Regulated intramembrane proteolysis (RIP) is an emerging paradigm in signal transduction. RIP is mediated by intramembrane-cleaving proteases (I-CliPs), which liberate biologically active nuclear or secreted domains from their membrane-tethered precursor proteins. The yeast Pcp1p/Rbd1p protein is a Rhomboid-like I-CliP that regulates mitochondrial membrane remodeling and fusion through cleavage of Mgm1p, a regulator of these essential activities. Although this ancient function is conserved in PARL (Presenilins-associated Rhomboid-like protein), the mammalian ortholog of Pcp1p/Rbd1p, the two proteins show a strong divergence at their N termini. However, the N terminus of PARL is significantly conserved among vertebrates, particularly among mammals, suggesting that this domain evolved a distinct but still unknown function. Here, we show that the cytosolic N-terminal domain of PARL is cleaved at positions 52–53 (α-site) and 77–78 (β-site). Whereas α-cleavage is constitutive and removes the mitochondrial targeting sequence, β-cleavage appears to be developmentally controlled and dependent on PARL I-CliP activity supplied in trans. The β-cleavage of PARL liberates Pβ, a nuclear targeted peptide whose sequence is conserved only in mammals. Thus, in addition to its evolutionarily conserved function in regulating mitochondrial dynamics, PARL might mediate a mammalian-specific, developmentally regulated mitochondrial-to-nuclei signaling through regulated proteolysis of its N terminus and release of the Pβ peptide.

Regulated intramembrane proteolysis, RIP, is a signaling mechanism that involves the generation of biologically active peptides from membrane-tethered precursor proteins (1). RIP was originally described as a mechanism requiring two sequential cleavages carried out by distinct proteases (2). The first protease cleaves the precursor protein close to a transmembrane helix (TMH), whereas the second cleavage, executed by an intramembrane-cleaving protease, or I-CliP, occurs within the TMH and releases a biologically active peptide. In the well-characterized case of the sterol regulatory element-binding protein (SREBP), cleavage by the I-CliP site-2 protease (3) results in the liberation of a nuclear transcription factor (2, 3). A similar process has also been demonstrated for Presenilin-1 (PS1) (4). Intramembrane cleavage of Notch and APP by PS1 results in the liberation of the intracellular domains of these proteins, NICD and AID/AICD, respectively (5, 6), which are targeted to the nucleus and regulate gene expression (7, 8).

The recent characterization of Rhomboid-1, a previously described regulator of Drosophila development (9, 10), has brought new insights into RIP. Indeed, Rhomboid-1 is an I-CliP that does not require a preliminary cleavage of its substrate, Spitz, and the single cleavage, which occurs inside a TMH, yields a secreted regulatory peptide (11). The Rhomboid family of serine I-CliPs consists of two subfamilies, denoted the RHO and PARL subfamilies (12), the latter being named after the Presenilins-associated Rhomboid-like protein (13). The conserved core of the Rhomboid family consists of six transmembrane helices. Bacterial and archaeal proteins of this family contain only the six conserved TMHs, whereas most of the eukaryotic members have an additional TMH that is located either upstream (PARL subfamily) or downstream (RHO subfamily) of the core (12).

The yeast member of the PARL subfamily, Pcp1p/Rbd1p, is a mitochondrial I-CliP that appears to regulate different mitochondrial activities through the cleavage of the dynamin-like GTPase Mgm1p, an essential mediator of mitochondrial fusion, and Cep1, a protein required for peroxide and toxic radical scavenging (14–16). PARL, the human ortholog of Pcp1p/Rbd1p, can rescue the phenotype of a yeast strain lacking Pcp1p/Rbd1p activity, therefore indicating that the role of the members of the PARL subfamily in mitochondrial protein processing is conserved in eukaryotes (16). However, Pcp1p/Rbd1p and PARL share no significant conservation in their N-terminal regions, whereas in vertebrates, particularly in mammals, this part of the protein is strongly conserved (12). This suggests
that the N-terminal portion of mammalian PARL could have a distinct, unknown function.

