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Expression of the Receptor Tyrosine Kinase Ret on the Plasma Membrane Is Dependent on Calcium*

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Mutations in the Ret receptor tyrosine kinase are responsible for a variety of human syndromes, including multiple endocrine neoplasia 2 and Hirschsprung's disease. Ret is expressed as a 150-kDa precursor form in the endoplasmic reticulum and a 170-kDa mature form at the plasma membrane. Here we show that expression of p170*ret* is dependent on calcium. Depletion of extracellular calcium completely blocks p170*ret* expression, which is not caused by a decrease in half-life of p170*ret* at the plasma membrane but by a defect in processing of p150*ret* into p170*ret*. This processing defect can be mimicked by treating the cells with thapsigargin, a drug that releases calcium from internal stores, indicating that reduction in luminal calcium is responsible for the processing defect. We propose that a relatively high concentration of luminal calcium is necessary for the proper folding of Ret in the endoplasmic reticulum.

Ret is a receptor tyrosine kinase expressed mainly in neural crest-derived cells. In most cell types Ret proteins are expressed as glycoproteins of 150 and 170 kDa. The p150*ret* and p170*ret* proteins differ in their subcellular localization. Cell fractionation experiments showed that both isoforms are present in membrane fractions (1). However, the 170-kDa isoform of Ret is present at the plasma membrane, because biotinylation of intact Ret-expressing cells results in biotinylation of only the 170-kDa isoform of Ret. The p150*ret* isoform is endoglycosidase H-sensitive, suggesting that p150*ret* is an incompletely processed form of Ret, present in the endoplasmic reticulum (2–4). Both forms of Ret are derived from a single polypeptide chain of approximately 120 kDa, as was shown by treatment of Ret-expressing cells with tunicamycin, an inhibitor of post-translational *N*-linked glycosylation (4).

Ret is part of a multi-component receptor for the transforming growth factor β-related neurotrophic factors glial cell line-derived neurotrophic factor (GDNF)[1] and neurturin. GDNF and neurturin do not bind to or activate Ret directly but only via the glycosylphosphatidylinositol-linked adapter molecules GDNF receptor α (also called TrnR1 and RETL1) and neurturin receptor α (also called (TrnR2 and RETL2), respectively (5–10). Stimulation of Ret-expressing cells by GDNF or neurturin results in activation of Ret tyrosine kinase activity (5–10) and Ret signal transduction (11, 12).

Mutations in the *ret* gene have been linked to several human syndromes, including multiple endocrine neoplasia 2A and 2B, familial medullary thyroid carcinoma, and Hirschsprung's disease (13). Hirschsprung's disease is a congenital disorder of the autonomic innervation of the gut (14). Mutational analysis has shown that specific point mutations in the intracellular domain of Ret result in impaired tyrosine kinase activity. Mutations in the extracellular domain of Ret result in dramatically reduced levels of p170*ret* expression at the plasma membrane (15, 16). Reduced levels of expression of functional Ret during embryonic development underlie the defects observed in Hirschsprung's disease patients, because in mice that completely lack Ret expression similar yet more severe symptoms are observed (17, 18).

In the extracellular domain of Ret, a cadherin-like domain of approximately 110 amino acids is present (19). In cadherins, this domain is repeated three or four times and mediates calcium binding and calcium-dependent homophilic interactions with cadherin molecules on other cells (20). Also in the cadherin-like domain in Ret, consensus calcium-binding sequences are present. Asai *et al.* (3) demonstrated that a point mutation in one of these sequences results in a dramatic reduction of p170*ret* expression at the plasma membrane, whereas p150*ret* expression is not affected. They hypothesized that mutation of the putative calcium-binding site might interfere with processing of Ret in the Golgi complex or with Ret transport to the plasma membrane. Alternatively, it is possible that this mutant of Ret is fully processed and transported to the plasma membrane but cannot be retained at the membrane in the absence of calcium binding.

We have investigated the role of calcium in cell surface expression of Ret. We found that calcium depletion completely abolishes cell surface expression of p170*ret*. Furthermore, we show by pulse-chase experiments that processing of p150*ret* into p170*ret* is strongly impaired under conditions of calcium depletion. Finally, we provide evidence that this impaired processing is due to improper processing of Ret in the endoplasmic reticulum (ER).

