

# Immunoglobulin-derived polypeptides enter the regulated secretory pathway in AtT-20 cells

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**Abstract** Constitutively secreted proteins have traditionally been believed to be excluded from the regulated secretory pathway. In this work we show that kappa light chain and Fc fragment, two markers of the constitutive pathway, are present in the regulated pathway in AtT-20 cells. They colocalize with the endogenous hormone ACTH and they exhibit stimulus-dependent secretion. The Fc fragment, which undergoes intracellular transport at the same rate as the ACTH precursor POMC, enters the forming secretory granules, however, it is partially lost during granule maturation. These observations show that classic constitutive secretory markers are not excluded from the regulated secretory pathway and that efficient sorting for regulated secretion occurs above a background of proteins which enter the granules without sorting.

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**Key words:** Protein sorting; Regulated secretion; Pituitary AtT-20 cell; Immunoglobulin

## 1. Introduction

In cells with a regulated secretory pathway, a subset of secretory proteins is packaged into membrane-bounded secretory granules for discharge by stimulus-dependent exocytosis whereas other secretory proteins are secreted continually by the constitutive pathway. Secretory granules originate from condensing vacuoles that are linked to the trans-Golgi network (TGN); they eventually separate to become immature granules where processing and condensation of the stored secretory products proceeds as the granules mature [1,2]. The budding of immature granules from the TGN is viewed by many investigators as a sorting step for regulated secretory proteins in which interactions between secretory proteins and future granule membranes largely exclude the entry of other itinerant proteins, particularly constitutively secreted proteins. However, in the past several years, it has been realized that immature granules also serve as an extension of the TGN where post-Golgi sorting activities continue. For example, certain exogenous regulated secretory proteins and lysosomal prohydrolases enter the immature granules but then are mostly removed in parallel with completing the packaging of the stored secretory proteins [3,4]. In view of this organization, it would appear that transport of proteins into con-

densing vacuoles and immature granules might not be as restricted to proteins for regulated secretion as originally envisioned.

While the role of immature granules in post-Golgi sorting has gained acceptance [3–8], it has not been clarified whether constitutive secretory proteins enter the immature granules on their way to the cell surface or are truly excluded. Proteins such as immunoglobulin (in particular the kappa light chain derivative), the soluble form of vesicular stomatitis virus glycoprotein (VSV-G), albumin, and heparin sulfate proteoglycan all have been used as markers of constitutive secretion, and it has been assumed that they do not enter the regulated pathway at all [9–12]. Our goal was to examine this issue directly. For this purpose, we selected polypeptides that are derivatives of immunoglobulin – the Fc fragment and the kappa light chain (KLC) of mouse immunoglobulin G. In contrast to truncated VSV-G [13] and heparin sulfate proteoglycan [10], the Fc fragment and KLC are not known to bind to membranes during intracellular transport. Therefore, according to the traditional thinking that constitutive secretory proteins lack sorting interactions and are transported by default, we thought that the immunoglobulin derivatives would be advantageous for avoiding interactions that potentially might influence their trafficking.

We have expressed cDNAs encoding the Fc fragment and KLC in the pituitary cell line AtT-20 which uses the regulated secretory pathway to export adrenocorticotrophic hormone (ACTH), endorphin and related polypeptides. Employing confocal immunofluorescence microscopy to examine steady-state distribution and biosynthetic labeling to follow stimulus-dependent secretion, we show that both immunoglobulin-derived polypeptides are readily detected in the regulated secretory pathway. These results demonstrate that sorting for regulated secretion is not an all or none decision and that there is a background of other types proteins in the regulated pathway.

## 2. Materials and methods

### 2.1. Antibodies, expression vectors and transfected cell lines

The antiserum JH93 against the NH<sub>2</sub>-terminus of ACTH was a kind gift of Drs. R. Mains and B. Eipper (Johns Hopkins University). The antiserum against the KLC of mouse immunoglobulin G was obtained from ICN Immunochemicals, Costa Mesa, CA. Genomic DNA encoding the KLC of mouse immunoglobulin G cloned into an expression vector has been used previously [9] and was the kind gift of Dr. Linda Matsuchi. A cDNA encoding the Fc domain of mouse immunoglobulin (a kind gift of Dr. Tim Reudelhuber) was cloned into the pRhR1100 vector [14]. AtT-20 cell lines from mouse pituitary were cultured and transfected as described previously [15] and stable cell lines were developed. The levels of expression of the Fc fragment and KLC were ≤10% of the level of expression of ACTH-related peptides.

