The prohormone convertases (PCs) are an evolutionarily ancient group of proteases required for the maturation of neuropeptide and peptide hormone precursors. In *Drosophila melanogaster*, the homolog of prohormone convertase 2, dPC2 (*amanilato*), is required for normal hatching behavior, and immunoblotting data indicate that flies express 80- and 75-kDa forms of this protein. Because mouse PC2 (mPC2) requires 7B2, a helper protein for productive maturation, we searched the fly database for the 7B2 signature motif PPNPCP and identified an expressed sequence tag clone encoding the entire open reading frame for this protein. dPC2 and d7B2 cDNAs were subcloned into expression vectors for transfection into HEK-293 cells; mPC2 and rat 7B2 were used as controls. Although active mPC2 was detected in medium in the presence of either d7B2 or r7B2, dPC2 showed no proteolytic activity upon coexpression of either d7B2 or r7B2. Labeling experiments showed that dPC2 was synthesized but not secreted from HEK-293 cells. However, when dPC2 and either d7B2 or r7B2 were coexpressed in *Drosophila* S2 cells, abundant immunoreactive dPC2 was secreted into the medium, coincident with the appearance of PC2 activity. Expression and secretion of dPC2 enzyme activity thus appears to require insect cell-specific posttranslational processing events. The significant differences in the cell biology of the insect and mammalian enzymes, with 7B2 absolutely required for secretion of dPC2 and zymogen conversion occurring intracellularly in the case of dPC2 but not mPC2, support the idea that the *Drosophila* enzyme has specific requirements for maturation and secretion that can be met only in insect cells.

Peptide hormones and neuropeptides are frequently synthesized as precursors that are biologically inactive until cleaved and modified through multiple posttranslational processing steps (reviewed in Ref. 1). Many of the proteases involved in these processing steps have been identified and are related to the yeast subtilisin-like endoprotease Kex2. These proprotein or prohormone convertases (PCs) cleave proproteins at pairs of basic amino acids or occasionally at monobasic sites (reviewed in Refs. 2 and 3). To date, seven vertebrate PCs have been reported, namely furin, PACE 4 (paired amino acid-cleaving enzyme 4), PC1/PC3, PC2, PC4, PC5/PC6, and PC7/lympoma PC/PC8. These proteases have different distributions. Although furin and PACE4 are expressed ubiquitously (4, 5), PC5/PC6 is found mostly in gastrointestinal tissues (6, 7). PC4 is expressed in testes (8), and PC7/lympoma PC/PC8 is found in lymphoid tissue (9, 10). On the other hand, PC1 and PC2 are most highly expressed in neuroendocrine cells (11–14). PC1 and PC2 are required in the processing of critical peptide hormone precursors and neuropeptide precursors such as proenkephalin (15, 16), proglucagon (17–20), proopiomelanocortin (21–24), and insulin (25–28). PC2 is unique among proprotein convertases in requiring association with the neuroendocrine-specific protein 7B2 (29) for maturation and activation (30–32).

After removal of the signal peptide, 7B2 is a 185-residue secretory protein (33, 34) composed of two different functional domains that interact with PC2: an N-terminal portion (21-kDa domain), which is involved in proPC2 maturation and activation (30, 35), and a C-terminal 31 amino acid peptide (CT peptide), which is a potent inhibitor of PC2 (36–38). The two domains are separated by a string of five basic amino acids, a recognition site for the proprotein convertase furin (39–41). In AtT-20 cells, 7B2 binds to proPC2 in the endoplasmic reticulum following folding of proPC2 and facilitates transport to the trans-Golgi network as well as the autocatalytic cleavage of the PC2 propeptide (42). Cellular proPC2 synthesis in the absence of 7B2 can also result in propeptide cleavage but yields inactive PC2 even in the presence of exogenously added 7B2 in vitro (30, 43). Recently, a 7B2 null mouse was shown to completely lack PC2 activity in brain extracts (44). This 7B2 null mouse confirms the requirement of vertebrate PC2 for 7B2 in an in vivo model.