MATERIALS AND METHODS

Cell Growth Conditions—The SKP2 cell line expressing Ret proteins has been described previously (11). Cells were grown in DF12 medium supplemented with 10% fetal calf serum and antibiotics. For culture under calcium-free conditions, calcium-free Dulbecco's modified Eagle's medium supplemented with 10% Chelex-treated (calcium-free) fetal calf serum was used. Cells were washed two times with calcium-free phosphate-buffered saline (PBS) prior to addition of calcium-free medium for culture under calcium-free conditions. For restoring physio-

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[1] The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ERKZ, extracellular signal-regulated kinase z; HERRet, human epidermal growth factor receptor-ret chimaeric receptor.

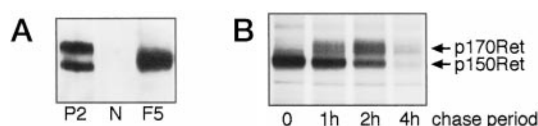

FIG. 1. **Expression of Ret and HERRet proteins in SK-N-MC-derived cell lines.** *A*, Ret is expressed as 150- and 170-kDa isoforms in SKP2 cells. Parental SK-N-MC cells (*N*), Ret-expressing SKP2 cells (*P2*), and HERRet-expressing SKF5 cells (*F5*) were lysed followed by immunoprecipitation with an anti-Ret antibody. Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting. Shown is the presence of p150^{ret} and p170^{ret} in SKP2 cells and p155HERRet in SKF5 cells but absence of Ret proteins from SK-N-MC cells. *B*, p150^{ret} is a precursor of p170^{ret}. Subconfluent cultures of SKP2 cells were labeled for 45 min with [^{35}S]methionine (pulse) followed by replacement of the pulse medium with medium containing cold methionine (chase). After different periods of time (as indicated), cells were lysed, and Ret proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The positions of p150^{ret} and p170^{ret} are indicated.

logical levels of extracellular calcium (1.8 mM), a stock solution of 1 M $CaCl_2$ was used. Thapsigargin was used at a concentration of 300 nM, and brefeldin A was used at a concentration of 5μg/ml. For the analysis of Ret expression, cells were lysed, and Ret proteins were immunoprecipitated and analyzed as described previously (24). ERK2 activation was measured by the mobility shift assay as described (24). This assay measures the appearance of a slower migrating form of active phospho-ERK by Western analysis.

[^{35}S]Methionine Labeling—Subconfluent SKP2 cultures were incubated for 45 min or 3 h in methionine-free medium containing 200 μCi of [^{35}S]methionine per dish. Cells were washed with PBS and incubated for different periods of time with unlabeled methionine-containing medium with or without calcium. Cells were lysed in RIPA buffer, and Ret proteins were immunoprecipitated as described previously (24). Immunoprecipitated proteins were separated by SDS-PAGE, and radioactive proteins were visualized by autoradiography.

Biotinylation of Ret Proteins—Subconfluent cultures of SKP2 cells were rinsed with PBS, followed by incubation with 0.5 mg/ml sulfo-NHS-biotin (Pierce) in calcium-containing PBS for 30 min at room temperature. Subsequently, cells were rinsed with PBS and incubated in calcium-containing or calcium-free culture medium at 37 °C. After different time periods, cells were lysed and Ret proteins were immunoprecipitated and analyzed by SDS-PAGE and Western blotting as described previously (24). For detection of biotinylated proteins streptavidin-conjugated horseradish peroxidase was used (Pierce).