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**Abbreviations:** POMC, pro-opiomelanocortin; ACTH, adrenocorticotrophic hormone; KLC, kappa light chain; TGN, trans-Golgi network; VSV-G, vesicular stomatitis virus G

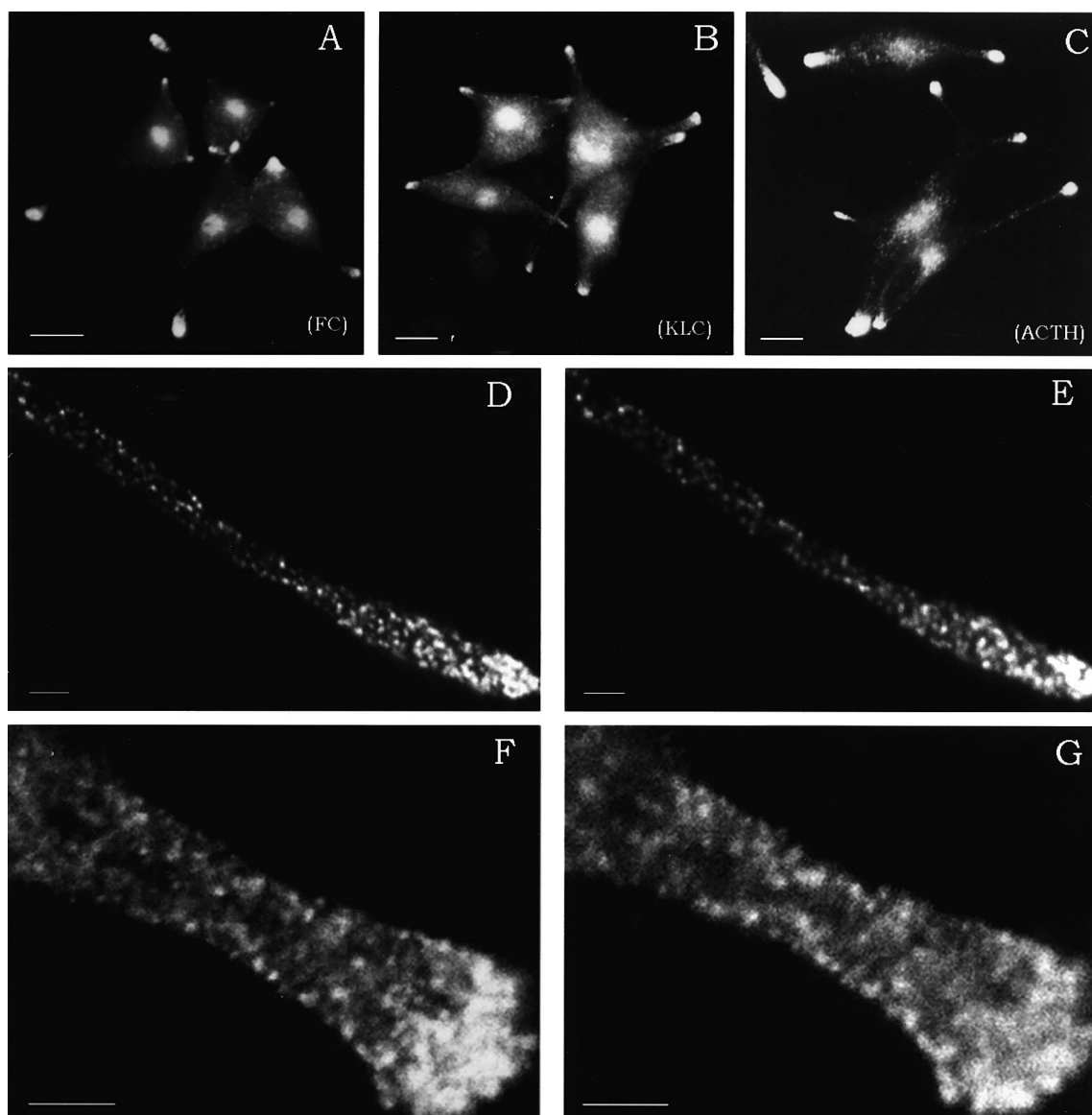


Fig. 1. Immunocytochemical localization of Fc fragment, KLC and ACTH in AtT-20 cells. AtT-20 cells expressing the Fc fragment (A) or KLC (B,C) were stained with anti-mouse IgG (A,B) or anti-ACTH antibody (C) as described in Section 2 and the localization was viewed by epifluorescence. Bar = 10  $\mu$ m. Cells expressing the Fc fragment (D,E) or KLC (F,G) were double labeled with anti-mouse IgG (D,F) or anti-ACTH antibody (E,G) and the fluorescence analyzed by confocal microscopy. A single optical section is presented in each panel. Bar = 2.5  $\mu$ m.