1 The abbreviations used are: PC, prohormone convertase; CHO, Chinese hamster ovary; CHO/mPC2 cells, CHO cells stably expressing mouse proPC2; dPC2, *C. elegans* prohormone convertase; CT peptide, C-terminal 31 residues of 7B2; hCT peptide, human CT peptide; dPC2, *Drosophila* prohormone convertase 2; d7B2, full-length *Drosophila* 7B2;endo H, endoglycosidase H; mPC2, mouse prohormone convertase 2; PAGE, polyacrylamide gel electrophoresis; r7B2, rat 7B2; S2, Schneider 2; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; PBS, phosphate-buffered saline.

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Neuropeptides play key roles in a wide range of functions in the metazoa (45). In insects, they are found in numerous neuronal cell types (reviewed in Ref. 46). Neuropeptide biosynthesis in Drosophila is largely undescribed with the exception of enzymes required for C-terminal α-amidation (47). In keeping with their important roles in neuropeptide synthesis, the functional domains of both PC2 and 7B2 are highly conserved throughout evolution. Homologs of PC2 and/or 7B2 have been described in invertebrates such as the mollusc Lymnaea stagnalis (48, 49), the worm Caenorhabditis elegans (50, 51), and Aplysia californica (52). Recently, a homolog of PC2 from Drosophila melanogaster has been identified and termed amonitilado; expression of this gene was found to be essential in the production of the movements that result in hatching (53), presumably to generate bioactive peptides required for this behavior. We wanted to determine whether amonitilado truly encoded a functional protease, implying protease control of behavior in Drosophila. Because PC2 activity is dependent on 7B2 expression in vertebrates, the question of whether Drosophila 7B2 can modulate PC2 activity was also of interest. To this end we identified and cloned 7B2 cDNA (described in Ref. 53) as a template with a 5'-GCTCTAGACCTCTGCGCGACCCG-3' sequence (5'-GCTCTAGACCTCTGCGCGACCCG-3'). This product was digested and ligated into the BamH I and XhoI sites of the pCDNA3 vector (Invitrogen, Carlsbad, CA), and the insert sequence was verified by sequencing. Drosophila PC2 in pCDNA3 was digested with BamHI, blunt-ended with Klenow fragment, digested with XhoI, and subcloned into the EcoRI and XhoI sites of the pAc5.1/V5-HisB vector. Drosophila 7B2 in pBluescript (obtained from Genome Systems) was subcloned directly into the EcoRI and XhoI sites of the pAc5.1/V5-HisB vector (Invitrogen).

Preparation of Recombinant Histagged d7B2—Recombinant His-tagged d7B2 was prepared using the QIAexpress system (Qiagen Inc., Valencia, CA). The DNA encoding d7B2 was generated by polymerase chain reaction using a terminal primer (5'-CGGGCGGATCT-TACCGGTGCAGTCCTAT-3') and a C-terminal primer (5'-CGGGCGGAAGTTTTAGGATAAAAAGGT-3'). The polymerase chain reaction fragment was cloned in pQE30 at the BamHI and HindIII restriction sites. The His-tagged d7B2 construct was verified by DNA sequencing. Proteins were expressed in Escherichia coli XL-1 Blue (Stratagene, La Jolla, CA), induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) and purified based on the guanidine-HCl extraction method (55). Briefly, cells from a 500-ml culture were lysed in 20 ml of buffer A (10 mM Heps, pH 7.5, containing 6 mM guanidine HCl) for 1 h in the cold room, centrifuged for 15 min at 10,000 × g, and loaded onto a nickel-nitrotriacetic acid column (1.5 ml of resin) previously equilibrated with buffer A and washed with buffer A using a FPLC protein liquid chromatograph. The denatured protein was renatured using a linear gradient of buffer A and buffer B (10 mM Heps, pH 7.5, and 0.1 M NaCl) at a flow rate of 0.4 ml/min over 130 min. After washing with 10 column volumes of 10 mM Heps, pH 7.5, containing 50 mM NaCl, proteins were eluted from the column with 30 ml of buffer C (100 mM Heps, pH 7.5, containing 250 mM imidazole, 50 mM NaCl, and 0.02% NaN3) at a flow rate of 0.3 ml/min. Fractions were stored at 70 °C; an aliquot of each was analyzed on a 15% SDS-PAGE gel using Coomasie staining. A predominant band was observed on a SDS-PAGE gel with the expected molecular mass and an estimated purity of greater than 90%.