RESULTS

p150^{ret} Is a Precursor of p170^{ret}—To study Ret processing and the role of calcium in Ret expression, we used SKP2 cells. The SKP2 cell line is derived from the human neuroepithelioma cell line SK-N-MC by stable transfection with a full-length Ret expression plasmid (11). In these cells, the 150- and 170-kDa isoforms of Ret are roughly equally expressed (Fig. 1*A* and Ref. 11). A precursor-product relationship between p150^{ret} and p170^{ret} was proposed previously (2–4) but has never been demonstrated. To substantiate the proposed precursor-product relation and to determine the kinetics of this process, we pulse-labeled SKP2 cells for 45 min with [^{35}S]methionine, followed by a chase for different time periods. Cells were lysed, and Ret proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1*B*, the 45-min pulse resulted in labeling of only the 150-kDa isoform of Ret. With time, p150^{ret} was converted into p170^{ret} as judged from the decrease in labeled p150^{ret} and the increase of labeled p170^{ret}. From this result we conclude that indeed p150^{ret} is a precursor of p170^{ret}. The estimated half-life of p150^{ret} is about 1 h, which is similar to the estimated half-life of p170^{ret}, explaining the equal expression of the two products in steady state (Fig. 1*A*).

p170^{ret} Expression Is Dependent on Extracellular Calcium—Mutation of a critical amino acid in the calcium-binding domain of Ret drastically reduces p170^{ret} expression (3), suggesting a role for calcium in plasma membrane expression of p170^{ret}. To investigate the role of calcium on Ret expression, we cultured SKP2 cells in the presence and absence of extracellular calcium. In the presence of extracellular calcium, the 150- and 170-kDa isoforms of Ret were expressed (Fig. 2*A*). When extracellular calcium was removed by replacement of the culture medium with calcium-free medium, p170^{ret} expression gradually decreased. After 4 hours of incubation in the absence of extracellular calcium, hardly any p170^{ret} expression could be detected. Under these conditions an increase in p150^{ret} expression was observed (Fig. 2*A*). When, after 16 h of growth in calcium-free medium, physiological levels of calcium were restored in the medium, p170^{ret} was re-expressed within 80 min (Fig. 2*B*). These results show that extracellular calcium is necessary for expression of p170^{ret} proteins in SKP2 cells.

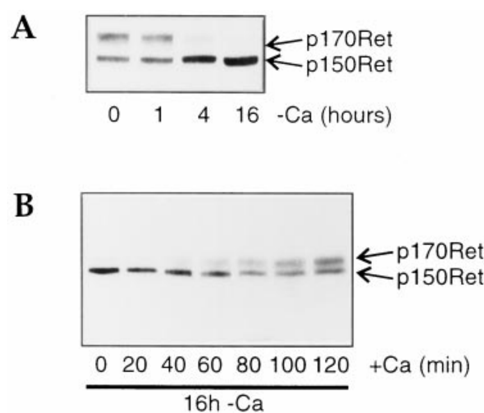

FIG. 2. **p170^{ret} expression is dependent on calcium.** *A*, p170^{ret} expression is down-regulated in the absence of extracellular calcium. Subconfluent cultures of SKP2 cells were washed twice with calcium-free PBS and incubated in calcium-free medium. After the indicated time periods cells were lysed, and Ret proteins were immunoprecipitated and analyzed by Western blotting. The positions of the 150- and 170-kDa isoforms of Ret are indicated. *B*, p170^{ret} is re-expressed after restoration of physiological levels of extracellular calcium. Subconfluent cultures of SKP2 cells were washed twice with calcium-free PBS and incubated in calcium-free medium for 16 h. Extracellular calcium levels were restored to physiological levels (1.8 mM), and the cells were incubated under these conditions for the indicated time periods. Cells were then lysed, and Ret proteins were immunoprecipitated and analyzed by Western blotting. The positions of p150^{ret} and p170^{ret} are indicated.

