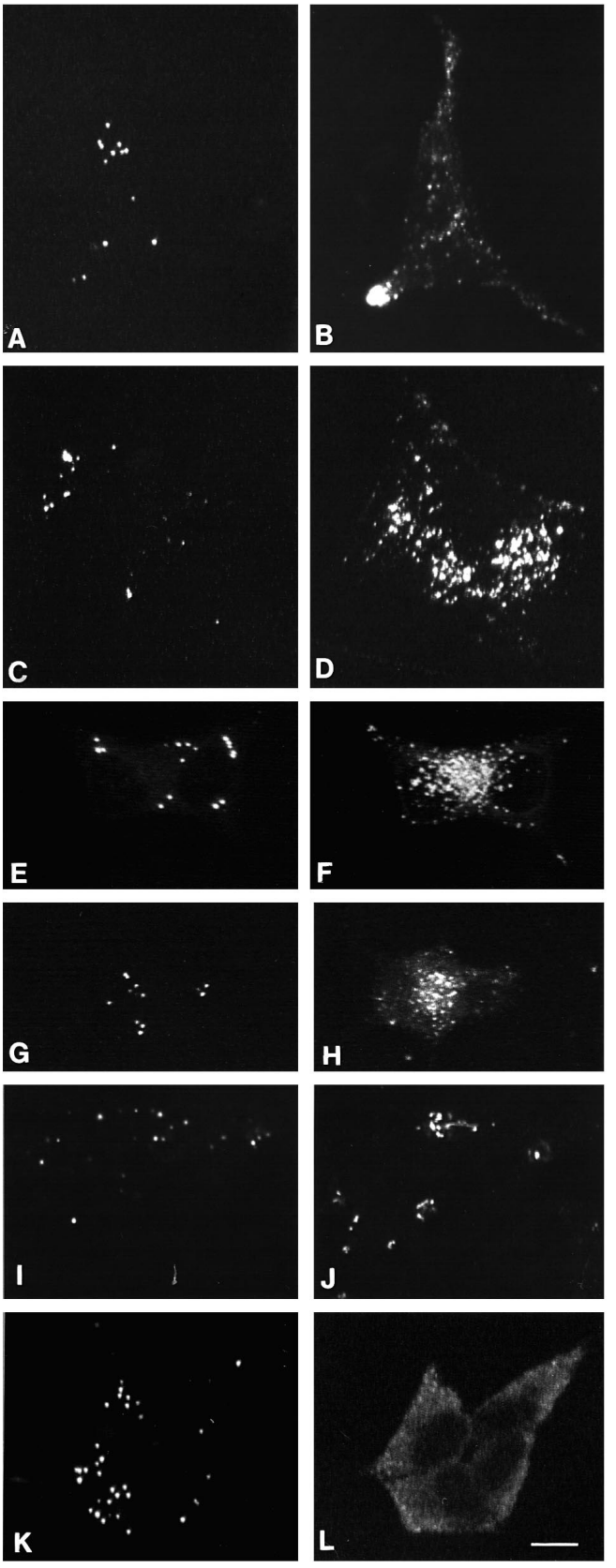

Fig. 5. Localization of FNΔIII$_{1-7}$ relative to other markers using double-label immunofluorescence. AtT-20 cells expressing FNΔIII$_{1-7}$ were fixed and processed for immunofluorescence as described in Materials and Methods. The fixed cells were stained with a combination of antibodies against FN (A,C,E,G,I,K) and ACTH (B), lysosomal membrane proteins LAMP-1 and LAMP-2 (F), mannose 6-phosphate receptor (H), TGN38 (J) and BiP (L). Cells shown in C and D were allowed to take up Texas Red-ovalbumin (3 mg/ml) for 3 hours before fixation. Fluorescence was examined by confocal microscopy. Each profile was obtained by optically sectioning through the cell at 1 μm intervals and superimposing the resulting images. Due to the relatively low concentration of FNΔIII$_{1-7}$ antigen in the Golgi region, the staining there is not visualized under these conditions. Bar, 10 μm.

secretory granules in AtT-20 cells but instead is found within structures with distinct characteristics and localization. FNΔIII$_{1-7}$ is also distinguished from the other recFNs by its ability to associate with other FN dimers during extracellular fibril formation. Therefore, packaging of recFNs into different compartments correlates with their differential abilities to form fibrils. As selective aggregation is thought to be an important factor in sorting of secretory proteins, it is possible that the recFNs also differ in their ability to form intracellular aggregates.

