Intrinsic susceptibility to misfolding of a hot-spot for Hirschsprung disease mutations in the ectodomain of RET

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Loss-of-function mutations in *RET* cause abnormal development of the enteric nervous system, a congenital condition known as Hirschsprung disease. Hirschsprung mutations in the extracellular domain of RET (RET^{ECD}) affect processing in the endoplasmic reticulum (ER) and prevent RET expression at the cell surface. We have investigated the processing and function of a series of Hirschsprung disease mutations affecting different biochemical properties of the RET^{ECD}. All mutations examined prevented the maturation of RET^{ECD} in the ER and abolished its ability to interact with the GDNF/GFRα1 ligand complex, indicating defects in protein folding. Immature forms of RET^{ECD} accumulating intracellularly associated with the ER chaperone Grp78/BiP and showed different degrees of protein ubiquitination. Maturation of RET^{ECD} mutants, including those deficient in Ca^{2+} binding and disulfide bridge formation, could be rescued by allowing protein expression to proceed at 30°C, a condition known to facilitate protein folding. Several of the mutants produced at 30°C regained their ability to bind to the GDNF/GFRα1 complex comparable to wild-type, demonstrating that the mutations affected RET^{ECD} folding but not function. Analysis of autonomous folding subunits in the RET^{ECD} indicated an intrinsic propensity to misfolding in three N-terminal cadherin-like domains, CLD1–3, which also concentrate the majority of Hirschsprung mutations affecting the RET^{ECD}. In agreement with this, expression and maturation of these subdomains was specifically improved at 30°C, identifying them as temperature-sensitive determinants in RET^{ECD}. Intriguingly, while production of human and mouse RET^{ECD} was suboptimal at 37°C compared with 30°C, expression of Xenopus RET^{ECD} was higher at 37°C, a non-physiological temperature for amphibians. The intrinsic susceptibility to misfolding of mammalian RET^{ECD} may be the result of a trade-off that helps to avoid an increased incidence of tumors, at the expense of a greater vulnerability to Hirschsprung disease.

INTRODUCTION

The receptor tyrosine kinase RET plays crucial roles in the development of the kidney and the enteric nervous system (1). RET serves as a receptor for members of the GDNF (glial cell line-derived neurotrophic factor) family, a small group of growth factor-like molecules with diverse activities in the nervous system and selected peripheral organs (1). RET is unable to bind GDNF on its own but can be activated in a complex with GFRα1 (GDNF family receptor alpha-1), a glycosyl phosphatidylinositol (GPI)-anchored protein that binds GDNF with high affinity (2,3). All members of the GDNF ligand famil utilize RET as a signal transducing receptor subunit, with specificity being determined by cooperation between RET and different members of the GFRα family of GPI-anchored receptors (4,5). Both gain- and loss-of-function mutations in the *RET* gene have been identified in human diseases. Mutations inducing constitutive dimerization and activation of the RET tyrosine kinase lead to congenital and sporadic cancers in neuroendocrine organs, including Multiple Endocrine Neuroplasias type 2A and 2B (MEN2A and 2B) and Familial Medullary Thyroid Carcinoma (FMTC) (6,7). Loss-of-function mutations in RET cause a dominant genetic disorder of neural crest development known as Hirschsprung disease, which results in a lack of neurons in distal segments of the enteric nervous system and colon aganglionosis (8).

Hirschsprung disease, also known as congenital aganglionic megacolon, is a common congenital abnormality leading to bowel obstruction. Hirschsprung disease is characterized by the absence of enteric ganglion cells in all or parts of the hind gut

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and occurs in 1/5000 live births (9). Hirschsprung disease is fatal if not treated. However, the so-called pull-through surgery allows survival of patients with only minor complications in most cases (10). Mutations in the *RET* gene are associated with Hirschsprung disease in up to 40% of familial cases and in about 5% of sporadic cases. Biochemical analyses performed on a fraction of Hirschsprung mutations in RET have indicated that they invariably result in loss of RET function by targeting its kinase activity (11,12), docking sites for intracellular signaling effectors (13), or residues in the RET extracellular domain (RET^{ECD}) (12,14). Hirschsprung mutations in the RET^{ECD} affect RET processing in the endoplasmic reticulum (ER) and prevent RET expression at the cell surface (8,11,14).

Recent molecular modeling of the RET^{ECD} has shown it to contain 4 N-terminal cadherin-like domains (CLD1–4) followed by a juxtamembrane cysteine-rich domain (CRD) (15). Cadherins comprise a large and divergent superfamily of Ca^{2+}-dependent cell adhesion molecules (16). The extracellular region of cadherins is formed by a variable number of repeated modules (cadherin domains) of about 110 residues, with Ca^{2+} binding sites in between each of the domains. Unlike bona fide cadherins, RET is thought to have only one Ca^{2+} binding site in between cadherin-like domains 2 and 3 (CLD2 and 3). RET binds Ca^{2+} directly and Ca^{2+} is required for its interaction with GDNF and GFRα1 as well as signaling (15,17). Depletion of extracellular Ca^{2+} impairs RET processing and insertion in the plasma membrane (18).

Several mutations affecting the RET^{ECD} of patients with Hirschsprung disease have been found to target residues involved in Ca^{2+} coordination, such as Arg-231 and Asp-267, thereby preventing Ca^{2+} binding and expression at the plasma membrane (11). Other mutations have been found in cysteine residues in the CRD, including Cys-609, Cys-618 and Cys-620, which also prevent expression of RET at the membrane. In several of these mutants, the unpaired cysteine induces RET dimerization by the formation of intermolecular disulfide bridges. Intriguingly, this results in the constitutive activation of mutant RET molecules which, due to misfolding, are retained intracellularly. These mutations cause a combined Hirschsprung/ MEN2A syndrome, a rare combination of gain- and loss-of-function phenotypes in a single mutant molecule (19). Other Hirschsprung mutations have been localized to N-linked glycosylation sites in RET^{ECD}, such as N361K and N394K. N-linked glycosylation plays an important role in protein folding and trafficking, and mutations that prevent glycosylation could therefore affect cell surface expression of a receptor.

During the last decade, retention in the ER of plasma membrane proteins affected by missense mutations has emerged as a common molecular mechanism for a number of genetic diseases, including cystic fibrosis (*CFTR* gene), long QT syndrome (*HERG* gene), familial hypercholesterolemia (*LDL* gene) and oculocutaneous albinism (*Tyrosinase* gene). Numerous mutations of diverse chemical nature have been found in these genes which invariably lead to protein misfolding and ER retention.

In the present study, we investigated the processing and function of a series of Hirschsprung disease mutants of the RET^{ECD}, delineated autonomous folding domains within RET^{ECD}, and examined the possibility that irregularities in the distribution of Hirschsprung mutations in the RET^{ECD} may be due to the existence of hot-spots of increased susceptibility to misfolding.

RESULTS

Missense mutations in RET^{ECD} of patients with Hirschsprung disease

To date, over 50 different missense mutations have been found in the RET^{ECD} of patients with Hirschsprung disease (Fig. 1). We have tentatively classified these mutations according to established or predicted effects on *N*-glycosylation (group I), Ca^{2+} coordination (group II), and disulfide bond formation (group III). The majority of the mutations, however, have no clear predictable effects on RET^{ECD} structure and include several conservative amino acid residue exchanges (group IV). Intriguingly, most of these mutations are located within the first three cadherin like domains (CLD1–3) of the RET^{ECD}, indicating a preferential susceptibility of this region of the molecule to Hirschsprung mutations (Fig. 1).