Processing of p150^{ret} Is Dependent on Calcium—A simple explanation for the lack of p170^{ret} expression in the absence of extracellular calcium is that binding of calcium to p170^{ret} is necessary for the stability of p170^{ret} at the cell surface. To address this possibility we have tested the effect of extracellular calcium on the half-life of p170^{ret} at the plasma membrane. To that end, plasma membrane proteins of SKP2 cells were labeled with biotin, using the membrane-impermeable reagent sulfo-NHS-biotin. After biotinylation, cells were incubated for different periods of time in the presence or absence of extracellular calcium. Cells were lysed and (biotinylated) Ret proteins were immunoprecipitated with an anti-Ret antiserum and visualized by Western blotting using enzyme-conjugated streptavidin. As shown in Fig. 3*A* (*top panel*) the half-life of p170^{ret} at the plasma membrane in the presence of calcium is approximately 1 h, in agreement with the measured half-life of p170^{ret} in the pulse-chase experiment presented in Fig. 1*B*. However, the same half-life for Ret was found when the cells were cultured in calcium-free medium. As a control we reprobed the blot with anti-Ret antiserum. As expected, new (nonbiotinylated) p170^{ret} is formed in the presence of calcium, whereas in the absence of calcium the decrease in biotinylated p170^{ret} is not replaced by newly synthesized Ret (Fig. 3*A*, *bottom panel*). Thus, the decreased cell surface expression of p170^{ret} in the

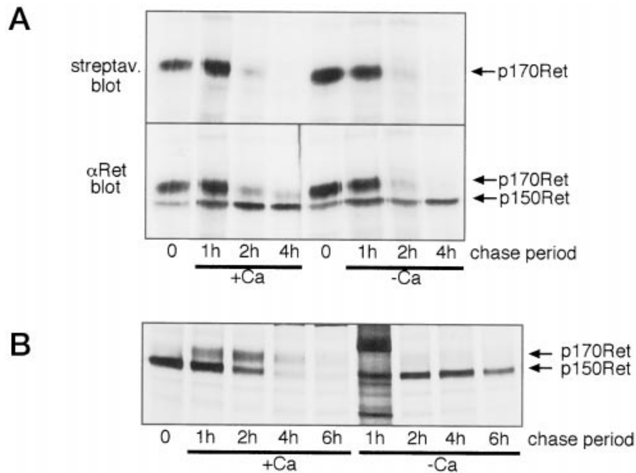

FIG. 3. **p150^{ret} processing but not p170^{ret} half-life is calcium-dependent.** *A*, p170^{ret} half-life is not decreased in the absence of extracellular calcium. Cell surface proteins of SKP2 cells were biotinylated, and cells were incubated for the indicated time periods in the presence (*+Ca*) or absence (*−Ca*) of extracellular calcium, as indicated. Ret proteins were immunoprecipitated and analyzed by SDS-PAGE and Western blotting using enzyme-conjugated streptavidin (*streptav. blot*, *upper panel*) and reprobed using an anti-Ret antibody (*αRet blot*, *lower panel*). The positions of p150^{ret} and p170^{ret} are indicated. *B*, Absence of extracellular calcium results in a block in p150^{ret} processing. Subconfluent cultures of SKP2 cells were labeled for 45 min with [^{35}S]methionine followed by replacement of the medium with medium containing cold methionine, in the presence (*+Ca*) or absence (*−Ca*) of calcium. After indicated periods of time cells were lysed, and Ret proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The positions of p150^{ret} and p170^{ret} are indicated.