To assess the propensity of intracellular recFNs to aggregate, we employed sedimentation analysis of detergent-solubilized cell extracts, as has been done in studies of aggregation for other proteins (e.g. see Colomer et al., 1994; Shennan et al., 1994; Kuliawat and Arvan, 1994). We selected the nonionic detergent Nikkol to solubilize the cells as it has been shown previously to give efficient solubilization and at the same time to maintain the integrity of insulin aggregates (Kuliawat and Arvan, 1994). Cells expressing different recFNs were pulse-labeled for 15 minutes and chased for 30 minutes (FN-C110) and 75 minutes (FNΔIII$_{1-7}$ and FN$_M$ΔIII$_{1-7}$) to allow newly synthesized proteins to enter the Golgi complex. Following solubilization, pre-cleared lysates were subjected to ultracentrifugation, and supernatant and pellet fractions were analyzed for recFNs. As shown in Fig. 9A, FNΔIII$_{1-7}$ is completely sedimentable, whereas 60% of FN$_M$ΔIII$_{1-7}$ and 79% of FN-C110 remained in the supernatant at pH 6.3, in the pH range suspected to exist in the TGN of endocrine cells (Anderson and Pathak, 1985). Under these conditions the membrane protein SCAMP (Brand et al., 1991) and the ER chaperone BiP (Bole et al., 1986) are found primarily in the supernatant, indicating that the cells are fully solubilized (Fig. 9B). Interestingly, only 10% of the endogenous ACTH was sedimentable (Fig. 9B), suggesting that either aggregates containing ACTH are labile under these conditions or ACTH is not aggregated in the granules of AtT-20 cells.

The sedimentation properties of intracellular recFNs were not affected by calcium ions (data not shown); however, the pH of the solubilization buffer had a clear effect. A large fraction of FN$_M$ΔIII$_{1-7}$ and FN-C110 remained in the supernatant at pH 7.5 while most of the FNΔIII$_{1-7}$ was in the pellet (Fig. 9A). Quantitation of the amounts of recFNs in the pellet shows that: 82% of FNΔIII$_{1-7}$, 17% of FN$_M$ΔIII$_{1-7}$, and 2% of FN-C110 are sedimentable at pH 7.5. FNΔIII$_{1-7}$ exhibited different sedimentation characteristics compared to FN$_M$ΔIII$_{1-7}$ and FN-C110 when cell lysates prepared at pH 6.3 were treated with 0.5 M sodium chloride. Sodium chloride only partially disrupted the FNΔIII$_{1-7}$ aggregates (67% sedimentable), whereas aggregation of FN$_M$ΔIII$_{1-7}$ and FN-C110 was almost completely prevented (8% of FN$_M$ΔIII$_{1-7}$ and 2% of FN-C110 were sedimentable; Fig. 9A).