Expression and maturation of RET^{ECD} carrying Hirschsprung mutations can be rescued at lower temperatures

In addition to the mature, fully glycosylated form of RET found on the cell membrane (with an apparent molecular weight of 170 kDa), previous work has established the existence of a glycosylation intermediate of higher electrophoretic mobility (apparent molecular weight of 150 kDa) (12,14). This form is normally retained within the ER and slowly transformed into mature RET upon ER export and further processing in the Golgi compartment. Mature and immature forms of 140 and 105 kDa, respectively, can also be distinguished in conditioned medium of Chinese hamster ovary (CHO) cells expressing the RET^{ECD} at 37°C (Fig. 2, wild-type). The presence of immature RET^{ECD} in conditioned medium is probably due to direct leakage from the ER as a result of overexpression. In agreement with this, treatment with Na butyrate, which boosts protein expression by enhancing transcription from the CMV promoter used in our constructs, invariably led to an increase in the recovery of immature RET^{ECD} from cell supernatants (Fig. 2). Stable lines of CHO cells expressing epitope-tagged RET^{ECD} constructs carrying representative Hirschsprung mutations from each of the four groups identified above were generated, and production of RET^{ECD} was assessed by western blotting of cell conditioned medium. None of the Hirschsprung mutant molecules analyzed produced any significant amount of mature RET^{ECD} protein at 37°C (Fig. 2, left column). Only immature RET^{ECD} could be observed, and three of the mutants (N361K, C609Y and G93S) required additional treatment with Na butyrate for any RET^{ECD} protein to be detected (Fig. 2, left column), suggesting rapid degradation of misfolded products. The absence of mature RET^{ECD} reflects the inability of the mutant proteins to pass through the ER-Golgi proofreading checkpoint, and suggests that the corresponding mutations cause misfolding of the RET^{ECD}.
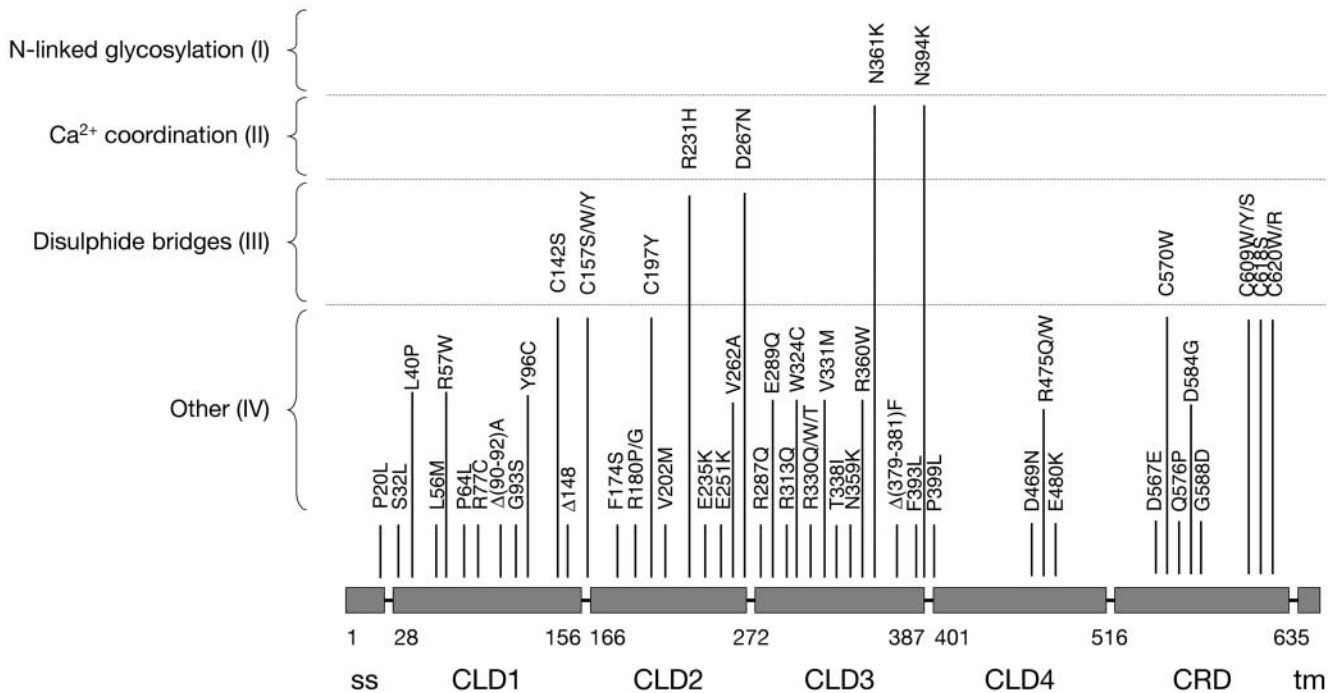
Figure 1. Missense mutations in RET[ECD] of patients with Hirschsprung disease. Diagram showing the approximate location of Hirschsprung mutations in the RET[ECD] classified according to established or predicted (i.e. part of an N–X–S/T consensus motif) effects on N-glycosylation (group I), Ca^{2+} coordination (group II), and disulfide bond formation (group III). The majority of the mutations, however, have no clear predictable effects on RET[ECD] structure and include several conservative amino acid residue exchanges (group IV). Alternative residues found at the same positions are indicated with slashes. 'Δ' denotes a deletion of the interval indicated between brackets, and is followed by the replacing residue. ss, signal sequence; CLD, cadherin-like domain; tm, transmembrane domain. The list of mutations were compiled from entries in the Expasy database (www.expasy.org/cgi-bin/niceprot.pl?P07949) and updated with entries from recent publications (43,47).

The detrimental effects of several missense mutations found in conformational or protein misfolding diseases, such as those in the *CFTR*, *HERG* and *Tyrosinase* genes, have been rescued by protein expression at a lower temperature, typically 30°C, a condition known to facilitate protein folding (20–22). We therefore investigated whether a similar treatment could affect the expression and maturation of RET[ECD] carrying Hirschsprung mutations. With the exception of G93S, all other mutants examined produced mature RET[ECD] as the only product in supernatants of cells grown at 30°C (Fig. 2, right column), indicating a complete rescue of protein folding at the lower temperature. Even after Na butyrate treatment, half of the mutants analyzed still predominantly produced the mature form of RET[ECD] (Fig. 2, right column), indicating that defects in N-linked glycosylation, Ca^{2+} coordination and disulfide bonding can in principle be compensated by facilitating protein folding at 30°C. Intriguingly, G93S is the only Hirschsprung mutation known that is capable of causing total colon aganglionosis in heterozygous form (23). Importantly, folding of the wild type RET[ECD] protein was also enhanced at the lower temperature, as indicated by our inability to detect immature RET[ECD] in supernatants of cells grown at 30°C even after Na butyrate treatment (Fig. 2, right column), and suggests an intrinsic susceptibility to misfolding of the wild-type RET molecule.

Analysis of lysates of CHO cell lines expressing wild-type and mutant RET[ECD] constructs showed that immature RET[ECD] was the predominant intracellular form of this protein (Fig. 3A).