Transient Transfection of Drosophila S2 Cells—Transient transfection of DPC2 and/or d7B2 into S2 cells was performed as described in the Drosophila expression system instruction manual from Invitrogen (Invitrogen, Carlsbad, CA). Briefly, 1 × 106 S2 cells per ml were seeded into a 35 mm plate in 2.5 ml complete Drosophila expression system medium and were grown overnight at 24 °C. A total of 19 μg DNA per plate were transfected in the presence of the CaCl2 and Heps-buffered saline (HBS) solutions provided by Invitrogen. Twenty-four hours after transfection, the cells were washed with Drosophila expression system serum-free medium and were incubated in 1 ml of Drosophila expression system serum-free medium overnight at 24 °C. The overnight medium was then used for PC2 activity assays and for Western blotting.

Transient Transfection, Metabolic Labeling, and Immunopurification—CHO cells expressing mPC2 (40) were split to 3 × 106 cells/well to a 12-well tissue culture plate on the day before transfection and incubated with an antiseraum (bleed 4, diluted 1:15000) against the C terminus of dPC2. Antisera were mixed in 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 5% nonfat dry milk, 0.1% Tween 20, and 0.02% NaN3 at 70 °C; an aliquot of each was analyzed on a 15% SDS-PAGE gel using Coomasie staining. A predominant band was observed on a SDS-PAGE gel with the expected molecular mass and an estimated purity of greater than 90%.
same method described above except that Drosophila expression system serum-free medium was used for the chase medium. Metabolic labeling of Drosophila S2 cells was performed at room temperature.

Preparation of Golgi-enriched Subcellular Fractions—Golgi-enriched subcellular fractions were prepared from CHO cells overexpressing mouse proPC2 (CHO/mPC2 cells) as described previously (42). Briefly, CHO/mPC2 cells were grown in four 850-cm² rollers for each membrane preparation. All of the following steps were performed on ice or at 4 °C. After washing with calcium-free PBS, cells were scraped in this buffer and pelleted at low speed. Cells were then resuspended in 6 ml of 0.25 M sucrose in 10 mM Tris, pH 7.4, and gently homogenized with a ball-bearing homogenizer. The homogenate was centrifuged for 20 min at 8,000 rpm, and the supernatant was pelleted at 56,000 rpm in a Beckman TL 100.4 rotor for 35 min. Pellets were resuspended in 1.5 ml of 1.15 M sucrose in 10 mM Tris, pH 7.4, and loaded on the bottom of a discontinuous sucrose gradient composed of 1.5 ml of 0.86 M sucrose in 10 mM Tris, pH 7.4, and 1.5 ml of 0.25 M sucrose. The gradient was centrifuged at 46,000 rpm in the same rotor for 140 min. Five fractions were collected, and each fraction was assayed using membrane markers as described previously (42).

In Vitro Activation of PC2—Golgi-enriched fractions (15 µg protein) were incubated with 200 nM of purified His-tagged 7B2s in 100 mM sodium acetate, pH 5, in the presence of 0.2% Triton X-100, 5 mM CaCl₂, and a protease inhibitor mixture composed of 1 µM trans-eugenol, a 1 µM pepstatin, 280 µM tosylphenylalanine chloromethyl ketone, and 50 µM 3-[(p-nitrophenyl) chloromethyl]ketone. Incubations were conducted at 37 °C for 6 h. PC2 activity was estimated using 200 µM Pyr-Glu-Arg-Thr-Lys-Arg methylcoumarinamide as a substrate as described below.

The identification of enzymatic activity as PC2-specific was assessed by measuring the extent of inhibition with 1 µM hCT peptide, a PC2-specific inhibitor that corresponds to the C-terminus of human 7B2 (36). Experiments were performed with Golgi fractions obtained from three different preparations.

PC2 Enzyme Assay—The PC2 enzyme assay was performed as described previously (36). Briefly, PC2 activity was measured in 100 mM sodium acetate, pH 5.0, containing 5 mM CaCl₂, 0.4% octyl glucoside, and a protease inhibitor mixture indicated above, using 25 µl of the 25% protein A-Sepharose bead slurry containing immunopurified proteinase inhibitor mixtures obtained from transfected cells. 1 µM hCT peptide (final concentration), a PC2-specific inhibitor (36), was added to some samples to assess specificity. The incubations were conducted at 37 °C using Pyr-Glu-Arg-Thr-Lys-Arg-methylcoumarinamide as a substrate; the fluorescence of the product was quantified by reference to a free aminomethylcoumarin standard.