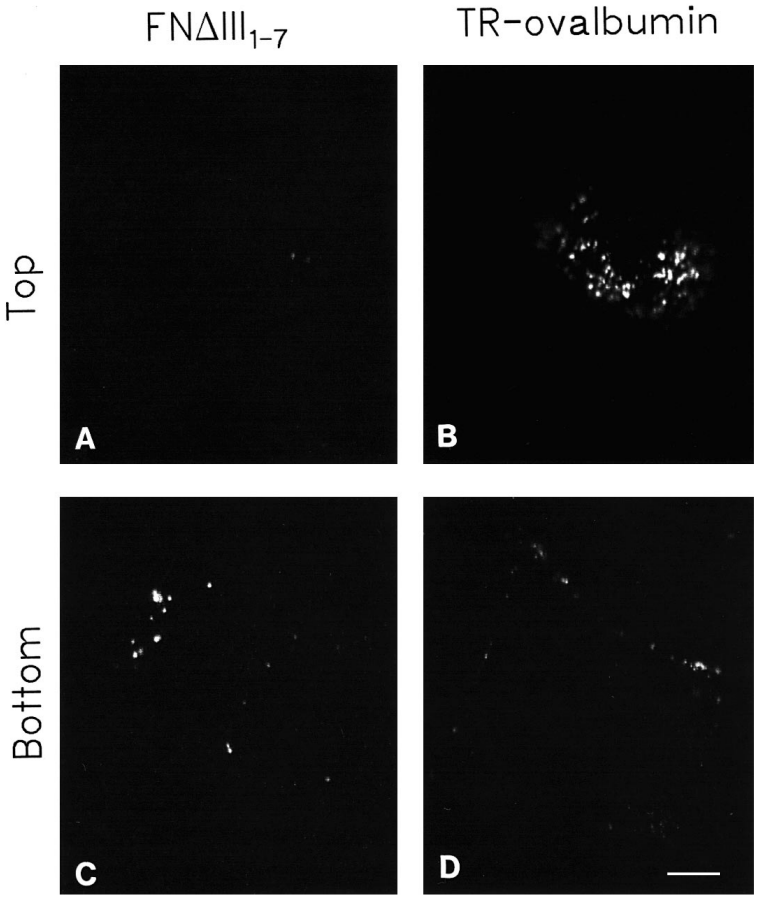

Fig. 6. Distribution of FNΔIII$_{1-7}$ structures and internalized Texas Red ovalbumin at the top and the bottom of the cell. A single optical section from the top (A, B) and bottom (C,D) of the same cell as in Fig. 4C,D is shown. (A,C) Staining with anti-FN antibody; (B,D) internalized Texas Red-ovalbumin. Bar, 10 μm.

Overall, the results obtained using the sedimentation analysis indicate that FNΔIII$_{1-7}$ aggregates very efficiently within the cell and is maintained within sedimentable complexes under a variety of conditions. In contrast, FN$_M$ΔIII$_{1-7}$ and FN-C110 are relatively disaggregated under the same conditions. Thus the ability to form aggregates correlates with the absence of FNΔIII$_{1-7}$ in the dense-core granules of AtT-20 cells and instead with its presence in the distinct organelles.

DISCUSSION

The most significant finding in our analyses of intracellular transport and sorting of recFNs in AtT-20 cells is the distinction in routing of the dimeric FNΔIII$_{1-7}$ versus monomeric FN$_M$ΔIII$_{1-7}$ and carboxy-terminal FN-C110. FNΔIII$_{1-7}$ forms sedimentable complexes and can segregate from the regulated pathway into distinct granule-like structures. In contrast, the other recFNs are less aggregated and can be stored in the dense-core granules. Thus, the secretory pathways taken by each recFN are strongly correlated with their ability to form intracellular aggregates.

Characterization of FNΔIII$_{1-7}$ organelles

FNΔIII$_{1-7}$ polypeptides expressed in AtT-20 cells are present in large structures located within the cytoplasm of the cell body that is closest to the substratum. These structures turn over more rapidly than the endogenous ACTH-containing granules and their secretion is not regulated by protein kinase A or C. These characteristics are strongly reminiscent of the organelles that form when vonWillebrand factor is expressed in pituitary AtT-20 cells (Wagner et al., 1991) and kidney CV-1 cells (Voorberg et al., 1993).

In contrast to the formation of vonWillebrand factor organelles in AtT-20 cells, where a single domain, the propolypeptide, is required for their formation, the formation of FNΔIII$_{1-7}$ organelles requires two features, dimeric structure and the N-terminal domain of FN. Because the same two features are necessary for efficient intermolecular associations (as judged by the ability to form extracellular matrix fibrils and intracellular aggregates), we suggest that such intermolecular associations are important in the formation of the FNΔIII$_{1-7}$ organelles. Similarly, intermolecular associations of vonWillebrand factor have been proposed to play a role in the formation of its organelles (Wagner et al., 1991; Voorberg et al., 1993), although it is not clear whether these interactions are covalent (multimerization) or noncovalent as in the case of FNΔIII$_{1-7}$. The correlation between the capacity for assembly into large structures and the formation of transport organelles containing the assembled proteins also holds for collagen, an extracellular fibril-forming protein (Leblond and Wright, 1981). In odontoblasts and osteoblasts fibrillar procollagen is packaged into granule-like organelles before it is secreted into the extracellular medium. Interestingly, these organelles are secreted relatively rapidly following their formation (<4 hours) and their secretion is not regulated (Weinstock and Leblond, 1974).