Thus, once fully processed, mature RET[ECD] is rapidly secreted and does not accumulate to any significant level inside the cell. In agreement with our observations in cell supernatants, higher levels of immature RET[ECD] protein accumulated intracellularly after incubation at 30°C and in the presence of Na butyrate (Fig. 3A).

Immature RET[ECD] interacts with the ER chaperone Grp78/BiP and is ubiquitinated

In order to identify proteins involved in the intracellular retention of the immature form of RET[ECD], we performed co-immunoprecipitation experiments on lysates from stable CHO cells expressing wild-type and Hirschsprung RET[ECD] constructs. The ER chaperone Grp78/BiP is known to be involved in the recognition of misfolded proteins in the secretory pathway, such as the low density lipoprotein LDL (24). We therefore tested the ability of the immature form of RET[ECD] to interact with this chaperone in CHO cells. As shown in Figure 3B, both wild-type and Hirschsprung mutant forms of immature RET[ECD] co-immunoprecipitated with Grp78/BiP to a varying degree. Increased levels of Grp78/BiP were associated with the immature forms of G93S and R475W mutants, whereas wild-type and N361K RET[ECD] co-immunoprecipitated Grp78/BiP to a lower degree (Fig. 3B).

We also investigated whether immature RET[ECD] proteins retained intracellularly were ubiquitinated. In agreement with
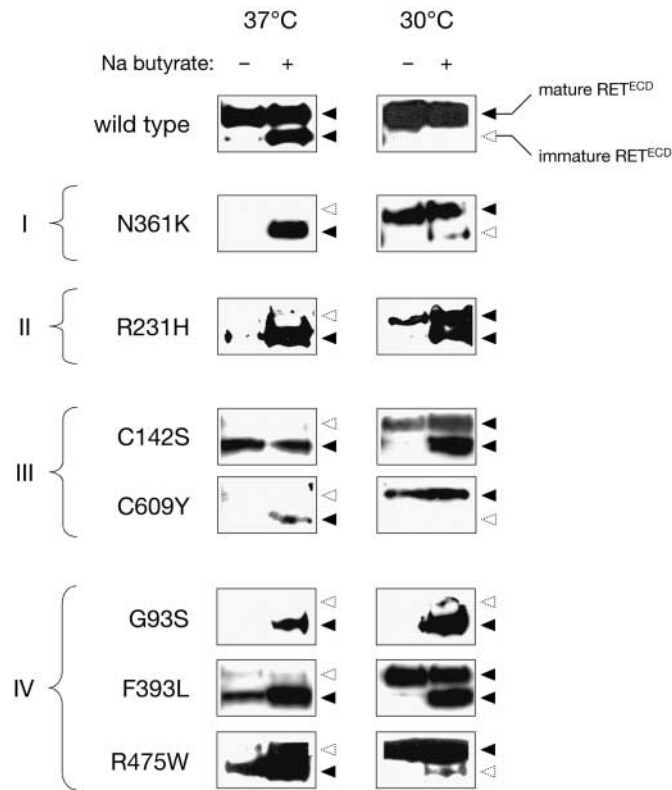
Figure 2. Expression and maturation of RET^{ECD} carrying Hirschsprung mutations can be rescued at lower temperatures. CHO cells stably expressing RET^{ECD} constructs carrying the indicated mutations were incubated for 2 days at 37 or 30°C and in the presence or absence of 2 mM sodium butyrate as indicated. RET^{ECD} proteins collected from cell supernatants were analyzed by immunoblotting with antibodies against the HA epitope tag. Examples of RET^{ECD} mutants from groups I, II, III and IV are shown. Arrowheads denote the presence (solid) or absence (empty) as well as the positions of mature and immature RET^{ECD} bands as indicated.

their high degree of association with Grp78/BiP, both G93S and R475W mutants showed the highest levels of ubiquitination (Fig. 3B). Lower levels were observed in the N361K mutant, and very low, but detectable levels of ubiquitination were also seen in the immature form of wild-type RET^{ECD} (Fig. 3B). Thus, similar to proteins involved in conformational or misfolding diseases such as CFTR, a fraction of newly synthesized RET is normally recognized as misfolded and degraded by the proteasome.

Functional rescue of RET^{ECD} Hirschsprung mutants produced at lower temperature

The functional capabilities of RET^{ECD} proteins recovered at 30°C was assessed in a solid-phase binding assay using immobilized GDNF/GFRα1 complex as target ligand. The binding activity of the N361K mutant was comparable to that of wild-type (Fig. 4A) indicating that glycosylation of this residue is not required for interaction with the GDNF/GFRα1 complex. On the other hand, RET^{ECD} carrying the R231H mutation, which disrupts Ca^{2+} binding, was unable to bind ligand (Fig. 4B), in agreement with previous observations
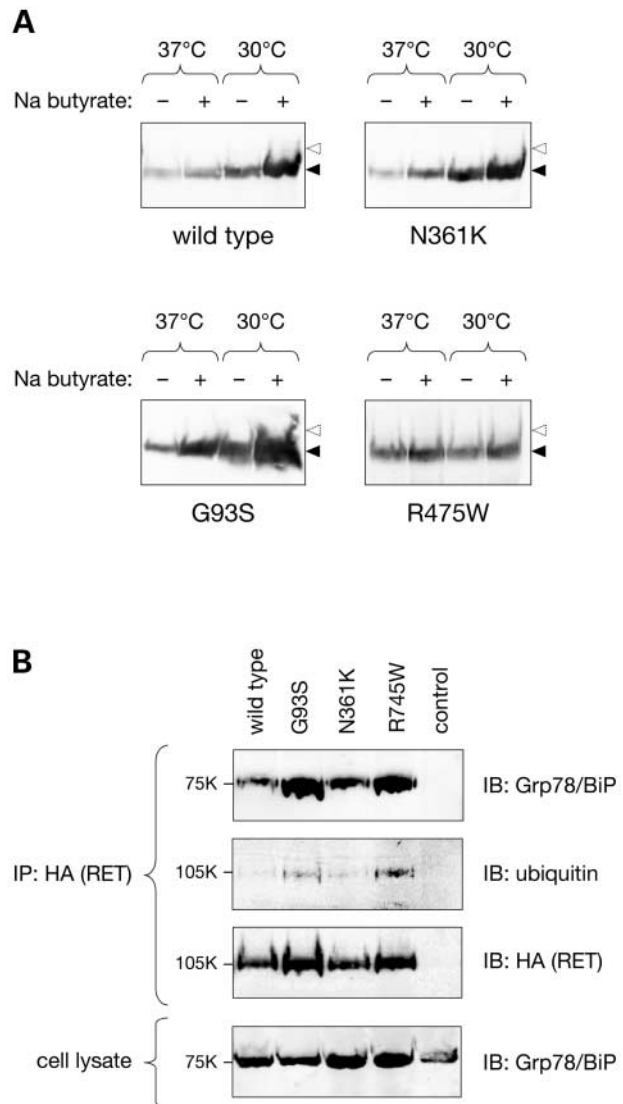

Figure 3. The immature forms of RET^{ECD} Hirschsprung mutants are ubiquitinated intracellularly and co-immunoprecipitate with the ER chaperone Grp78/BiP. (**A**) Intracellular expression of wild type and mutant RET^{ECD} proteins. Lysates of CHO cells stably expressing RET^{ECD} constructs carrying the indicated mutations that were incubated at 37 or 30°C and in the presence or absence of 2 mM sodium butyrate were analyzed by immunoblotting with antibodies against the HA epitope tag. In all cases, the predominant intracellular form of RET^{ECD} corresponds to the immature protein. (**B**) RET was immunoprecipitated (IP) using anti-HA antibodies from lysates of CHO cells lines stably expressing wild-type and RET^{ECD} mutants N361K, G93S and R475W. A lysate of parental CHO cells was used as control. Immunoblots (IB) were probed with antibodies against the ER chaperone Grp78/BiP, ubiquitin or the HA epitope tag present in the RET^{ECD} constructs. In the lower panel, 20 μg aliquots of the total cell lysates were probed with anti-Grp78/BiP antibodies.

indicating the requirement of Ca^{2+} coordination for ligand binding by RET (15,17). Thus, Ca^{2+} binding appears to be dispensable for the folding of RET^{ECD} at 30°C, but nevertheless is required to stabilize the protein in a conformation competent for ligand binding.