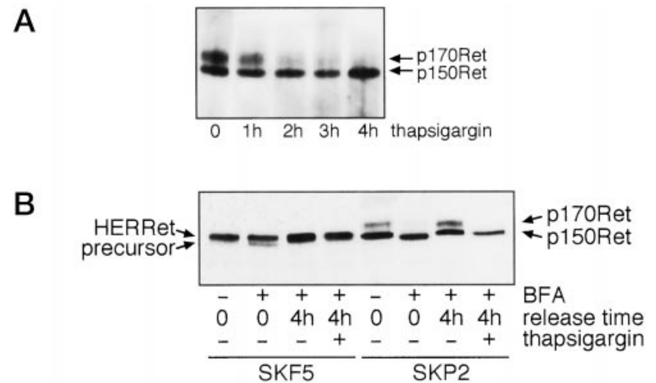

FIG. 4. **Thapsigargin specifically block p150^{ret} processing.** *A*, depletion of internal calcium stores with thapsigargin inhibits p170^{ret} expression. Subconfluent cultures of SKP2 cells were incubated in normal, calcium-containing medium. To the medium 300 nM thapsigargin was added, followed by lysis of the cells after indicated time periods. Ret proteins were immunoprecipitated and analyzed by Western blotting. The positions of the 150- and 170-kDa isoforms of Ret are indicated. *B*, thapsigargin specifically inhibits Ret but not HERRet processing. Subconfluent cultures of SKF5 and SKP2 cells were incubated for 2 h with brefeldin A (*BFA*, 5 μg/ml) and were released in the presence and absence of thapsigargin (300 nM) as indicated. Subsequently, the cells were lysed, and Ret/HERRet proteins were immunoprecipitated and analyzed by Western blotting. The positions of the 150- and 170-kDa isoforms of Ret and the HERRet fusion receptor are indicated.

absence of extracellular calcium cannot be explained by a decrease in the half-life of cell surface-expressed p170^{ret}.

An alternative explanation for the absence of p170^{ret} at the cell surface may be that processing of p150^{ret} is very sensitive to the levels of intracellular calcium, a level that will be affected when the cells are cultured in calcium-free medium. To test this hypothesis we have performed pulse-chase experiments similarly as presented in Fig. 1*B*. In contrast to the result obtained in the presence of extracellular calcium, p150^{ret} was not processed into p170^{ret} when the chase was performed in the absence of extracellular calcium (Fig. 3*B*). Moreover, under these conditions there was no decrease in the amount of labeled p150^{ret} during the first 4 h and only a small decrease after 6 h. Clearly, in the absence of extracellular calcium processing of p150^{ret} is strongly reduced, resulting in an increased half-life of p150^{ret} and absence of p170^{ret} at the plasma membrane.

Reduced Levels of Calcium in the ER Inhibit p150^{ret} Processing—One possible explanation for the lack of p150^{ret} processing in the absence of extracellular calcium is that calcium is necessary for the proper folding of the extracellular domain of Ret. This folding process takes place in the ER, and proteins not properly folded are retained in the ER and subsequently, after a delay, degraded ("quality control", for review see Ref. 21). To test this possibility we have incubated SKP2 cells with thapsigargin. Thapsigargin is a toxin that specifically inhibits calcium ATPases in the ER membrane (22). As a result of this inhibition, calcium levels in the ER are reduced. Immunoprecipitation and Western blotting of Ret proteins from thapsigargin-treated SKP2 cells showed that in the presence of 300 nM thapsigargin p170^{ret} expression decreased with similar kinetics as upon removal of extracellular calcium (Fig. 4*A*). This result suggests that the calcium concentration in the ER determines whether p150^{ret} is processed.

To show that the thapsigargin-induced block in processing is specific for the extracellular domain of Ret, we used the SKF5 cell line. This cell line is also a clonal derivative of the SK-N-MC cell line but stably expresses a chimeric receptor consisting of the transmembrane and extracellular domain of the human epidermal growth factor receptor fused to the intracellular domain of Ret (HERRet) (23, 24). HERRet proteins are detected by Western blotting as a single band of 155 kDa (Fig. 1*A* and Refs. 23 and 24), suggesting a rapid processing of the precursor protein. However, when SKF5 cells were treated with the toxin brefeldin A, which induces a collapse of the intermediate compartment and the Golgi complex and thereby induces a block in protein processing at the level of the ER (25), this precursor form of HERRet was revealed (Fig. 4*B*). This faster migrating protein could not be labeled with sulfo-NHS-biotin, whereas the 155-kDa form of HERRet was efficiently biotinylated, establishing their localization in the cell and at the plasma membrane, respectively (data not shown). When brefeldin A was removed, the precursor protein was processed into the mature form of HERRet, both in the presence and absence of thapsigargin (Fig. 4*B*). Treatment of SKP2 cells with brefeldin A also inhibited p150^{ret} processing (while p170^{ret} protein down-regulation continued). Release of the cells from the brefeldin A block in the absence of thapsigargin restored p170^{ret} expression, but, as expected, release of the cells in the presence of thapsigargin did not lead to p150^{ret} processing (Fig. 4*B*). These results show that the block in p150^{ret} processing under reduced calcium concentrations in the ER is specific for the extracellular domain of Ret.