## 2.2. Metabolic labeling, stimulated secretion and quantitation of radiolabeled polypeptides

AtT-20 cells expressing the Fc fragment or KLC or mock transfected cells were plated in 24-well plates with  $1 \times 10^5$  cells per well. Cells were radiolabeled with 0.15–0.25 mCi/ml of Expre<sup>35</sup>S<sup>35</sup>S labeling mix (New England Nuclear, Boston, MA) as indicated for individual experiments and chased with DME medium containing excess cold methionine and cysteine. Medium containing secreted proteins was collected, and cells were lysed at the end of incubation in the presence of protease inhibitors as already described [3]. Radiolabeled polypeptides were immunoadsorbed from the media and from cell lysates (solubilized in radioimmune precipitation buffer [15]) and were subjected to SDS-PAGE. Radioactively labeled pro-opiomelanocortin (POMC) and its derivatives were assessed by scintillation counting of eluted gel slices or by phosphorimager analysis (ImageQuant software, Molecular Dynamics, Inc., Sunnyvale, CA) of Tricine gels [3]. Radioactively labeled Fc fragment and KLC were determined by phosphorimager analysis of gel bands [3].

## 2.3. Immunofluorescence microscopy

For immunofluorescence microscopy, transfected cells were plated and cultured on coverslips [16]. Samples were fixed 1 h at room temperature in 3% formaldehyde in 0.12 M sodium phosphate, pH 7.4, washed in PBS and permeabilized and quenched 20 min in 0.2% Triton X-100, 1 M glycine in PBS. Following blocking for 30 min in 16% goat serum in PBS, the cells were stained 1–3 h at room temperature (or overnight at 4°C) with anti-mouse IgG (Fc fragment specific for the detection of Fc fragment) diluted in the blocking solution. Subsequently, they were washed three times with PBS and stained 1 h at room temperature with Cy3-conjugated secondary antibody. In double labeling experiments the cells were subsequently incubated with biotinylated anti-ACTH antibody followed by Cy5-conjugated avidin. After washing three times in PBS, the specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA) for viewing by fluorescence. Confocal microscopy was performed with a Zeiss LSM 410 microscope (Carl Zeiss, Inc., Thornwood, NY), and images were collected using the appropriate filters, a laser attenuation in the range of 3–30 and a scan time of  $\leq 2$  s.

### 3. Results

#### 3.1. Detection of Fc fragment and KLC in secretory compartments marked by ACTH using double label immunofluorescence

Using stable cell lines transfected with cDNAs for the Fc fragment and KLC, we employed conventional and confocal immunofluorescence microscopy to compare the distributions of the Fc fragment and KLC with that of ACTH. The images obtained are shown in Fig. 1. Low power images (Fig. 1A–C) show accumulation of the exogenous proteins and endogenous ACTH at the tips of cellular processes and in the perinuclear (Golgi) region within the cell bodies. In the processes that emanate from the cell bodies the extent of overlap of each exogenous protein and ACTH is impressive (Fig. 1D–G). This overlap is particularly apparent for the Fc fragment (Fig. 1D,E). Although at several points in the images, the relative intensities of staining of the Fc fragment and KLC differ from that of ACTH, the general impression from the corresponding patterns is that both the Fc fragment and KLC accumulate within compartments that are storage sites for ACTH. Thus both exogenous secretory proteins mark the regulated secretory pathway.

#### 3.2. Stimulation of secretion of Fc fragment and KLC

To address the presence of the transfected proteins in the regulated pathway biochemically, we measured stimulus-dependent secretion following a long-term biosynthetic labeling. Using this approach we found that secretion of both exogenous proteins was stimulated with 8Br-cAMP confirming their presence in the granule pool (Fig. 2A,B). Quantitation of the bands shown in Fig. 2 indicated that the secretion of Fc was stimulated 3-fold, corresponding to a net release of  $15 \pm 3\%$  ( $n=5$ ) of total radiolabeled Fc (Fig. 2A). The stimulation of secretion of KLC was lower, yet reproducible, with a fold stimulation of 1.5 and a net stimulated release of  $5 \pm 1\%$  ( $n=3$ ) of total radiolabeled KLC (Fig. 2B). The stimulated release of the endogenous granule marker, ACTH, under the same experimental conditions was 35% for the clone expressing the Fc fragment and 44% for the clone expressing KLC (data not shown).