Mature and folded RET^{ECD} carrying the C142S mutation was incapable of interacting with the GDNF/GFRα1 complex
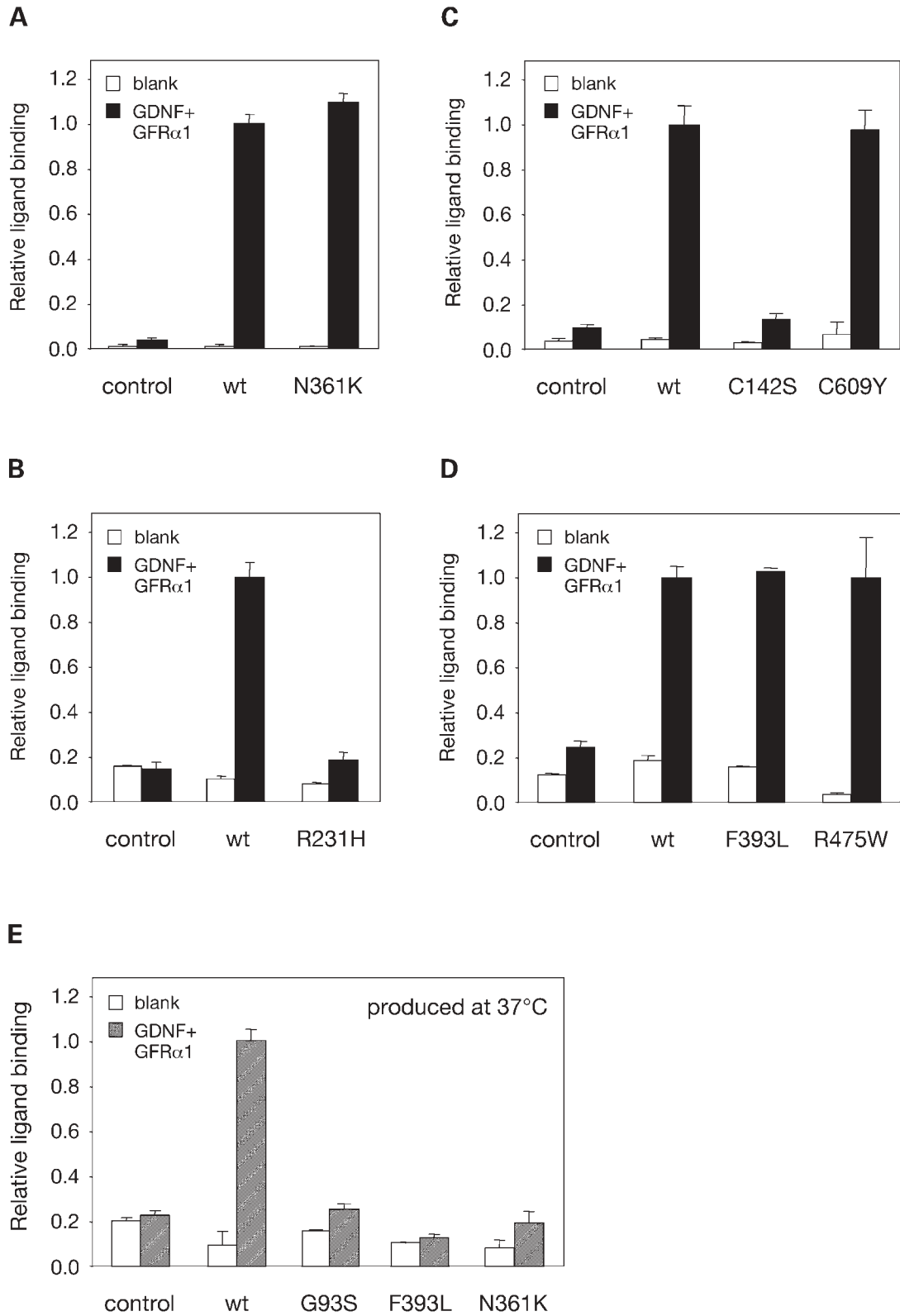
Figure 4. Functional rescue of RET^ECD Hirschsprung mutants produced at lower temperature. Solid phase binding assay of RET^ECD Hirschsprung mutants. Wells were coated with recombinant purified GDNF/GFRα1-Fc complex (solid bars) or PBS (open bars) and subsequently blocked with low-fat milk. HA-tagged RET^ECD mutant and wild-type proteins harvested from CHO cell supernatants were allowed to bind, washed and subsequently detected with anti-HA antibodies. Results in each panel were normalized to the binding of wild-type RET^ECD. 'Control' denotes supernatant from mock-transfected CHO cells. (**A**)–(**D**) Results obtained with proteins produced at 30°C. For (**E**), proteins were expressed at 37°C.

(Fig. 4C), indicating that this residue may either be directly involved in ligand binding or contributing to stabilize the ligand-binding conformation of RET^{ECD}, perhaps by forming an inter-domain disulfide bridge. In support of this notion, Cys-142 is predicted to be exposed in the surface according to a recent molecular model of RET CLD1 (15) (but see Discussion below). In contrast, the mature form of the RET^{ECD} mutant C609Y produced at 30°C was indistinguishable from wild-type in its ability to interact with the GDNF/GFRα1 complex (Fig. 4C), indicating that, despite its importance for folding at 37°C, this residue is not involved in the contact of RET with its ligands.

The mature forms of RET^{ECD} mutants F393L and R475W recovered at 30°C were also found to be able to interact with the GDNF/GFRα1 complex (Fig. 4D), indicating that these positions are not crucial for ligand binding. As expected, immature, misfolded forms of RET^{ECD} Hirschsprung mutants produced at 37°C were unable to interact with the GDNF/GFRα1 complex (Fig. 4E), confirming that immature RET^{ECD} forms are non-functional. These included mutations that could be rescued at 30°C, such as F393L and N361K, as well as the G93S mutant, which remained misfolded and unable to bind ligand at either temperature (Fig. 4E and data not shown). It remains possible that, in addition to its detrimental effects on protein folding, the G93S mutation may also affect ligand binding.

Together, these results indicate that Hirschsprung mutations which do not disrupt key structural features, such as Ca^{2+} coordination, do not have a direct functional effect on the ability of RET to bind its ligands but rather affect the folding of the RET protein at 37°C, and can therefore be rescued if biosynthesis is allowed to proceed at a lower temperature.