Aggregation-dependent diversion of nonextracellular matrix secretory proteins from the conventional secretory pathways

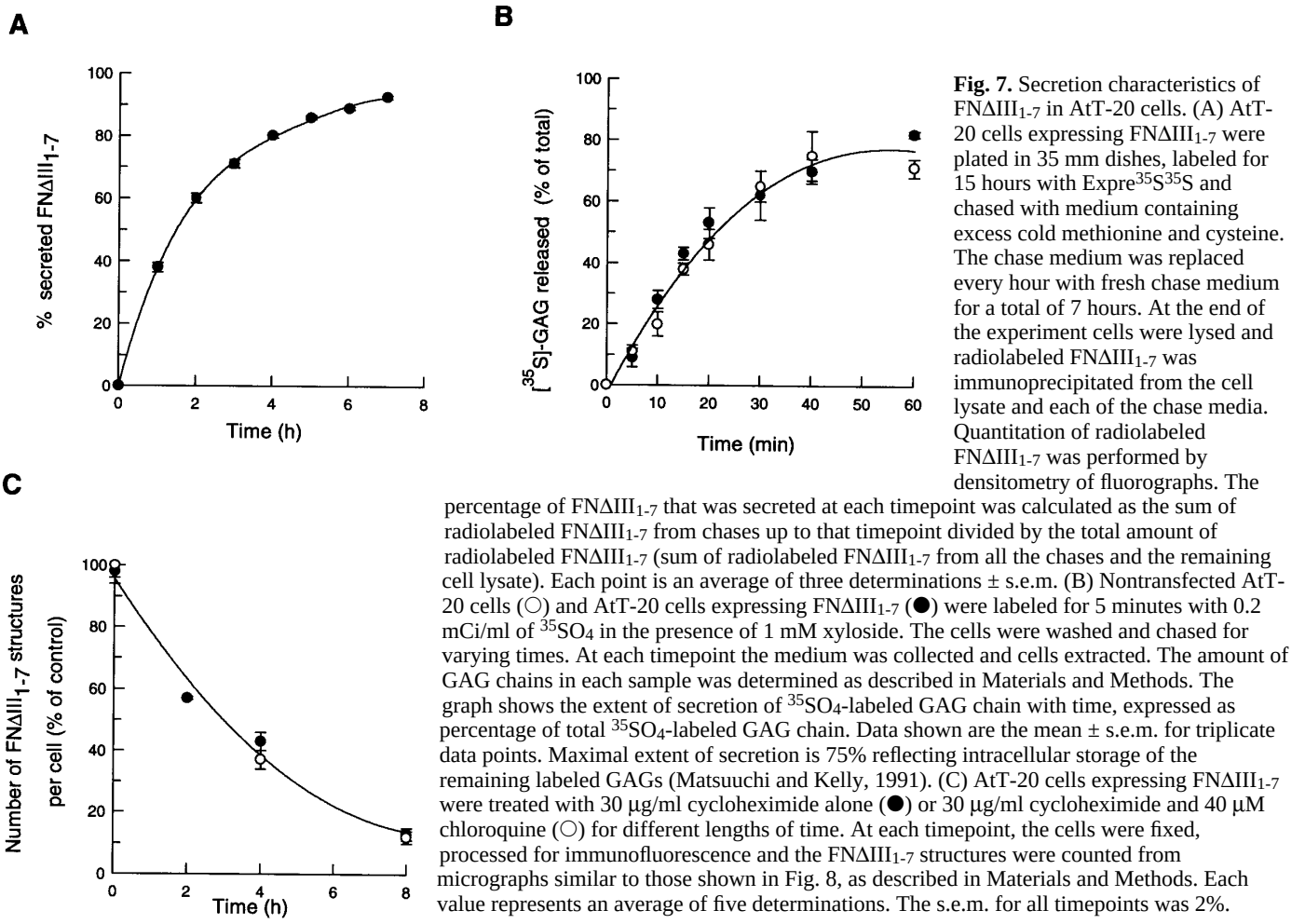

Fig. 7. Secretion characteristics of FNΔIII$_{1-7}$ in AtT-20 cells. (A) AtT-20 cells expressing FNΔIII$_{1-7}$ were plated in 35 mm dishes, labeled for 15 hours with Expre^{35}S^{35}S and chased with medium containing excess cold methionine and cysteine. The chase medium was replaced every hour with fresh chase medium for a total of 7 hours. At the end of the experiment cells were lysed and radiolabeled FNΔIII$_{1-7}$ was immunoprecipitated from the cell lysate and each of the chase media. Quantitation of radiolabeled FNΔIII$_{1-7}$ was performed by densitometry of fluorographs. The percentage of FNΔIII$_{1-7}$ that was secreted at each timepoint was calculated as the sum of radiolabeled FNΔIII$_{1-7}$ from chases up to that timepoint divided by the total amount of radiolabeled FNΔIII$_{1-7}$ (sum of radiolabeled FNΔIII$_{1-7}$ from all the chases and the remaining cell lysate). Each point is an average of three determinations ± s.e.m. (B) Nontransfected AtT-20 cells (○) and AtT-20 cells expressing FNΔIII$_{1-7}$ (●) were labeled for 5 minutes with 0.2 mCi/ml of $^{35}SO_4$ in the presence of 1 mM xyloside. The cells were washed and chased for varying times. At each timepoint the medium was collected and cells extracted. The amount of GAG chains in each sample was determined as described in Materials and Methods. The graph shows the extent of secretion of $^{35}SO_4$-labeled GAG chain with time, expressed as percentage of total $^{35}SO_4$-labeled GAG chain. Data shown are the mean ± s.e.m. for triplicate data points. Maximal extent of secretion is 75% reflecting intracellular storage of the remaining labeled GAGs (Matsuuchi and Kelly, 1991). (C) AtT-20 cells expressing FNΔIII$_{1-7}$ were treated with 30 μg/ml cycloheximide alone (●) or 30 μg/ml cycloheximide and 40 μM chloroquine (○) for different lengths of time. At