#### 3.3. Analysis of secretory sorting of the Fc fragment

As judged by the percent stimulation of secretion, the Fc fragment and KLC accumulate in the granules to a much lower extent than the endogenous hormone ACTH. We

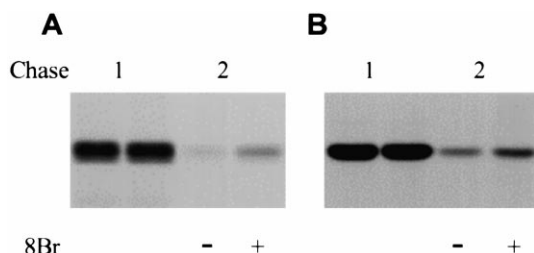


Fig. 2. Stimulation of secretion of Fc fragment and KLC. AtT-20 cells expressing the Fc fragment and KLC were labeled with 0.15 mCi/ml of Expre<sup>35</sup>S<sup>35</sup>S labeling mix for 15 h. The cells were first chased for 5 h with DMEM in the absence of secretagogue (chase 1) and then in the absence (–) or the presence (+) of 5 mM 8-Br-cAMP (chase 2) for 1 h. Immunoprecipitated Fc fragment (A) and KLC (B) from the chases are shown.

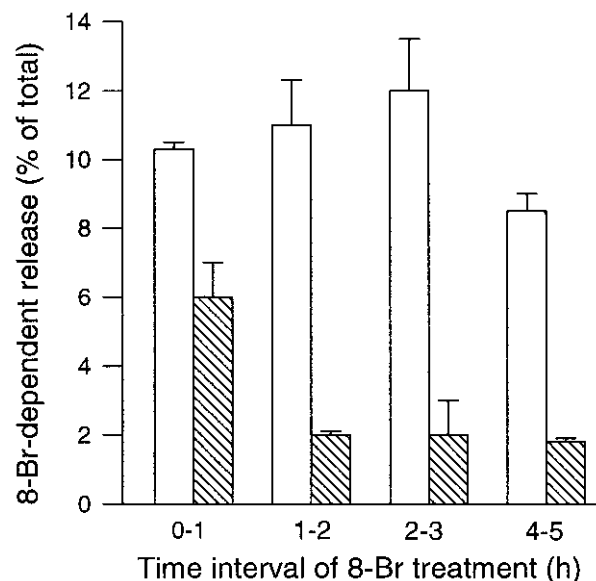


Fig. 3. Time course of stimulation of Fc fragment and of ACTH. Duplicate wells of cells expressing the Fc fragment were pulse-labeled for 15 min with 0.25 mCi/ml of Expre<sup>35</sup>S<sup>35</sup>S labeling mix and chased for 0, 1, 2 or 4 h. Subsequently the medium was replaced with fresh medium in the absence (basal) or the presence (stimulated) of 5 mM 8Br-cAMP (8-Br) and the cells were incubated for an additional 1 h. Following immunoprecipitation and quantitation of radiolabeled polypeptides, 8-Br-cAMP-dependent release was calculated as the amount of radiolabeled protein in stimulated secretion minus the amount of radiolabeled protein in basal secretion, expressed as percent of total radiolabeled protein. For ACTH, the values represent the radioactivity present in mature ACTH peptides (ACTH, glycosylated ACTH and  $\alpha$ -melanotropin-sized material) expressed as percent of total POMC-related peptides (each corrected for the number of methionines). ACTH, open bars; Fc fragment, hashed bars. Error bars correspond to S.E.M.

were curious whether the lower storage resulted from their less efficient entry into granules or their less efficient retention in granules during maturation (as compared to ACTH).

Initially we evaluated the endoplasmic reticulum (ER) exit rates for the Fc fragment and KLC because efficient ER exit is a prerequisite for simple analysis of transport through the post-Golgi compartments. The ER exit rates were estimated by monitoring the rate of unstimulated secretion [15]. We measured a half-time of unstimulated secretion of 30 min for Fc fragment which is very similar to the half-time of ER exit that has been reported previously for POMC in AtT-20 cells [17]. In contrast, the half-time of unstimulated secretion of KLC was 4 h, indicating a slow rate of exit from the ER. Thus only the Fc fragment was used in subsequent studies of secretory sorting.