Here, we show that the N-terminal domain of PARL is cleaved in two unique sites. The first cleavage is constitutive and removes the mitochondrial import peptide, whereas the second cleavage, which depends on the I-CliP activity of PARL supplied in trans and is developmentally regulated, liberates Pβ, a nucleus-targeted peptide. Thus, the PARL case represents a new type of RIP in which the putative signaling moiety is part of the I-CliP itself.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfections—Cell lines HEK 293, neuro-2a (N2a), HeLa, and COS-1 were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 μg/ml streptomycin, and 10 μg/ml penicillin. Cells were transfected at 20–40% of confluence with FuGENE (Roche). Cortical primary cultures were prepared from newborn brain of rats as described (17).

Immunoblot and Immunoprecipitation Assays—Forty-eight to seventy-two hours after transfection, cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, and 0.2% Nonidet P-40, pH 7.6) containing a protease inhibitor mixture (Roche Applied Science). Lysate was spun at 10,000 × g for 10 min, and the supernatant was recovered for immunoblot or immunoprecipitation analysis. For immunoprecipitation analysis, an antibody was added, and the immunoprecipitation reaction was incubated at 4°C for 12 h with gentle rocking. Immunocomplexes were captured by the addition of protein A/G-agarose beads (Pierce) and incubation at 2 h at room temperature. Beads were washed three times in wash buffer I (50 mM Tris, 500 mM NaCl, 2 mM EDTA), and 0.2% Nonidet P-40, pH 7.6), washed three times in wash buffer II (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40, and 0.1% SDS, pH 7.6), and resuspended in 1 μl of Laemmli loading buffer. Samples were heated at 85°C for 2 min and separated at 4–12% polyacrylamide–SDS gel (Invitrogen). Proteins were transferred onto nitrocellulose membranes and probed with the specified antibodies as described (18, 19). Immunoblots were developed using the SuperSignal kit (Pierce).

Mitochondria Protease Protection Assay—Mitochondria-enriched preparations were obtained from one Petri dish containing confluent HEK 293 cells transfected with the PARL-HF construct (FLAG-tagged PARL, with an HA tag). Cells were washed in ice-cold Dulbecco’s modified phosphate-buffered saline and disrupted with 10–20 strokes of a Teflon-coated Dounce homogenizer in 0.6 ml of homogenization buffer, which consisted of 0.1 M sucrose, 10 mM MES, 10 mM Hepes, pH 7, 100 mM KCl, 10 mM KH2PO4, 3.5 mM MgCl2, and 1 mM EDTA. Mitochondria-enriched fractions were obtained by differential centrifugation. The pellet was resuspended in 100 μl of homogenization buffer and incubated at 37°C for 10 min, followed by 20-μl aliquots subjected to protease-K or trypsin (Sigma) digestion (1 μg) for 30 min at room temperature.

Antibody Preparation—For the preparation of the anti-PNT antibody (directed against a PARL peptide located near the N terminus), a 12-amino acid-long peptide spanning amino acids 54–66 of PARL was synthesized, purified by high pressure liquid chromatography, conjugated to bovine serum albumin, and used to immunize New Zealand rabbits according to standard 90-day protocols for antiserum production (Covance). Antisera (bleed 2 and 3) was used and diluted 1:1000 and 1:100 for immunoblotting and immunoprecipitation, respectively. The antibody used for immunocytochemical analysis was obtained by peptide-affinity purification of the antisera and used 1:2000 (1:1200 in immunoblot). The specificity of the anti-PNT immunostaining was addressed by pre-adsorbing the antisera with a recombinant GST-PARL N-terminal fusion protein. Anti-PNT immunostaining was abolished when the antibody was incubated 1–4 h at 4°C with lysate of Escherichia coli cells expressing a recombinant GST-PARL N-terminal (amino acids 1–120 of PARL) fusion protein (10).