Autonomously folding subunits in RET^{ECD}

Crystallographic and bioinformatical studies of the extracellular regions of receptor tyrosine kinases, such as our previous analysis of the RET^{ECD} (15) or the experimentally determined structures of FGFR (25–27) and Erb (28–33) receptors, have indicated a modular arrangement of structurally discrete domains. This modularity, however, does not necessarily imply that individual domains are capable of folding independently from each other. Folding of some individual domains in multidomain proteins has been shown to require the presence of other domains from the same molecule acting as intramolecular chaperones (34). The preferential localization of group IV Hirschsprung mutations within the CLD1, 2 and 3 of RET^{ECD} prompted us to examine the folding behavior of these domains, independently or in various combinations, and in comparison to other regions of the RET^{ECD} (Fig. 5). As above, we expressed constructs of the RET^{ECD} in stable lines of CHO cells and their production was assessed by western blotting of cell conditioned medium. Because of their heterogeneous sizes, mature and immature forms of truncated RET^{ECD} fragments could not be distinguished in all cases by simply assessing differences in electrophoretic mobility as performed above for full-length RET^{ECD} variants. We therefore took advantage of the differential sensitivity to Endoglycosidase H (Endo H) of folded and misfolded glycoproteins en route from the ER to the Golgi. Upon exit from the ER,

correctly folded glycoproteins lose sensitivity to Endo H as carbohydrates of higher complexity are added in the Golgi complex. Misfolded glycoproteins, however, are retained in the ER and remain Endo H sensitive. As noted above, over-expression can cause leakage of misfolded, Endo H sensitive glycoproteins from the ER to the cell supernatant, where they may also be detected along with correctly folded, Endo H resistant proteins. All glycoproteins, folded and misfolded, are sensitive to deglycosylation by peptide:*N*-glycosidase F (PNGase F).

Of the individual domains in RET^{ECD}, only CLD3 and CRD were able to fold autonomously and generate mature, Endo H-resistant proteins (Fig. 5). Although CLD1 and CLD2 could not fold independently, together they generated a correctly folded protein (Fig. 4), suggesting intramolecular chaperone-like interactions between these two domains. In support of this notion, no mature RET^{ECD} lacking only CLD1 could be obtained, although deletion of both CLD1 and CLD2 allowed high levels of expression of correctly folded protein (Fig. 4). N-terminal truncations within loop regions of CLD1 generated only low levels of Endo H-resistant proteins (Fig. 5). Likewise, CLD4 could only generate a correctly folded protein when expressed together with the C-terminal CRD, but not with N-terminal domains (Fig. 5), suggesting another interdomain interaction that may be important for the folding of RET^{ECD}. The importance of correctly defined interdomain boundaries for folding was illustrated by the analysis of a construct encompassing CLD1, 2 and 3 that was unable to produce correctly folded protein unless 12 additional residues from the linker region downstream of CLD3 were added (Fig. 5).

Interestingly, N-terminally truncated constructs lacking CLD1 and CLD2 were expressed at much higher levels than the full length protein (Fig. 5). Thus, while full-length RET^{ECD} produced 100–200 µg/l protein in serum-free cell supernatant after 4 days at 37°C, CLD3–CLD4–CRD and CLD4–CRD accumulated at 2–5 mg/l under similar conditions. On the other hand, constructs of CLD1–CLD2, CLD1–CLD2–CLD3 and CLD3 alone produced much lower levels, i.e. 10–50 µg/l of supernatant. Together, these results indicate that an intrinsic difficulty in the folding and production of CLD1 and CLD2 constrains the level of RET^{ECD} expression in the cell. Interestingly, these are the same domains that, together with CLD3, concentrate the majority of Hirschsprung mutations affecting the RET^{ECD}.

Temperature-sensitive determinants in RET^{ECD}

We next investigated the effects of lower temperature on the expression levels of full-length RET^{ECD} and various RET^{ECD} domains. After 4 days in serum-free medium, the level of expression of full-length RET^{ECD} was approximately 10-fold higher at 30°C compared with that at 37°C (Fig. 6), indicating that expression of human RET^{ECD} is suboptimal at the physiological temperature, a feature also observed for the secretion of the ectodomain of tyrosinase (22). For comparison, the extracellular domain of the rat neural cell adhesion molecule (NCAM^{ECD}), which consists of regular immunoglobulin (Ig) and fibronectin III (FnIII) domains, was studied under the same conditions. NCAM has recently been shown to function as an alternative signaling receptor for GDNF family
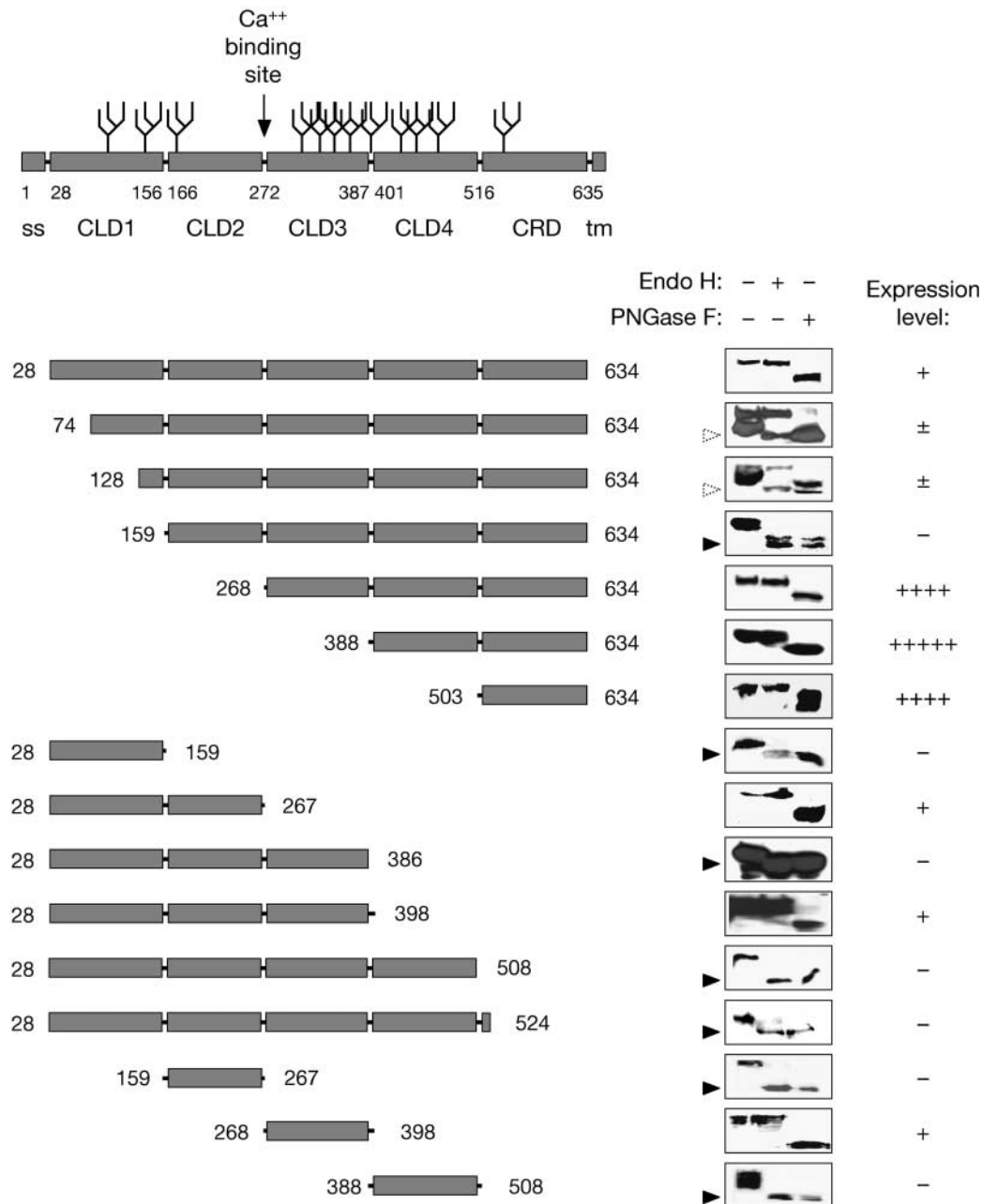
Figure 5. Autonomously folding subunits in RET^{ECD}. N- and C-terminal truncations of HA-tagged RET^{ECD} constructs were produced in supernatants of stably transfected CHO cells grown at 37°C and subjected to deglycosylation as indicated. RET^{ECD} proteins were detected with an anti-HA antibody. Sensitivity to Endo H results in a faster migrating band (solid arrowheads) indicative of a misfolded glycoprotein. Partial Endo H sensitivity is denoted by an open arrowhead. A diagram of the RET^{ECD} with amino acid residue positions is shown at the top. The approximate positions of known and predicted glycosylation sites are indicated. Abbreviations are as in Figure 1. Relative expression levels of each construct are indicated to the right and varied from very low ($-$) to very high ($+++++$).