each timepoint, the cells were fixed, processed for immunofluorescence and the FNΔIII$_{1-7}$ structures were counted from micrographs similar to those shown in Fig. 8, as described in Materials and Methods. Each value represents an average of five determinations. The s.e.m. for all timepoints was 2%.

has been documented for the zymogens in the pancreas (Tooze et al., 1989) and the β-subunit of the thyrotropic hormone (Noda and Farquhar, 1992), both of which form ER-derived granules under certain conditions. In the former case, the transport of zymogens from the ER to the Golgi is inhibited and the zymogens are covalently crosslinked with aberrant disulfide bonds. In the latter case, granules formed in the ER are converted to lysosomal structures with lysosome-like membrane and content. In contrast to the zymogens, conversion of FNΔIII$_{1-7}$ from the Endo H sensitive to the Endo H resistant form was rapid and efficient. Further, the presence of a substantial intracellular Endo H resistant pool, comparable to that found for vonWillebrand factor expressed in AtT-20 cells (Wagner et al., 1991), suggests that FNΔIII$_{1-7}$ accumulates in a post-Golgi compartment. Combined with the lack of colocalization with BiP and no evidence for intermolecular disulfide bond formation, other than dimerization (Anna Castle, unpublished observations), these findings suggest that the FNΔIII$_{1-7}$ structures do not represent ER-derived intracisternal granules or accumulation within the ER/Golgi intermediate compartment. Since the FNΔIII$_{1-7}$ structures contain neither lysosomal membrane proteins nor content, they do not resemble the ER-derived lysosomal pathway described for thyrotrophs. Consistent with this conclusion, inhibition of lysosomal degradation with chloroquine had no effect on the turnover of the FNΔIII$_{1-7}$ structures. Thus the FNΔIII$_{1-7}$ structures most likely correspond to a post-Golgi, non-degradative compartment, similar to procollagen granules and Weibel-Palade bodies.