Immunoprecipitation—For immunoprecipitation under denaturing conditions, cells were boiled for 5 min in 0.1 ml of boiling buffer (50 mM sodium phosphate, pH 7.4, 1% SDS, 50 mM β-mercaptoethanol, and 2 mM EDTA). Cells were then diluted with 0.9 ml of AG buffer (0.1 M sodium phosphate, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, and 0.9% NaCl). For non-denaturing immunoprecipitation, cells were extracted as described previously (42). 500 µl of each sample were preincubated with 0.1 ml of 20% protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) hydrated, washed with AG buffer at 4 °C for 1 h, and then centrifuged. 15 µl of each sample were then washed three times with AG buffer, were then added, and the samples were rocked at 4 °C for 1 h. The beads were washed twice with AG buffer, once with 0.5 M NaCl in PBS, and twice with PBS. Immunoprecipitates were resuspended in Laemmli sample buffer and analyzed using 8.8% SDS-PAGE. After electrophoresis, gels were fixed with 25% methanol containing 10% acetic acid for 30 min and exposed to a Storm PhosphorImager screen and analyzed with ImageQuant.

RESULTS

Drosophila Tissues Express PC2 Protein—To assess the molecular mass of dPC2 in insect tissues, polyclonal antiserum against dPC2 was prepared using a synthetic peptide corresponding to the last 10 residues of dPC2 coupled to keyhole limpet hemocyanin. By Western blot analysis (n = 10), we detected two prominent dPC2 immunoreactive bands of 80 and 75 kDa (Fig. 1a) in insect tissues. The larger band co-migrated with proPC2 synthesized in Drosophila S2 cells (data not shown). A prominent 35-kDa band was also present, presumably representing a C-terminal fragment of dPC2, because the antiserum is directed toward this terminus. A 45-kDa N-terminally truncated protein has been observed in mammalian PC2-expressing tissues (43); the significance of this cleavage event is unknown, but it would be expected to destroy catalytic activity. Preimmune serum used at the same dilution did not detect any of these bands (results not shown), suggesting that these bands indeed represent three different forms of Drosophila PC2. The anterior section of third instar contained much more material than did posterior sections. From analysis of dissected tissues, we determined that the source of most larval signals was the anterior midgut, although material was also detected in CNS and salivary gland (data not shown).

Drosophila PC2 was found in larval, pupal, and adult developmental stages but was rarely detected in adult heads (Fig. 1b).

A 7B2-like Sequence Is Present in Drosophila—Because 7B2 is co-expressed with PC2 in mammalian tissues and because PC2 expression is known to be critical to PC2 activity, we searched for this protein in the D. melanogaster expressed sequence tag data base using the PPNPCP consensus sequence, conserved in all known 7B2s (51). A cDNA encoding 261 amino acids was identified that contained this sequence; it was obtained from Genome Systems and sequenced from both directions. A notable feature is the conservation of the 86–121-residue sequence containing a proline-rich region, the minimum sequence required for r7B2 formation (35). Conservation of a CT peptide-like sequence in the C terminus is also apparent (Fig. 2). Interestingly, the encoded protein appeared to lack a furin consensus sequence for cleavage into the 21-kDa and CT peptide domains; it is the second 7B2 sequence cloned thus far that lacks this feature (Lymnaea 7B2 also lacks a furin consensus cleavage site; Ref. 49). The lack of this site in two species may imply that furin cleavage into two domains is not required for 7B2 function. Drosophila 7B2 also contains an inhibitory CT peptide-like sequence containing the highly conserved heptapeptide V/1DNPY/FILGQ as well as the KK pair observed in all 7B2s cloned thus far (Ref. 51; reviewed in Ref. 56).