To further support the notion that the defect in processing is due to retention of p150^{ret} in the ER, we have allowed p150^{ret} to reach the Golgi compartment prior to calcium depletion. To that end we first pulse-labeled SKP2 cells with [^{35}S]methionine in the presence of extracellular calcium at 37 °C for 45 min. This resulted in labeling of only p150^{ret} (Fig. 1*B*). Subsequently, the cells were chased for 2 h at 20 °C. At this temperature no processing of proteins in the Golgi complex occurs, and thus folded p150^{ret} protein will accumulate in the Golgi complex (Fig. 5, *lane 1*). Next, we treated the cells with brefeldin A (5 μg/ml) for 10 min at 37 °C followed by 30 min at 37 °C in the presence or absence of extracellular calcium. As a consequence

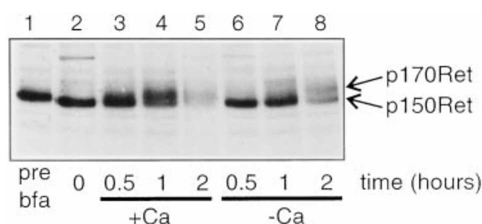

FIG. 5. **p150^{ret} proteins that have been exported from the ER can be processed into p170^{ret} in the absence of extracellular calcium.** Subconfluent cultures of SKP2 cells were pulse labeled with [^{35}S]methionine for 45 min at 37 °C. Subsequently the cells were chased for 2 h at 20 °C to allow processing of p150^{ret} up to the Golgi complex in the presence of extracellular calcium (*lane 1*, *pre bfa*). Cells were then incubated for 10 min at 37 °C with 5 μg/ml brefeldin A (*bfa*) followed by an additional incubation for 30 min at 37 °C in the presence or absence of extracellular calcium. Brefeldin A was then removed, and the cells were incubated for indicated time periods in the presence (*+Ca*) or absence (*−Ca*) of extracellular calcium. Finally, the cells were lysed, and Ret proteins were immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography. Indicated are the positions of the 150- and 170-kDa isoforms of Ret.

proteins trapped in the Golgi complex that have already passed the quality control of the ER will be returned to the ER. After this treatment a small decrease in the size of the p150^{ret} proteins was observed, most likely as a result of trimming of glycan moieties, which normally occurs in the Golgi complex but which apparently did not occur at 20 °C (Fig. 5, *lane 2*). Processing of this returned p150^{ret} was analyzed in the presence or absence of extracellular calcium. In the presence of extracellular calcium, p150^{ret} proteins that were returned to the ER were normally processed into p170^{ret}, showing that after removal of brefeldin A, protein processing returns to normal (Fig. 5, *lanes 3–5*). However, in the absence of extracellular calcium, p150^{ret} was also processed into p170^{ret} (Fig. 5, *lanes 6–8*). This result suggests that once p150^{ret} proteins have passed the ER quality control, reduction of calcium can no longer prevent processing, providing evidence that calcium affects an early step in p150^{ret} processing.

Inhibition of p150^{ret} Processing Abrogates Ret but Not HERRet Signal Transduction—GDNF stimulation of Ret-expressing cells results in p170^{ret} tyrosine phosphorylation and activation of different signal transduction pathways (11). From our results it can be predicted that SKP2 cells cultured in the presence of thapsigargin no longer respond to GDNF stimulation because of the absence of p170^{ret} expression. Indeed, although under normal culture conditions GDNF induced p170^{ret} tyrosine phosphorylation and ERK2 activation, GDNF stimulation of thapsigargin-treated SKP2 cells no longer resulted in p170^{ret} tyrosine phosphorylation or in ERK2 activation (Fig. 6). Similar results were obtained when SKP2 cells were grown in the absence of extracellular calcium prior to GDNF stimulation (data not shown).