Constructions—For bacterial expression we used the pGEX-4 vector (Amersham Biosciences), whereas for mammalian expression we used the pcDNA3 (Invitrogen) and the pEGFP-N1 or pEGFP-C1 (Clontech) vectors. Note that the pEGFP-N1 vector used in this study was mutagenized at the methionine residue situated just downstream of the poly-A signal to avoid possible co-expression of GFP with small PARL-GFP fusion proteins (i.e. PARL-(52–77)-GFP), because a Kozak sequence embeds this methionine residue. The amino acid sequence encoded by the FLAG-tagged PARL (PARL-F) construct in the proximity of the PARL C terminus is GPKKGKGGSKDYKDDDK (FLAG peptide is underlined). The amino acid sequence encoded by the PARL-HF construct in the proximity of the PARL TMH1 is PPVEETVPYDVPDYAPGPYPIRLS (HA peptide is underlined).

Protein Sequencing—For N-terminal sequencing of immunoprecipitated proteins by Edman degradation, HEK 293 cells were transfected with the PARL-F construct (Fig. 1A). Two days later, cells were lysed in radioimmuno precipitation assay buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 150 mM NaCl) in the presence of a protease inhibitor mixture (Roche Applied Science). Cell lysate was immunoprecipitated with mouse anti-FLAG-conjugated agarose beads (Sigma), washed, and resuspended in Laemmli buffer. Immunoprecipitated proteins were run on a 4–12% MOPS gel, blotted on polyvinylidene difluoride membranes, and visualized by enhanced chemiluminescence. Protein bands with a molecular weight corresponding to those of the predicted MAMP and PACT proteins were excised from the blot, and their N-terminal sequences were analyzed on an Applied Biosystems Procise cLC494 protein sequencer. Approximately 1 pmol of the protein was loaded on the sequencer. A standard program using liquid phase trifluoroacetic acid was employed for sequencing. The phenylthiodyanthion-derivatives were determined by comparison with phenylthiodyanthion standards (Applied Biosystems) and analyzed on-line on a capillary separation system (ABI 140D) at the start of a sequence analysis.

Immunocytochemistry—Rat neuronal primary cultures were fixed in 4% paraformaldehyde and 0.1 mM phosphate buffer and incubated in rabbit anti-PARL (1:2000), mouse anti-HA rabbit (1:2000), mouse anti-β-tubulin (TuJ-1, 1:1000), or mouse anti-neuronal nuclear protein (NeuN, 1:2000; Chemicon) and visualized using fluorescence-tagged secondary antibodies (Alexa 488 and/or Alexa 546, Molecular Probes). HEK 293 cells were transfected, fixed, and washed in PBS or, for the hypo-osmotic treatment, in water. For confocal immunofluorescence analysis, cells were incubated with mouse anti-mitochondrial oxidative phosphorylation complex III (OxPhos, 1:500; Molecular Probes), rabbit anti-PARL, and mouse anti-HA (1:1000, Covance) and visualized as described above. For electron microscopy analysis, cells were incubated with mouse anti-HA followed by 1-nm gold-conjugated secondary antibody (Aurion) incubation and subsequent silver intensification as described (20). Sections were dehydrated, embedded, cut using Ultra-Cut, and examined using a Tecnai 12 electron microscope.