ligands in collaboration with GFRα1 (35). Previous work had shown that Ig domains are rapidly and efficiently processed through the ER (36). In contrast to the RET^{ECD}, NCAM^{ECD} was more efficiently expressed at 37°C than at 30°C (Fig. 6), indicating that the rate of NCAM^{ECD} expression is controlled by the overall metabolic activity of the cell. Temperature-sensitive determinants in RET^{ECD} were mapped by studying the levels of expression of different RET^{ECD} constructs. Similar to full-length RET^{ECD}, fragments containing CLD1, CLD2 and

CLD3 also showed a pronounced increase in expression at 30°C compared with 37°C (Fig. 6), identifying these domains as the temperature-sensitive determinants in RET^{ECD}. Temperature sensitivity was drastically reduced upon deletion of CLD1 and CLD2 [Fig. 5, fragment CLD(3–4)–CRD] and completely eliminated in CLD4–CRD, which, like NCAM^{ECD}, showed higher levels of expression at 37°C compared with 30°C (Fig. 6). Combined, these results indicate that CLD1, CLD2, and to a lesser extent CLD3, constitute folding
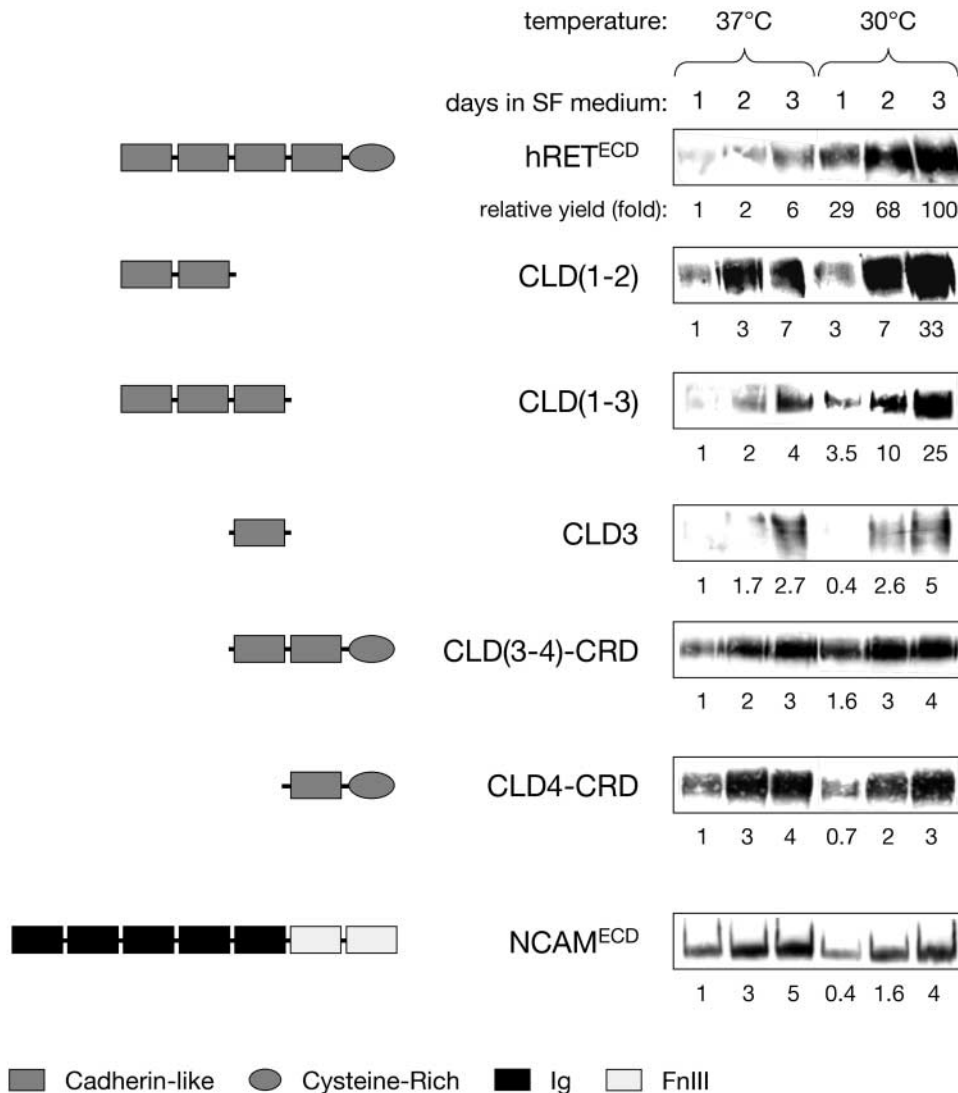
Figure 6. Temperature-sensitive determinants in RET[ECD]. CHO cells stably expressing full length human RET[ECD] (hRET[ECD]), or the indicated RET[ECD] fragments, or the ectodomain of rat NCAM were cultured at 30 or 37°C and conditioned medium was collected at the indicated time points. Relative expression levels were quantified by immunoblotting and are indicated relative to those obtained after 1 day incubation at 37°C. Diagrams of RET[ECD] and rNCAM constructs are shown to the left. SF, serum-free; Ig, immunoglobulin domain; FnIII, fibronectin III domain.

bottlenecks in the processing and expression of RET[ECD] through the early secretory pathway.