On the basis of localization, the lack of stimulated release and the turnover time of 3 hours, the FNΔIII$_{1-7}$ structures constitute a compartment distinct from the ACTH storage granules. Although the FNΔIII$_{1-7}$ structures are secreted without regulation, the kinetics of their secretion suggest they are also distinct from the rapidly secreted ($t_{\frac{1}{2}}$ =15 minutes) constitutive vesicles. The overall kinetics of secretion of FNΔIII$_{1-7}$ polypeptides ($t_{\frac{1}{2}}$ = 90 minutes) are more rapid than would be expected if FNΔIII$_{1-7}$ was secreted solely via the FNΔIII$_{1-7}$ structures ($t_{\frac{1}{2}}$=3 hours) but they are slower than would be expected for secretion via the constitutive pathway, for which the rate-determining step is the exit from the ER ($t_{\frac{1}{2}}$=40 minutes for FNΔIII$_{1-7}$). Therefore, FNΔIII$_{1-7}$ is most likely transported by both constitutive vesicles and FNΔIII$_{1-7}$ organelles.

The segregation of FNΔIII$_{1-7}$ into two types of constitutively secreted vesicles suggests that AtT-20 cells have the capacity for multiple secretory pathways. Thus, in addition to sorting between the regulated and constitutive pathways, secretory proteins may also be carried in different types of constitutive vesicles. Rivas and Moore (1989) have suggested that one type of vesicle may be localized to the cell body and the other type to the tips of cellular processes. The structures containing FNΔIII$_{1-7}$ and probably vonWillebrand factor (Wagner et al.,