RESULTS

PARL N Terminus Is Cleaved at the β-Site through a Self-regulated Mechanism—To address the role of the PARL N-terminal domain, a lysate of HEK 293 cells transfected with a PARL-F construct was immunoprecipitated with antibodies raised against a PARL peptide located near the N terminus (designated anti-PNT) and with antibodies against the FLAG epitope (Fig. 1A). With the anti-PNT antibody, two immunoprecipitated bands of ~42 and ~37 kDa were detected (Fig. 1B); we reasoned that these bands should correspond to the intact PARL and to the form with the mitochondrial targeting sequence (MTS) cleaved-off, respectively. We named this second form of the PARL protein MAMP, after mature mitochondrial PARL, and the cleavage site that originates it was designated α-site. PARL insertion in the mitochondria appeared to be an efficient process, as indicated by the low amount of unprocessed, full-length PARL (Fig. 1B). Unexpectedly, a third, specific, smaller protein of ~33.5 kDa was immunoprecipitated with the anti-FLAG antibody. The formation of this smaller, PARL-derived protein was abrogated by mutation of the amino acid residues that form the predicted catalytic site of Rhomboid-like proteases (Fig. 1, A and C) (11, 12, 16). In contrast, the cleavage of the putative MTS, which produced MAMP, was not affected by the same mutations (Fig. 1C). Given the location of the NPT and FLAG epitopes in the constructs used for these experiments, we hypothesized that the 33.5-kDa band was the product of a more distal cleavage of the PARL N terminus (Fig. 1A). We designated this PARL derivative PACT, after PARL C terminus, and the cleavage site that originates it was denoted β-site. We observed increasing accumulation of PACT starting at 48 h post-transfection in HEK 293 cells (Fig. 1D). Similar results were obtained in the murine neuroblastoma N2a cell
FIG. 1. Self-regulated cleavage of PARL. A, a schematic of the domain organization of PARL and the constructs used in transfection experiments, including the epitopes, catalytic residues, α- and β-cleavage sites, and the molecular mass (MW) of the different processed forms of PARL. B, in transfected HEK 293 cells, native, full-length PARL is processed at two sites (α and β). The anti-FLAG antibody immunoprecipitates only two forms of PARL, whereas the anti-PNT antibody immunoprecipitates only two forms (PARL and MAMP). The 33.5-kDa missing band (PACT) therefore does not contain the PNT epitope. C, PARL β-cleavage is self-regulated. In transfected HEK 293 cells, β-cleavage occurs to wild-type PARL but not to mutants lacking serine intramembrane-cleaving protease activity (H221G, S277G, and H335G). D and E, PARL β-cleavage is regulated. In panel D, β-cleavage increases as cells become more confluent (see increased amount of PACT). In panel E, β-cleavage is observed in HEK 293 and murine N2a cell lines but not in HeLa and COS-1 cells (see lack of PACT in HeLa and COS cells). WB, Western blot; WT, wild type.

To gain further insight into the mechanism of the self-regulated cleavage of PARL, we co-transfected HEK 293 cells with the inactivated PARL-HF S277G mutant and the wild-type or the H335G mutant PARL. The formation of PACT from PARL required for the β-cleavage of its N terminus could be supplied in trans. We conclude that β-cleavage is either executed by an unknown protease (PARLase) that is activated via a PARL-catalyzed cleavage or by PARL itself through an intermolecular reaction. However, the latter appears to be a remote possibility because the β-site is not embedded within a TMH, an essential requirement in all known RIP systems (1, 4, 11, 15, 16).

β-Cleavage of PARL Generates Pβ, a Small Nuclear Targeted Peptide—In several RIP signaling systems, released cytosolic fragments of the cleaved membrane-bound precursor proteins (e.g. AID/AICD) (6) are transported into the nucleus where they regulate gene expression (1, 7, 8). Therefore, we hypothesized that the N-terminal peptide generated from the self-regulated β-cleavage of PARL could be a nucleus-targeted molecule. To
test this possibility, we transfected HEK 293 and HeLa cells with constructs expressing different portions of the N-terminal domain of PARL fused C-terminally (GFP-PARL) or N-terminally to GFP (PARL-GFP; Fig. 3A).

Fluorescent microscopy analysis of HEK 293 cells transfected with a construct expressing PARL-(1–52)-GFP showed exclusive mitochondrial localization of this protein, suggesting that the mitochondrial targeting sequence of PARL is located within the region spanning the first 52 amino acids and the conserved -H9251-cleavage site of PARL N terminus (Fig. 2B). This region is predicted to have an amphiphilic α-helical structure, which is typical of mitochondrial targeting sequences, and is identified as an MTS using the MITOPROT program (21). Mitochondrial targeting was abrogated in GFP-PARL constructs that contained GFP at the N terminus, indicating that the N-terminal position of the MTS is critical (Fig. 3A).