Temperature sensitivity of RET[ECD] expression in warm- and cold-blooded verebrate species

Is the temperature sensitivity of RET[ECD] an intrinsic property of all RET molecules or does it have any relationship to the body temperature of the organism? In order to address this question, the extracellular regions of mouse and *Xenopus* RET were isolated and expressed as above. Expression was compared in supernatants of stably transfected CHO cells grown at 37 and 30°C. Similar to human RET[ECD], the extracellular domain of mouse RET also accumulated at higher levels when expressed at a lower temperature (Fig. 7). In contrast, *Xenopus* RET[ECD] expression was higher at 37°C

compared with 30°C (Fig. 7), behaving in this regard as a protein with a production rate limited only by overall cellular metabolic activity, i.e. similar to rNCAM[ECD] (Fig. 7). Although *Xenopus* RET[ECD] accumulated to higher levels than human or mouse RET[ECD] at 37°C, the three proteins were expressed at comparable levels at 30°C (data not shown). Thus, temperature sensitivity appears to be a property of RET[ECD] from warm-blooded vertebrate species, suggesting it may have evolved to limit the overall level of RET expression in organisms with a body temperature of 37°C.

DISCUSSION

The role of the ER as a site of control of protein expression has been the subject of intense investigation during recent years (37,38). Quality control systems operate at different levels in
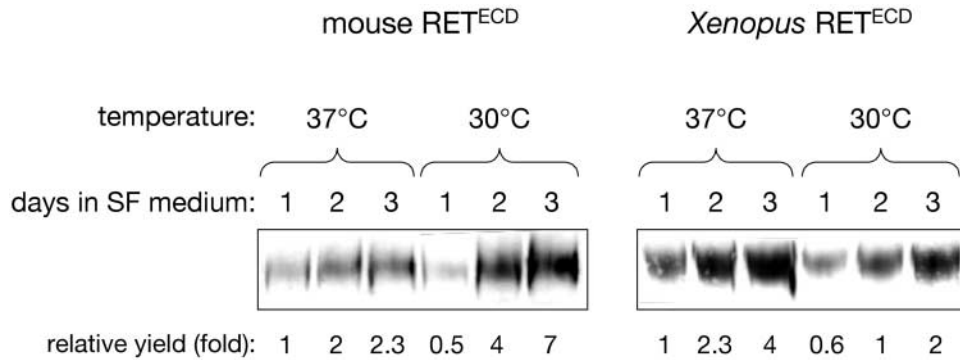
mouse RET^ECD   Xenopus RET^ECD

Figure 7. Temperature sensitivity of mouse and *Xenopus* RET^ECD expression. Serum-free supernatants from stably transfected CHO cells expressing the ecto-domain of either mouse or *Xenopus* RET^ECD (mRET^ECD and xRET^ECD, respectively) were collected at the indicated time points and analyzed by immunoblotting as above. Relative expression levels were quantified and are indicated relative to those obtained after 1 day incubation at 37°C.

the ER to ensure that secretory and membrane-associated proteins fold properly and acquire their native tertiary and quaternary structures. Several ER retention signals have been identified that keep the different subunits of multimeric integral membrane proteins in the ER until they are correctly folded and assembled (39). In addition to their intrinsic lack of function, misfolded proteins, particularly multidomain proteins and protein complex subunits, can interfere with normal cellular function by sequestering other proteins in unproductive complexes. Guaranteeing the quality of protein folding is a costly process for the cell. In the case of the CFTR, for example, only about one-third of all synthesized molecules ever reach the cell surface (40). Folding of certain membrane proteins would appear to be just at the edge of the capacity of the cell and therefore particularly susceptible to perturbations, such as overexpression, temperature, oxidative stress and mutations (41). Such hypersensitivity may help to explain why there are so many individual disease-associated point mutant forms of these types of proteins. A recent study analyzed the thermodynamic properties of a range of proteins involved in misfolding-based pathologies. In most cases, there was no significant relationship between the spectrum of mutations observed and their predicted effects on the thermo-dynamic stability of the protein in question (42), suggesting that events leading to correct folding versus misfolding arise early in the secretory pathway and may be under kinetic control (41). For these type of proteins, small differences between the efficiencies of competing folding and misfolding pathways may help to explain why the yield of correctly folded protein can be enhanced by merely lowering the temperature.

Our analysis of the RET^ECD indicate that RET has a relatively high propensity to misfolding, placing it firmly in the group of proteins involved in so-called conformational or misfolding diseases. In keeping with this notion, immature forms of RET^ECD accumulating intracellularly associated with the ER chaperone Grp78/BiP and showed different degrees of protein ubiquitination. Within the RET^ECD, CLD1, 2 and 3 appeared by several criteria intrinsically difficult to fold correctly, which may help to explain the increased frequency of Hirschsprung disease mutations observed within these three domains. The fact that most RET^ECD mutants could be rescued by expression at a lower temperature supports the notion that the mutations

affect the differential efficiencies of energetically nearby folding and misfolding pathways rather than the overall stability or function of the RET molecule. These observations have important implications for the pathogenesis of misfolding-related diseases and may help to explain why several mutations found in these patients appear to be associated with genetic lesions at other loci. For example, a recent report identified several mutations in the RET^ECD of patients with short-segment Hirschsprung disease that co-segregated with two other loci at 3p21 and 19q12 (43). In the light of our findings, it could be speculated that some of the genes implicated in those regions might encode factors participating in the RET^ECD folding pathway.

The analysis of the temperature-sensitivity of RET^ECD mutants provided a direct means to discriminate effects on expression, folding and function. Using this approach, we found that the majority of RET^ECD Hirschsprung mutants analyzed had a temperature-reversible protein phenotype, i.e. they were stable and functional if produced at 30°C, indicating that this method can be useful for the study of structure–function relationships in RET^ECD. In our hands, for example, another glycosylation mutant that has been linked to Hirschsprung disease, N394K, could be produced and secreted as a mature and folded protein at either temperature, and was fully functional (S. Kjær and C.F. Ibáñez, unpublished data), indicating that this mutation may not be a Hirschsprung determinant but is more likely to be a polymorphism. Replacement of Lys for Asp-300, a residue involved in Ca^{2+} coordination, has been shown to disrupt Ca^{2+} binding by RET (44), although this mutation has not been found in Hirschsprung patients. In our hands, this mutant behaved like the R231H mutation that has been linked to the disease in that it produced mostly immature RET^ECD at 37°C but fully folded protein at 30°C, supporting the requirement of Ca^{2+} coordination for RET^ECD folding at physiological temperatures. Similar to R231H, mature RET^ECD carrying the D300K mutation produced at 30°C was unable to interact with ligand (S. Kjær and C.F. Ibáñez, unpublished data), providing further evidence of the importance of Ca^{2+} binding for the stabilization of a conformation competent for ligand binding.