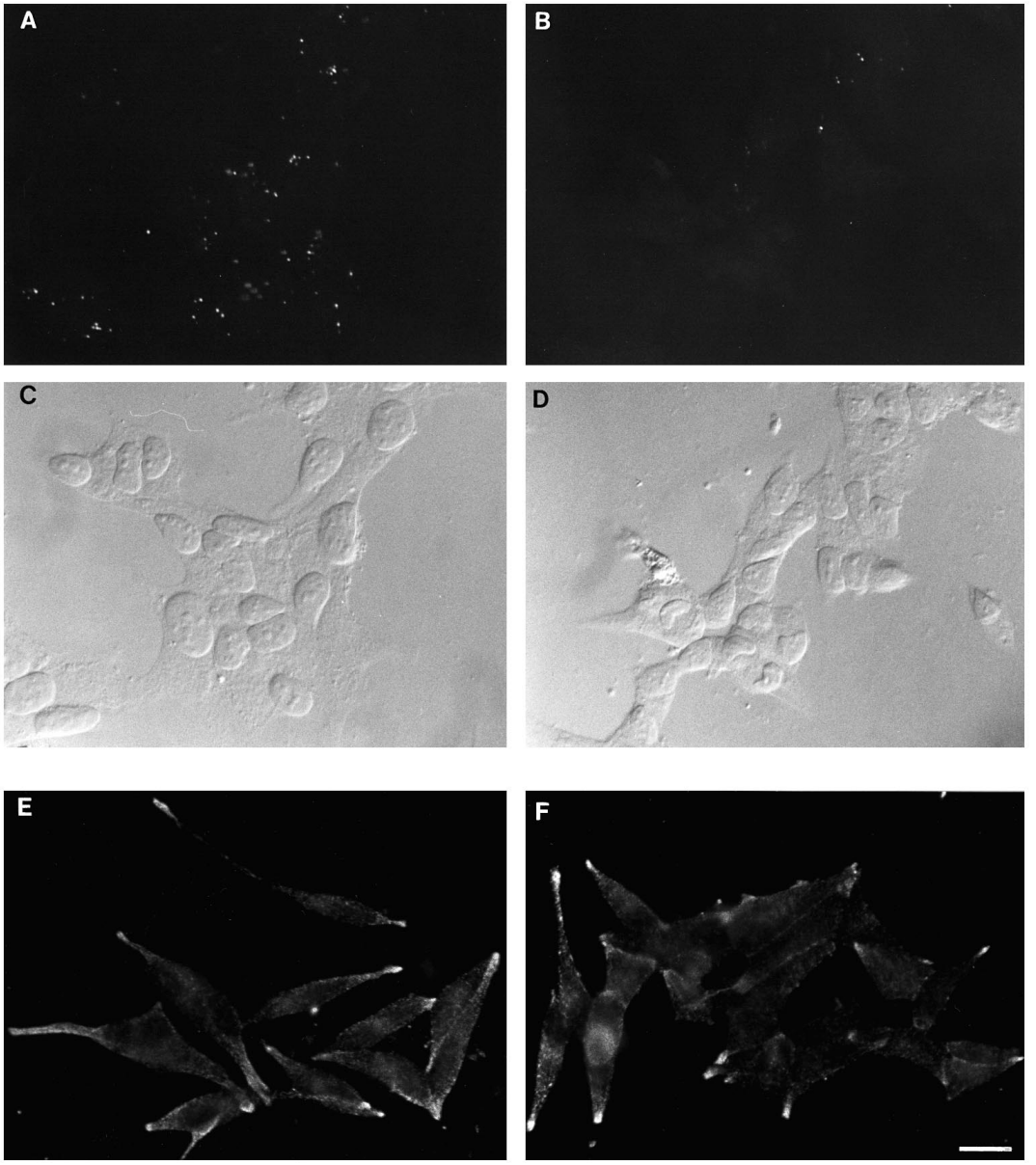

Fig. 8. Effect of cycloheximide on the FNΔIII$_{1-7}$ structures and on ACTH-containing granules. AtT-20 cells expressing FNΔIII$_{1-7}$ were incubated in the absence (A,C,E) or presence (B,D,F) of 30 μg/ml of cycloheximide for 8 hours and processed for immunofluorescence. The cells were stained with an antibody against FN (A,B) or against ACTH (E,F). (C,D) The DIC images of cells corresponding to A,B. Bar, 10 μm.

1991) may represent an additional pathway that segregates proteins that are assembled into very large arrays or aggregates from the bulk of secretory traffic.

Aggregation of recFNs and implications for sorting in the regulated pathway

There is general agreement that selective aggregation among secretory proteins is a key determinant in their packaging in dense-core secretory granules (Arvan and Castle, 1992; Bauerfeind and Huttner, 1993). Regulated proteins may selectively aggregate in the *trans*-Golgi network as a prerequisite to granule formation or protein-protein associations may be responsible for selective retention of regulated proteins following entry into the forming granule. In addition to aggregation, specific targeting information may also play a role in insuring accurate sorting of secretory proteins. Although it is now clear that regulated proteins do not possess a general sorting signal, as was originally proposed (Burgess and Kelly, 1987; Chung et al., 1989), specific domains may participate in receptor-mediated sorting of regulated proteins (Tamm et al., 1993; Arrandale and Dannies, 1994) or recognition of the aggregates in the *trans*-Golgi (Chanat et al., 1994).

Using sedimentation analysis we have shown that intracellular FNΔIII$_{1-7}$ has the ability to undergo highly efficient noncovalent aggregation that is only partially disrupted by higher pH and high ionic strength. Given the propensity of FNΔIII$_{1-7}$ for efficient aggregation under a variety of conditions, even at higher pH levels, it is likely that associations may occur relatively early in the pathway, where the pH is thought to be less acidic. In contrast, the less avid and acidic pH-dependent associations of FN$_M$ΔIII$_{1-7}$ and FN-C110 suggest that these recFN are transported in a relatively disaggregated form but may aggregate in the more acidic environment of the *trans*-Golgi network and the secretory granule. This distinction could be significant in the sorting of recFNs, as formation of aggregates early in the secretory pathway may lead to exclusion from the