In contrast, a PARL-GFP construct that encompassed the region between the α- and β-cleavage sites, (PARL-(53–77)-GFP), which is conserved only in vertebrates, showed a clear nuclear localization (Fig. 3, C and D). Because monopartite and bipartite nuclear localization signals (NLS) are found both at the N and C termini of nuclear proteins and their function is often unaffected by their location within a fusion protein (see also GFP-PARL constructs in Fig. 3A) (22), we suspected that the region between the α- and β-sites might harbor an NLS. Although this sequence does not contain a canonical monopartite or bipartite nuclear localization signal, it contains three closely spaced doublets of positively charged amino acids (spanning amino acids 54–65), the first and second of which are conserved in vertebrates, whereas the third one is mammalian-

**Fig. 2. Identification of the α- and β-cleavage sites in the PARL N terminus.** A, epitope mapping of PARL α- and β-cleavage sites. Both cleavages occur at the N terminus of PARL, before the first TMH of PARL. B, multiple alignments of the N-terminal regions of PARL orthologs from animals. The alignment was generated using the MACAW program (32); regions without statistically significant similarity to mammalian PARLs are shown in lowercase letters. Conserved amino acid residues are shaded. The putative nuclear localization signal is shown in blue, and the rest of the sequences between the α and β sites are shown in red. The PNT epitope is underlined. The beginning of TMH1 is marked with asterisks. The cleavage sites are indicated with arrowheads, and the N-terminal sequences obtained by Edman degradation of MAMP and PACT are shown above the alignment. The mutations that abrogated MAMP cleavage are marked with black circles, and the mutations that had no effect on this cleavage are denoted with empty circles. C, mutations around the β-site block β-cleavage. Shown is an immunoblot (IB) of lysates of HEK 293 cells transfected with the indicated PARL-F mutant (see also Fig. 2B). D, PARL cleavage occurs in trans. HEK 293 cells were co-transfected with different ratios of constructs expressing the wild-type (WT) PARL and the inactive PARL-HF S277G mutant. The inactive S277G mutant is cleaved, indicating that cleavage is supplied in trans through the co-transfected wild-type PARL. WB, Western blot.
In this study and their subcellular localization. Figure 3 represents the PARL-GFP and GFP-PARL fusion proteins used in this study.

**FIG. 3.** Pβ/H9252 contains a nuclear localization signal. A, schematic representation of the PARL-GFP and GFP-PARL fusion proteins used in this study and their subcellular localization. Mitoch., mitochondria.

**B.** The PARL mitochondria targeting sequence is localized within amino acids 1–52. Shown is confocal microscopy analysis of HEK 293 cells transfected with pEGFP-N1 PARL-(1–52) and stained with MitoTracker (MT, red) to visualize mitochondria. C and D, Pβ contains a nuclear localization signal. Fluorescent microscopy analysis of HEK 293 cells expressing PARL-(52–77)-GFP and GFP-PARL-(20–96) fusion proteins (wild-type and mutants at the putative nuclear localization signal) and associated with the removal of the mitochondrial targeting sequence, whereas the β-cleavage is self-regulated and associated to the generation of a nuclear targeting peptide. We designated this nucleus-targeted peptide spanning amino acids 53–77 of PARL Pβ. Accordingly, Pβ is predicted to be a 25-amino acid-long nuclear peptide produced by self-regulated β-cleavage of MAMP.

**PARL β-Cleavage Is Developmentally Regulated**—To investigate β-cleavage of endogenous PARL, we performed immunohistochemical analysis on rat neuronal primary cultures using an antibody raised against a peptide encompassing a sequence located between the α- and β-cleavage sites (anti-PNT; Figs. 1A and 2B). This antibody specifically recognized, by immunohistochemical analysis, PARL-HF in the mitochondria of transfected HEK 293 cells (Supplemental Fig. 2, in the on-line version of this article). In addition, it recognized GFP-PARL-(20–96) in the nuclei of transfected HEK 293 cells (not shown). Specific anti-PNT immunostaining was detected in the nuclei and the mitochondria (Fig. 4A and B). Interestingly, however, immature neurons showed primarily nuclear immunostaining, whereas differentiated neurons had largely mitochondrial immunostaining. The degree of neuronal differentiation was assessed by means of well established morphological criteria, such as the size of the soma and the dendritic arborization pattern (24) (Fig. 4A, right panel), as well as by co-immunostaining with anti-βIII tubulin (TuJ-1) and anti-neuronal nuclear protein (NeuN), markers of early and late differentiation, respectively (Fig. 4B). These findings suggest that, during neuronal differentiation, PARL is cleaved to generate Pβ, which is then targeted to the nucleus (Fig. 4B). Upon differentiation, however, β-processing stops or greatly decreases, as indicated by the anti-PNT mitochondrial labeling and, conversely, by the lack of nuclear immunostaining in differentiated neurons (Fig. 4B, right panel). Thus, in vivo β-cleavage of PARL appears to be under developmental control, and nuclear accumulation of Pβ might be linked to neuronal development.