To examine the relative efficiency of entry and retention of exogenous polypeptides, we used the approach where pulse radiolabeled cells were tested for stimulated secretion following short (1 h) and long (5 h) chase incubations [3]. As a first approximation, stimulated secretion at the early chase time corresponds to release from immature granules, whereas stimulated secretion at the late chase time corresponds to release from more mature granules. The data presented in Fig. 3 show that the percent stimulation of Fc fragment is lower than that of ACTH at all time points examined. In addition, the percent stimulation of Fc fragment decreases with increasing chase incubations whereas the percent stimu-

lation of ACTH remains relatively constant suggesting that the Fc fragment is poorly retained in the granules relative to ACTH.

#### 4. Discussion

Over the past few years, evidence has grown that the immature granule contains several types of proteins other than those identified with regulated secretion. This situation has been difficult to reconcile with the more classical view that entry into the regulated secretory pathway requires a sorting signal or the ability to interact with a protein having a sorting signal [1,10,18,19]. A major factor in sustaining the classical view is the notion that constitutively secreted proteins lack sorting signals and therefore cannot enter the regulated secretory pathway. Our results show that two polypeptides, KLC and the Fc fragment of mouse immunoglobulin, that mainly undergo constitutive secretion are included in the regulated secretory pathway.

The finding that KLC has access to the secretory granules in AtT-20 cells contrasts somewhat with a previous study which reported lack of stimulus-dependent secretion but found accumulation of KLC in the tips of cellular processes of AtT-20 cells [9]. Our results clearly show a small but reproducible stimulation of secretion of KLC. The low stimulation of secretion over the high background of unstimulated secretion (due to slow drainage from the ER) might explain why a negative conclusion regarding the presence of KLC in the granules was reached before. An additional strong argument for the presence of KLC in the granules is the demonstration by confocal analysis of coincidental immunolabeling of KLC and the granule marker ACTH (Fig. 1F,G).

The realizations that immunoglobulin polypeptides have access to the regulated pathway but are stored with lower efficiency than the endogenous ACTH lead to a number of implications regarding post-Golgi sorting of secretory proteins. The most evident implication is that proteins without sorting signals may enter forming granules to the extent that there is accessible volume. Accessible volume may reflect the relative rates of several parallel processes including the condensation of proteins for regulated secretion, the separation of condensing vacuoles from the TGN and the formation of other TGN-derived pathways. Already, it has been shown quite clearly that lysosomal prohydrolases have access to the granule pool [4,7]. In view of our results on KLC and the Fc fragment, we suggest that previous reports of regulated secretion of hepatic apolipoprotein A1 [20], IGF-1 [13], parathyroid hormone-related protein [21], certain forms of fibronectin [22] and gp80-clusterin [23] do not reflect the presence of a sorting signal but merely inclusion in the accessible volume of the regulated pathway.

Second, the low levels of storage of immunoglobulin polypeptides relative to endogenous ACTH suggest that efficient storage of regulated proteins requires a mechanism for enhancing their entry into and/or their retention within granules above the background of unsorted proteins. The possible enhancement mechanisms could include interactions with sorting receptors as has been postulated periodically [1,18,24,25] or selective aggregation of regulated secretory proteins during granule formation and maturation [26–28]. It is clear that the ACTH peptides are more efficiently retained within the granules (Fig. 3) than the immunoglobulin polypeptides suggesting

that a retention mechanism is operating at this level. However, further experimentation will be required to determine whether the ACTH precursor has enhanced access to the granule or whether the lower storage of immunoglobulin polypeptides is simply a consequence of their efficient removal from the granule pool. Since the immunoglobulin polypeptides appear to enter the granules without sorting, the observed difference in the degree of storage of Fc fragment and KLC is likely to reflect differing contributions of two opposing processes: removal from granules and propensity to associate with granule content proteins.

Finally, the finding that the Fc fragment and KLC are present within the regulated secretory pathway implies that proteins that are truly excluded from the pathway may be subject to interactions that 'sort' them for other destinations. Indeed, since the truncated VSV-G protein and heparin sulfate proteoglycan are both known to interact with membranes [9,13] they may be precluded from simply accessing the available volume in different branches of post-Golgi transport. In conclusion, it is now clear that constitutively secreted proteins are able to travel along the regulated pathway unless specifically restricted from doing so.