To confirm that the extracellular (calcium-binding) domain of Ret is responsible for the effect of thapsigargin on Ret expression and signaling, we again used the SKF5 cell line. Stimulation of SKF5 cells with epidermal growth factor resulted in HERRet autophosphorylation and ERK2 activation (Fig. 6), which is in agreement with previous results (24). In contrast to p170^{ret} expression in SPK2 cells, culture of SKF5 cells in the presence of thapsigargin did not influence HERRet expression (Fig. 6). Moreover, epidermal growth factor-induced HERRet autophosphorylation and ERK2 activation were also not influenced by thapsigargin treatment (Fig. 6). Similar results were obtained when SKF5 cells were grown in the absence of extracellular calcium prior to epidermal growth factor stimulation (data not shown). These results show that the

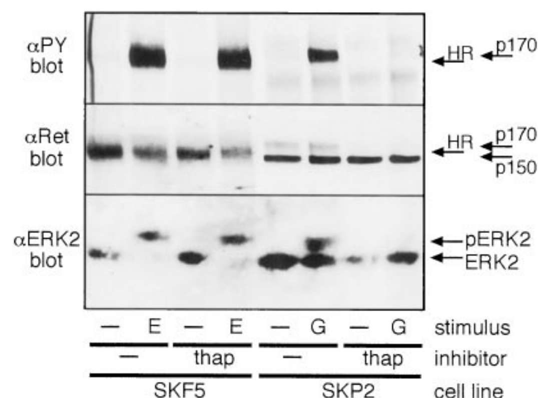

FIG. 6. **Ret function, but not HERRet function, is decreased after thapsigargin treatment of the cells.** Subconfluent cultures of SKF5 and SKP2 cells were serum starved overnight and were treated for 4 h with 300 nM thapsigargin (*thap*) as indicated. Cells were then stimulated with 100 ng/ml GDNF (*G*) (SKP2 cells) or 40 ng/ml epidermal growth factor (*E*) (SKF5 cells) for 5 min followed by lysis of the cells. *Top* and *middle panel*, p170^{ret} but not HERRet autophosphorylation after ligand stimulation is absent after thapsigargin treatment because of the absence of p170^{ret} expression. Ret or HERRet proteins were immunoprecipitated and analyzed by Western blotting using an anti-phosphotyrosine antibody (*top panel*) and an anti-Ret antibody (reprobe of the blot) (*middle panel*). Indicated are the positions of p155HERRet (*HR*) (*left-most four lanes*) and p150^{ret} and p170^{ret} (*p150* and *p170*) (*right-most four lanes*). *Lower panel*, Thapsigargin treatment blocks ligand-induced Ret-mediated but not HERRet-mediated ERK2 activation. After lysis of the cells a sample of the lysate was kept aside and was used for analysis of ERK2 activation. Indicated are the positions of the active (pERK2) and inactive (ERK2) forms of the kinase.

extracellular domain of Ret is responsible for the lack of p170^{ret} expression and signaling under reduced calcium conditions.

DISCUSSION

We have analyzed Ret expression in the stably transfected cell line SKP2, in which Ret is expressed as 150- and 170-kDa isoforms (11). Whereas p170^{ret} is a functional receptor located at the cell surface, p150^{ret} is insensitive to GDNF-stimulation and is located in the ER (3, 11). Using pulse-chase experiments we now established previous proposals that p150^{ret} is a precursor of p170^{ret} (2–4). The half-life of both isoforms is approximately 1 h, which means that there is a relatively slow conversion of p150^{ret} into p170^{ret} but a relatively rapid down-regulation of p170^{ret} from the plasma membrane as compared with for instance the platelet-derived growth factor or insulin receptor (26, 27).

Based on the observation that a mutation in the putative calcium-binding domain of Ret leads to aberrant expression of p170^{ret} (3), we have investigated the role of calcium in p170^{ret} expression. We observed that depletion of extracellular calcium results in down-regulation of p170^{ret} but not p150^{ret} expression. This provided us with an excellent system for studying in further detail the reason for the aberrant cell surface expression of p170^{ret}. Using pulse-chase experiments we found that calcium is necessary for proper processing of p150^{ret}. Furthermore, by depleting intracellular calcium stores with thapsigargin, we could show that ER luminal calcium is responsible for this proper processing, compatible with the reported localization of p150^{ret} proteins in the ER (3). Moreover, we provide evidence that once p150^{ret} proteins have left the ER, calcium is no longer necessary for proper processing. The finding that calcium affects processing of Ret in the ER is supported by the notion that the extracellular domain of Ret is responsible for the calcium sensitivity.