**PARL β-Cleavage Occurs on the Cytosolic Side of the Mitochondrial Outer Membrane**—The nuclear localization of the Pβ peptide prompted us to investigate the topology and localiza-
tion of PARL in the mitochondria to determine how Pβ/H9252, upon its liberation, could reach the nucleus. To this end, HEK 293 cells were transfected with the PARL-HF construct, followed by immunogold anti-HA electron microscopy analysis. Specific anti-HA labeling was detected on the cytosolic side of the outer membrane of the mitochondria, indicating that the N terminus of PARL protruded into the cytosol (Fig. 5A).

**Fig. 4. PARL β-cleavage is developmentally controlled, and Pβ is a nuclear peptide.** Anti-PNT immunolabeling of primary cultures of rat cortical neurons at different developmental stages is shown. The anti-PNT antibody was raised against a peptide located between the PARL α- and β-cleavage sites (see Figs. 1A and 2B) and can therefore recognize both the mitochondrial form of PARL (MAMP) and the Pβ peptide. A, nuclear immunostaining is observed in an undifferentiated neuron (yellow arrowhead), whereas a prevalent mitochondrial immunostaining is observed in a more differentiated neuron (blue arrowhead). 4’,6-Diamidino-2-phenylindole (DAPI) staining shows the nuclei of these cells (red; false color). Note the differences in the morphology of the two neurons (right panel); the undifferentiated neuron (yellow arrowhead) has small soma and limited dendritic arborization, whereas the differentiated neuron has a larger soma and extensive arborization (blue arrowheads). B, left panel shows neurons co-immunostained with anti-PNT (green) and anti-TuJ-1, an antibody that recognizes a cytosolic neuronal protein that is expressed at an early phase of neuronal differentiation (red; note also the limited arborization of the cells, depicted by the yellow arrowheads). Right panel shows a neuron co-immunostained with anti-PNT (green; note the mitochondria staining) and anti-NeuN (red), an antibody that recognizes a nuclear neuronal protein and a marker for late phases of neuronal differentiation.

**Fig. 5. PARL β-cleavage occurs at the cytosolic side of the mitochondria outer membrane.** A, PARL is inserted into the outer and inner mitochondria membranes. HEK 293 cells were transfected with the PARL-HP construct and either subjected to hypo-osmotic treatment (lower panel) or not (upper panel) prior to immunogold labeling with an anti-HA antibody. Immunolabeling was then visualized by electron microscopy. Gold particles on the cytosolic side of the outer membrane of the mitochondria indicate PARL insertion in this membrane and localization of its N termini to the cytosol (see Fig. 1A). B, β-cleavage occurs at the cytosolic side of the mitochondrial outer membrane. Protease protection assay of mitochondria prepared from PARL-HF-transfected cells is shown (see Fig. 1A). The protease treatment greatly decreases the amount of MAMP and PACT but does not affect OxPhos, a protein inserted in the mitochondrial inner membrane. The disappearance of PACT indicates that β-cleavage occurs at the cytosolic side of the mitochondrial outer membrane.

