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 p170ret is dependent on calcium. Depletion of extracellular calcium completely blocks p170ret expression, which is not caused by a decrease in half-life of p170ret at the plasma membrane but by a defect in processing of p150ret into p170ret. 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 p150ret and p170ret 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 p150ret isoform is endoglycosidase H-sensitive, suggesting that p150ret 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) and neurturin 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 p170ret 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 p170ret expression at the plasma membrane, whereas p150ret 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 p170ret. Furthermore, we show by pulse-chase experiments that processing of p150ret into p170ret 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 SK2 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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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; HERRet, human epidermal growth factor receptor-ret chimera receptor; 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).

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RESULTS

p150ret Is a Precursor of p170ret—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. 2). Subconfluent cultures of SKP2 cells were labeled for 45 min with [35S]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 S-PAGE and autoradiography. The positions of p150ret and p170ret are indicated.

p170ret is a precursor of p150ret. 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. 2A). When extracellular calcium was removed by replacement of the culture medium with calcium-free medium, p170ret expression gradually decreased. After 4 hours of incubation in the absence of extracellular calcium, hardly any p170ret expression could be detected. Under these conditions an increase in p150ret expression was observed (Fig. 2A). When, after 16 h of growth in calcium-free medium, physiological levels of calcium were restored in the medium, p170ret was re-expressed within 80 min (Fig. 2B). These results show that extracellular calcium is necessary for expression of p170ret proteins in SKP2 cells.

Processing of p150ret Is Dependent on Calcium—A simple explanation for the lack of p170ret expression in the absence of extracellular calcium is that binding of calcium to p150ret is necessary for the stability of p170ret at the cell surface. To address this possibility we have tested the effect of extracellular calcium on the half-life of p170ret 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. The positions of p150ret and p170ret are indicated.

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 (Fig. 2A). A precursor-product relationship between p150ret and p170ret was proposed previously (2–4) but has never been addressed. To address this possibility we have tested the effect of extracellular calcium on the half-life of p170ret in SKP2 cells.
absence of extracellular calcium cannot be explained by a decrease in the half-life of cell surface-expressed p170ret.

An alternative explanation for the absence of p170ret at the cell surface may be that processing of p150ret 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. 1B. In contrast to the result obtained in the presence of extracellular calcium, p150ret was not processed into p170ret when the chase was performed in the absence of extracellular calcium (Fig. 3B). Moreover, under these conditions there was no decrease in the amount of labeled p150ret during the first 4 h and only a small decrease after 6 h. Clearly, in the absence of extracellular calcium processing of p150ret is strongly reduced, resulting in an increased half-life of p150ret and absence of p170ret at the plasma membrane.

Reduced Levels of Calcium in the ER Inhibit p150ret Processing—One possible explanation for the lack of p150ret 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 p170ret expression decreased with similar kinetics as upon removal of extracellular calcium (Fig. 4A). This result suggests that the calcium concentration in the ER determines whether p150ret 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. 4B). 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. 4B). Treatment of SKP2 cells with brefeldin A also inhibited p150ret processing (while p170ret protein down-regulation continued). Release of the cells from the brefeldin A block in the absence of thapsigargin restored p170ret expression, but, as expected, release of the cells in the presence of thapsigargin did not lead to p150ret processing (Fig. 4B). These results show that the block in p150ret 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 p150ret in the ER, we have allowed p150ret to reach the Golgi compartment prior to calcium depletion. To that end we first pulse-labeled SKP2 cells with [35S]methionine in the presence of extracellular calcium at 37 °C for 45 min. This resulted in labeling of only p150ret (Fig. 1B). 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 p150ret 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
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 p150ret 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 p150ret was analyzed in the presence or absence of extracellular calcium. In the presence of extracellular calcium, p150ret proteins that were returned to the ER were normally processed into p170ret, showing that after removal of brefeldin A, protein processing returns to normal (Fig. 5, lanes 3–5). However, in the absence of extracellular calcium, p150ret was also processed into p170ret (Fig. 5, lanes 6–8). This result suggests that once p150ret proteins have passed the ER quality control, reduction of calcium can no longer prevent processing, providing evidence that calcium affects an early step in p150ret processing.

Inhibition of p150ret Processing Abrogates Ret but Not HERRet Signal Transduction—GDNF stimulation of Ret-expressing cells results in p170ret 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 p150ret expression. Indeed, although under normal culture conditions GDNF induced p170ret tyrosine phosphorylation and ERK2 activation, GDNF stimulation of thapsigargin-treated SKP2 cells no longer resulted in p170ret 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 lack of p170ret 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 p170ret is a functional receptor located at the cell surface, p150ret is insensitive to GDNF-stimulation and is located in the ER (3). Using pulse-chase experiments we now established previous proposals that p150ret is a precursor of p170ret (2–4). The half-life of both isoforms is approximately 1 h, which means that there is a relatively slow conversion of p150ret into p170ret but a relatively rapid down-regulation of p170ret 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 p170ret (3), we have investigated the role of calcium in p170ret expression. We observed that depletion of extracellular calcium results in down-regulation of p170ret but not p150ret expression. This provided us with an excellent system for studying in further detail the reason for the aberrant cell surface expression of p170ret. Using pulse-chase experiments we found that calcium is necessary for proper processing of p150ret. 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 p150ret proteins in the ER (3). Moreover, we provide evidence that once p150ret 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
Further research is necessary to show whether modulation of luminal calcium concentration is involved in the regulation of Ret expression in vivo. However, insositol 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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