Whether Cys-142, affected by the C142S Hirschsprung mutation, does in fact engage in disulfide bridge formation

remains unclear. In the model structure of the CLD1 of RET^{ECD}, this residue appears exposed but not in proximity to any other unpaired cysteine residue (15). Cys-142 is conserved only among mammalian species but is replaced by various other residues in other vertebrate variants and in *Drosophila* RET. Intriguingly, a similar pattern is found in Cys-217, a cysteine residue exposed on the surface of the CLD2 in mouse, rat and human RET^{ECD}, but absent in RET variants from non-mammalian species (15). Interdomain cystine bonds are rare, but one has recently been found in the human IL-6 receptor (45). Unlike the C142S mutation, however, replacement of Ser for Cys-217 did not affect the maturation of RET^{ECD} nor its ability to bind ligand (S. Kjær and C.F. Ibáñez,, unpublished data). Because similar effects are expected after mutation of either cystine residue from a given disulfide bond, these observations would argue against the possibility of an interdomain disulfide bridge between Cys-142 and Cys-217. As a side note of potential relevance to mechanisms of onco-genic RET activation, neither the C609Y or C634R mutants, the latter being the most frequent RET mutation found in MEN2A, form disulfide-linked dimers when expressed as soluble ectodomains (S. Kjær and C.F. Ibáñez, unpublished data), although they readily do so in the context of the full-length RET molecule. This suggests that membrane anchoring of RET is required for the formation of RET dimers via this mechanism, a prerequisite to its activation in MEN2A tumors. Unlike C609Y, the C634R mutant is readily produced and folded as a mature protein, indistinguishable in this respect from wild-type RET^{ECD} (S. Kjær and C.F. Ibáñez, unpublished data). In agreement with this, the C634R mutation has not been found in patients with Hirschsprung disease.

The expression levels of *Xenopus* and mammalian RET^{ECD} were comparable at 30°C, but differed markedly at 37°C. Expression of mature *Xenopus* RET^{ECD} was higher at 37°C than at 30°C, in agreement with its production being only limited by overall cellular activity. In contrast, lower levels of human and mouse RET^{ECD} were produced at 37°C compared to 30°C, reflecting the intrinsic susceptibility to misfolding of mammalian RET^{ECD}. In support of this notion, some positions in the native sequence of *Xenopus* RET, such as for example Leu-32 and Met-56, contain residues which are only found in Hirschsprung variants of human RET. Interestingly, the body temperature of *Xenopus* frogs is normally in the range of 20–23°C. Why would expression of human and mouse RET^{ECD} be suboptimal at the physiological temperature of those species? As recently pointed out by Ellgaard and Helenius (38), during evolution proteins have been optimized for function, but not necessarily for folding. In the case of RET, however, the fact that the *Xenopus* variant can be efficiently produced and folded at a temperature that is not physiological for that organism suggests instead that the folding efficiency of mammalian RET may be under evolutionary constrains. Previous work has shown that high levels of RET expression can result in constitutive activation in the absence of ligand (46). Considering that RET is in fact a proto-oncogene, it is therefore possible that the intrinsic susceptibility to misfolding of human and mouse RET^{ECD} may be the result of a trade-off that helps to avoid an increased incidence of tumors and cancer, at the expense of a greater vulnerability to Hirschsprung disease.

MATERIALS AND METHODS

Plasmid constructs

Protein-coding DNA sequences of human, mouse and *Xenopus* RET^{ECD} were amplified by PCR with primers containing *Sfi*I and *Not*I sites for directional cloning into the pSecTag2AHA mammalian expression vector (Invitrogen), modified to contain an additional hemagglutinin (HA) tag (YPYDVPDYA) at the C-terminus of the secreted protein. c-Myc-(EQKLISEEDLN) and 6× His-tags were also included to aid detection and purification of RET^{ECD} constructs. Point mutations were introduced by the QuickChange method (Stratagene). All expression constructs were verified by automated DNA sequencing.

Cell culture, transfection and selection of stable cell lines

CHO cells were maintained in an atmosphere of 5% CO$_2$ at 37°C in DMEM medium (Life Technologies Inc.) supplemented with 10% FCS, 2 mM glutamine and 60 μg/ml Gentamycin. Stable cell-lines were generated by transfection of cells using calcium phosphate. Forty-eight hours post-transfection, Hygromycin B (Invitrogen) was added to a final concentration of 800 μg/ml. Colonies of stably transfected CHO cells normally appeared within a week. The colonies were picked and expanded in 96-well plates. The positive clones were identified by immunoblotting with anti-HA antibodies (Covance).

Antibodies

A polyclonal rabbit antibody was generated using a highly purified preparation of a RET^{ECD} construct containing CLD3 and 4 and the CRD produced in CHO cells. The anti-HA antibody was from Covance. The anti-mouse–HRP conjugated antibody was from DAKO.

Deglycosylation of RET^{ECD} expressed in mammalian cells

Cell lines expressing RET^{ECD} fragments were expanded and shifted to serum-free medium at 80% confluency after several rinses with PBS to remove contaminating serum proteins. The serum-free medium was left for 72–96 h and subsequently concentrated 5- to 10-fold by ultracentrifugation (Amicon). To investigate the type of glycosylation (and thereby the state of maturation) of the protein, 50 μl of concentrated conditioned medium were subjected to deglycosylation using Endo-H or PNGase F (New England Biolabs) as indicated. Subsequently, deglycosylated proteins were resolved on SDS–PAGE gels and transferred to PVDF membranes (Amersham) by electroblot-ting. Upon blocking with 5% skimmed milk powder in PBS, RET^{ECD} fragments were analyzed by immunoblotting with anti-HA antibodies. Immunoblots were developed using enhanced chemiluminiscence (ECL; Pierce).

Immunoblotting and immunoprecipitation

Stably transfected CHO cells were maintained as described above in 10 cm dishes. To determine the expression yield in time-course experiments at either 30 or 37°C, cells were grown to 80% confluency and subsequently washed twice with PBS and serum-free DMEM medium, supplemented with 2 mM glutamine and buffered with 1 mM Hepes. Supernatants of cells maintained at either 30 or 37°C in the presence or absence of 2 mM sodium butyrate were collected once every 24 h for 3 days. To determine the relative yields, 50 µl of conditioned medium was subjected to SDS–PAGE and immunoblotting as described above. Relative yields were determined by quantification with ImageQuant software (Molecular Dynamics). For immunoprecipitation studies, cell monolayers from one 10 cm plate were lysed in 1 ml of ice-cold lysis buffer (50 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA and 1% NP-40 detergent) in the presence of a protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and immunoprecipitated overnight at 4°C with 2 µg of anti-HA monoclonal antibody. Immunocomplexes were captured by addition of Gamma-bind beads (Pharmacia). After five washes, immunoprecipitates were resuspended in SDS-sample buffer, fractionated by SDS–PAGE, transferred to a PVDF membrane and probed with antibodies against ubiquitin (P4D1, Santa Cruz), Grp78/BiP (PA1-014, Affinity Bioreagents) or the HA epitope tag.

Binding assays

To determine the ligand binding capacity of RET^{ECD} and its variants, a solid phase receptor ELISA was established. Wells of an ELISA plate were coated overnight at 4°C with 50 ng recombinant GDNF produced in bacteria (Peprotech) precomplexed in 100 µl PBS with 250 ng of GFRa1-Fc fusion protein (R&D Systems). Blank wells were set up by omitting GDNF/GFRa1 and proceeding directly to the blocking step. After washing and blocking with 5% skimmed milk, conditioned medium containing various RET^{ECD} mutants was added to the wells. The amounts of the different RET^{ECD} proteins added was equilibrated by prior quantitative immunoblotting assay using anti-HA and anti-Myc antibodies. CHO cell supernatants containing RET^{ECD} proteins were incubated for 1 h at room temperature, washed and detected with anti-HA antibodies and anti-mouse-HRP conjugated antibodies. The ELISA reaction was developed by addition of TMB substrate (Pierce).

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