**Fig. 6. A model for the PARL signaling.** A signal triggers the I-CliP activity of PARL, resulting in the cleavage of either a hypothetical protease (PARLase, depicted in orange) or a regulator thereof. This cleavage activates PARLase that, in turn, cleaves the N terminus of PARL (MAMP), protruding into the cytosol, at the β-site, thus releasing the Pβ peptide. Pβ is then targeted to the nucleus, thanks to its nuclear localization signal, where it participates in nuclear activities.
the cells were subjected to hypo-osmotic treatment, which partially disrupts mitochondrial structure and unveils masked epitopes, some immunolabeling was also detected in the inner membrane (Fig. 5A, lower panel). This finding dovetails with the recent observation that OPA1 (25), the mammalian ortholog of Mgm1p and therefore a probable substrate of PARL, is also present in both mitochondrial membranes (26). These findings were further supported by the results of a protease protection assay on mitochondrial preparations obtained from PARL-HF-transfected HEK 293 cells. This experiment showed that PACT is sensitive to protease digestion (Fig. 5B), thus locating β-cleavage on the outer membrane. Given the topology of PARL (Fig. 5A), we conclude that β-cleavage occurs at the cytosolic side of the outer mitochondrial membrane and that Pβ is released directly to the cytosol, from where it can move to the nucleus (Fig. 6).

**DISCUSSION**

In this study we report that the N-terminal domain of PARL is cleaved at two closely spaced sites, the α- and β-site at positions 52–53 and 77–78, respectively. Whereas the α-cleavage is constitutive and appears to comprise the removal of the mitochondrial targeting sequence, β-cleavage, which was not previously suspected to exist, is regulated and depends on PARL I-CliP activity supplied in trans. These findings suggest that β-cleavage is not simply an alternative cleavage of the mitochondrial import peptide but a distinct event that appears to occur consecutively with the α-cleavage. The β-cleavage generates Pβ, a small peptide that contains a highly effective nuclear localization signal (Fig. 3, C and D) and, in vivo, is targeted to the nuclei (Fig. 4). This conclusion is further supported by the fact that β-cleavage occurs on the cytosolic side of the mitochondrial outer membrane (Fig. 5B).

The small predicted size of Pβ virtually rules out the possibility that this peptide has an enzymatic activity. It seems likely therefore that Pβ functions through a specific interaction(s) with a nuclear factor(s). Small peptides can have potent biological activity, such as hormonal responses (27) and apoptosis (28). Given that Pβ accumulates in the nuclei of immature neurons, this peptide could contribute to regulation of nuclear activities, e.g. by interacting with a transcription factor(s) during neuronal differentiation (Fig. 6).

The apparent "invention" of Pβ and, consequently, of a putative new signaling function of PARL, appears to be a case of "gene sharing" whereby a gene product is recruited, in a specific lineage of descent, for a new function that is often unrelated to the original one (29). Many crucial biological functions, e.g. the role of cytochrome c in programmed cell death, evolved in similar ways (30), and this phenomenon seems to be more common than originally suggested (31). Our data suggest that the β-cleaved signaling function of PARL is a vertebrate-specific or, possibly, mammalian innovation, as suggested by the notable conservation of the Pβ NLS as well as its distal sequence among mammals and, to a lesser extent, other vertebrates, but not between vertebrates and insects (Fig. 2B and Supplemental Fig. 1, found in the on-line version of this article).

The results presented here suggest that, in addition to its evolutionarily conserved activity in regulating mitochondrial dynamics, mammalian PARL was recruited to execute a novel mitochondria-to-nucleus signaling function. Characteristically, in regard to RIP systems (1, 4), this activity appears to be subject to elaborate regulation. Indeed, the release of the Pβ peptide, the putative effector molecule of the PARL signaling, is self-regulated, likely needs the activity of a second protease (PARLase; Fig. 6), and appears to be under developmental control.

It has been recently shown that I-CliPs can be substrates for intramembranous proteolysis (33). Now we show yet an additional and novel type of RIP, which involves a self-regulated, non-intramembranous cleavage of an I-CliP that results in the release of a nuclear-targeted peptide. To our knowledge, this is the first case of RIP where the putative signaling moiety is part of the I-CliP itself.

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