In the ER, newly synthesized proteins are folded, and it has been shown for several proteins that high ER luminal calcium is necessary for proper folding (28, 29). Calcium could be either

necessary for the many calcium-binding proteins (chaperones) involved in protein folding (30–32), or alternatively, calcium is directly involved in the three-dimensional structure of the newly synthesized protein, as shown for cadherins (33, 34). A protein is exported from the ER only if a protein is properly folded ("quality control", for review see Ref. 21). The observed inhibition of p150^{ret} processing when ER luminal calcium is diminished is therefore most likely due to the failure to fold properly. This was further supported by the following observation: retention of p150^{ret} in the ER was diminished when Ret proteins were allowed to be folded in the presence of calcium and to be exported from the ER to the Golgi. When folded p150^{ret} proteins were subsequently returned from the Golgi to the ER using brefeldin A, they were no longer dependent on calcium for processing into p170^{ret}. Clearly, calcium affects p150^{ret} processing in the ER and does not influence processing after export from the ER.

The extracellular domain of Ret contains a cadherin-like domain of approximately 110 amino acids (1, 19). In cadherins, this domain is repeated three or four times and mediates calcium binding and calcium-dependent homophilic interactions with cadherin molecules on other cells (20). For correct folding of cadherin molecules, calcium binding is also essential (33, 34). It is therefore likely that calcium directly affects the folding of this domain. Indeed, a mutation of a putative calcium-binding site in Ret also inhibits p150^{ret} processing (3). However, despite the use of different methods, we were not able to convincingly show binding of calcium to full-length Ret proteins or to glutathione *S*-transferase-Ret fusion proteins containing the putative calcium-binding domain of Ret (data not shown). Most likely, the affinity of this domain for calcium is very low, explaining not only the absence of detectable calcium binding but also the drastic effect of mild calcium depletion on p170^{ret} expression. An alternative explanation for the inhibition of p150^{ret} processing during extracellular calcium depletion is that calcium-binding chaperones are inhibited. This latter possibility is less obvious, because in general these chaperones have a very high affinity for calcium (22, 35–37) not likely to be affected by the mild methods of calcium depletion used in this study.

Reduced levels of functional Ret during embryonic development most likely underlie the defects observed in Ret-linked Hirschsprung's disease, because in mice completely lacking Ret expression, similar yet more severe symptoms are observed (17, 18). Indeed, point mutations in the intracellular domain of Ret, as observed in Hirschsprung's disease, result in impaired Ret tyrosine kinase activity (16, 38, 39). Interestingly, Ret-linked Hirschsprung's disease is also caused by a variety of point mutations in the extracellular domain of Ret. These mutations result in dramatically reduced levels of p170^{ret} expression at the plasma membrane without affecting the presence of the 150-kDa form (15, 16). Furthermore, the severity of the disease, as indicated by the length of the aganglionic segment, correlates with the degree of impairment of p170^{ret} expression (15). These results indicate that the extracellular domain of Ret is very sensitive for mutational alterations that either affect Ret processing or retention of Ret at the plasma membrane. Our results indicate that protein processing, most likely protein folding, is a delicate step in p150^{ret} processing that needs high levels of calcium in the ER to occur. Even under these conditions processing of p150^{ret} is slow, taking at least 1 h to proceed. The similarity between the aberrant expression of p170^{ret} in Hirschsprung's disease associated with mutations in the extracellular domain of Ret and the aberrant expression of p170^{ret} in the absence of calcium may indicate that the extracellular domain mutations also cause a defect in proper folding of p150^{ret}.

Further research is necessary to show whether modulation of luminal calcium concentration is involved in the regulation of Ret expression *in vivo*. However, inositol 3-phosphate-mediated release of calcium from internal stores is a common event after receptor stimulation, and perhaps this may affect processing of Ret. In addition, gradients of extracellular calcium do exist, for instance in the skin where calcium influences keratinocyte differentiation (40, 41).

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