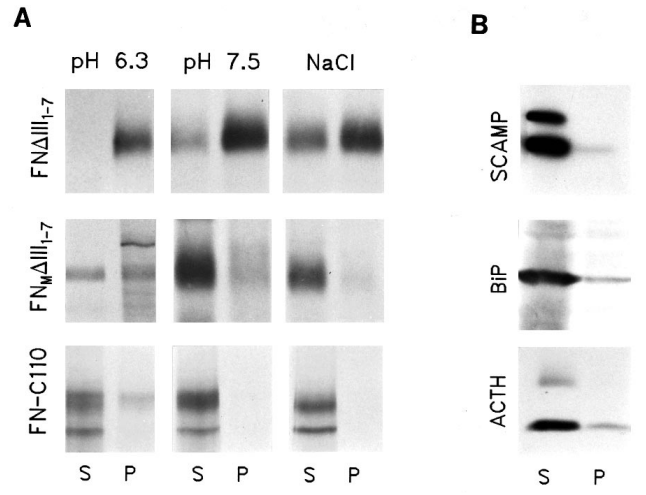

Fig. 9. Sedimentation analysis of intracellular $FN\Delta III_{1-7}$, $FN_M\Delta III_{1-7}$ and FN-C110. (A) AtT-20 cells expressing $FN\Delta III_{1-7}$, $FN_M\Delta III_{1-7}$ and FN-C110 were pulse-labeled for 15 minutes with Tran^{35}S-label and chased for 30 (FN-C110) or 75 minutes ($FN\Delta III_{1-7}$, $FN_M\Delta III_{1-7}$). Cells were solubilized at pH 6.3 or pH 7.5 as described in Materials and Methods, and subjected to centrifugation at 200,000 ***g*** for 30 minutes. To test the effect of ionic strength, cell lysates at pH 6.3 were supplemented with 0.5 M NaCl and then subjected to sedimentation (NaCl lanes). recFNs were immunoprecipitated from equivalent fractions of the supernatants (S) and pellets (P), and subjected to SDS-PAGE and fluorography. In all cases >90% of each recFN was recovered from supernatant and pellet fractions. The sharp band above $FN_M\Delta III_{1-7}$ in the (P) lane at pH 6.3 and the bands below FN-C110 in the (S) lanes result from nonspecific binding to the antibody. (B) Equivalent fractions of the supernatant and pellet samples from (A) were analyzed by western blotting with antibodies against SCAMP and BiP. ACTH in the supernatant and pellet was immunoprecipitated from ^{35}S-labeled samples obtained under (A). Both, the nonglycosylated (lower band) and glycosylated (upper, more diffuse band) are visible in the immunoprecipitates.

storage granules. These observations also imply that the ability to aggregate does not alone guarantee sorting into the dense-core granules. Our results suggest that cells have the capacity to distinguish between different types of aggregates and route them to different pathways.

The routing of $FN\Delta III_{1-7}$ away from the regulated pathway cannot be explained by receptor-mediated sorting, as FN receptors are not expressed by AtT-20 cells (J.E. Schwarzbauer, unpublished observations). It appears that in this case the efficient and pH-independent aggregation of $FN\Delta III_{1-7}$ is responsible for sorting to a different pathway. Significantly, interference with efficient aggregation of recFNs (by removing either one of the two necessary domains) leads to inclusion in the regulated pathway, not merely to constitutive secretion. The entry of recFNs into granules may be mediated by a targeting signal that is obscured by aggregation or they may enter passively without sorting, as is the case for proinsulin (Kuliawat and Arvan, 1994). The actual level of storage of recFNs is modest compared to that of ACTH or insulin in AtT-20 cells, suggesting that other mechanisms, such as specific signals or efficient retention mechanisms, are responsible for the higher level of storage of the neuroendocrine peptides.

In summary, we have demonstrated that recFN has the capacity to enter different pathways of secretion depending on the extent of its intracellular aggregation. Thus the secretory pathways taken by FN in different cell types may be determined by intracellular interactions with itself and other proteins in the secretory pathway, and the extent to which such interactions lead to aggregation.

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