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#### References

- [1] Kelly, R.B. (1985) *Science* 230, 25–32.
- [2] Thiele, C., Gerdes, H.H. and Huttner, W.B. (1997) *Curr. Biol.* 7, R496–R500.
- [3] Castle, A.M., Huang, A.Y. and Castle, J.D. (1997) *J. Cell Biol.* 138, 45–54.
- [4] Kuliawat, R. and Arvan, P. (1994) *J. Cell Biol.* 126, 77–86.
- [5] Fernandez, C.J., Haugwitz, M., Eaton, B. and Moore, H.H. (1997) *Mol. Biol. Cell* 8, 2171–2185.
- [6] Klumpermann, J., Kuliawat, R., Griffith, J.M., Geuze, H.J. and Arvan, P. (1998) *J. Cell Biol.* 141, 359–371.
- [7] Kuliawat, R., Klumpermann, J., Ludwig, T. and Arvan, P. (1997) *J. Cell Biol.* 137, 595–608.
- [8] Dittie, A., Thomas, L., Thomas, G. and Tooze, S. (1997) *EMBO J.* 16, 4859–4870.
- [9] Matsuuchi, L., Buckley, K.M., Lowe, A.W. and Kelly, R.B. (1988) *J. Cell Biol.* 106, 239–252.
- [10] Tooze, S.A., Flatmark, T., Tooze, J. and Huttner, W.B. (1991) *J. Cell Biol.* 115, 1491–1503.
- [11] Rosa, P., Weiss, U., Pepperkok, R., Ansorge, W., Niehrs, C., Stelzer, E.H.K. and Huttner, W.B. (1989) *J. Cell Biol.* 109, 17–34.
- [12] Mitra, A., Song, L. and Fricker, D.L. (1994) *J. Biol. Chem.* 269, 19876–19881.
- [13] Schmidt, W.K. and Moore, H.-P.H. (1994) *J. Biol. Chem.* 269, 27115–27124.
- [14] Stahl, L.E., Wright, R.L., Castle, J.D. and Castle, A.M. (1996) *J. Cell Sci.* 109, 1637–1645.
- [15] Castle, A.M., Stahl, L.E. and Castle, J.D. (1992) *J. Biol. Chem.* 267, 13093–13100.
- [16] Castle, A.M. and Castle, J.D. (1993) *J. Biol. Chem.* 268, 20490–20496.
- [17] Moore, H.-P.H. and Kelly, R.B. (1985) *J. Cell Biol.* 101, 1773–1781.
- [18] Cool, D.R., Normant, E., Shen, F., Chen, H.-C., Pannel, L., Zhang, Y. and Loh, Y.P. (1997) *Cell* 88, 73–83.

- [19] Kromer, A., Glombik, M.M., Huttner, W.B. and Gerdes, H.H. (1998) *J. Cell Biol.* 140, 1331–1346.
- [20] Fennewald, S.M., Hamilton, R.L. and Gordon, J.I. (1988) *J. Biol. Chem.* 263, 15568–15577.
- [21] Plawner, L.L., Philbrick, W.M., Burtis, W.J., Broadus, A.E. and Stewar, A.F. (1995) *J. Biol. Chem.* 270, 14078–14084.
- [22] Castle, A.M., Schwarzbauer, J.E., Wright, R.L. and Castle, J.D. (1995) *J. Cell Sci.* 108, 3827–3837.
- [23] Appel, D., Pilarsky, R., Graichen, R. and Koch-Brandt, C. (1996) *Eur. J. Cell Biol.*, 142–149.
- [24] Chung, K.-N., Walter, P., Aponte, G.W. and Moore, H.-P.H. (1989) *Science* 243, 192–197.
- [25] Arrandale, J.M. and Dannies, P.S. (1994) *Mol. Endocrinol.* 8, 1083–1090.
- [26] Colomer, V., Kicska, G.A. and Rindler, M.J. (1996) *J. Biol. Chem.* 271, 48–55.
- [27] Leblond, F.A., Viau, G., Laine, J. and Lebel, D. (1993) *Biochem. J.* 291, 289–296.
- [28] Chanat, E. and Huttner, W.B. (1991) *J. Cell Biol.* 115, 1505–1519.