Drosophila 7B2 Can Confer Activatability upon Mouse proPC2—To assess the functional conservation of d7B2, we examined the ability of Drosophila 7B2 (d7B2) to produce active PC2 from mouse proPC2 using two different functional
tests, one in vivo and one in vitro. In the first series, we transiently transfected into CHO/mPC2 cells: 1) the backbone vector as a control; 2) rat 27-kDa 7B2 (r27 kDa); 3) rat 21-kDa 7B2 (r21 kDa); or 4) d7B2 (full length; corresponding to rat 27-kDa 7B2). The overnight conditioned medium was then tested for PC2 activity. Drosophila 7B2 was capable of activating mouse proPC2 in CHO/mPC2 cells, although not as efficiently as r27 kDa and r21 kDa 7B2 (Table I). The difference in potency between rat and Drosophila 7B2s may be due to species specificity or to differential expression of 7B2s in transfections; however, we tested for the latter possibility by coimmunoprecipitation with PC2 antisera and observed roughly comparable expression of all 7B2s (results not shown).

Although the mechanism of facilitation of proPC2 activation by 7B2 is unknown, we have previously observed that proPC2 activation can be reconstituted in Golgi-enriched fractions by the addition of recombinant 7B2 (42). To study the ability of d7B2 to facilitate mPC2 activation in an in vitro system, we purified recombinant His-tagged d7B2 from E. coli and assayed its intrinsic activity by incubating recombinant d7B2 with a Golgi membrane-enriched fraction from CHO/mPC2 cells; this Golgi fraction is enzymatically inactive unless exposed to recombinant 7B2 (42). Recombinant d7B2, which corresponds to rat 27 kDa 7B2, was 12% as active on Golgi-derived mouse proPC2 as compared with recombinant r21 kDa 7B2 (Table II). The enzymatic activity generated by d7B2 was completely inhibited by 1 μM hCT peptide 1–31, a potent and specific inhibitor of PC2, suggesting that it indeed results from mouse proPC2 activation. A comparison of PC2 activity in the presence of recombinant d7B2 and r27 kDa 7B2 confirmed that d7B2 was able to facilitate the activation of Golgi-derived mouse proPC2 (Table II). The apparently lower activity of d7B2 compared with r21 kDa 7B2 is likely to be at least in part an artifact caused by inhibition of the inhibitory CT peptide sequence present in the d7B2 construct but lacking in the r21 kDa 7B2 construct. Indeed, purified His-tagged d7B2 inhibited purified recombinant mPC2 with an IC50 of 3 μM (data not shown), supporting the ability of d7B2 to inhibit mPC2. The ability of d7B2 to facilitate the activation of mouse proPC2 is not surprising because the polypyrrole-containing segment critical for conferring facilitation of proPC2 activation (amino acids 86–121 in rat 7B2; Ref. 35) is highly conserved in the Drosophila 7B2 molecule (see the alignment of 7B2s in Fig. 2).

Drosophila PC2 Is Inactive When Expressed in CHO/r7B2 Cells—To examine the enzymatic activity of dPC2, plasmids encoding either mPC2 or dPC2 were transiently transfected into CHO cells overexpressing r21 kDa 7B2 (CHO/r7B2) cells. The overnight conditioned medium from each transfection was assayed for PC2 activity in vitro, as has been previously described for mPC2 (30). Surprisingly, medium obtained from cells transfected with dPC2 plasmid was completely enzymatically inactive, although medium obtained from control mPC2-transfected cells exhibited PC2 activity (Fig. 3). Several possibilities exist to explain these results. One explanation is that dPC2 may not be well expressed or secreted in CHO/r7B2 cells. Alternatively, dPC2 may exhibit a strict requirement for its own Golgi fraction, for an insect cell line host, or both, to exhibit activity.

Drosophila proPC2 Is Synthesized in HEK-293 Cells but Is Enzymatically Inactive and Cannot Be Secreted—We assessed whether dPC2 was well expressed from our mammalian expression vector using pulse-chase analyses after transient co-transfection with d7B2 or r7B2 in HEK-293 cells; we used HEK-293 cells because of their high protein expression levels in transient transfections. After transfection, cells were labeled with [35S]Met/Cys for 20 min and chased for 2 h in methionine/cysteine-containing medium. Labeled cells and chase media were then subjected to immunoprecipitation under denaturing or non-denaturing conditions. As shown in Fig. 4a, dPC2 co-transfected with d7B2, r21 kDa, or r27 kDa 7B2 was not secreted into the medium, which also tested negatively for PC2 activity (data not shown). This apparent lack of secretion of dPC2 in HEK-293 cells is consistent with the absence of dPC2 activity in CHO/r7B2 cell medium (Fig. 3). In contrast, mPC2 cotransfected with either r27 kDa 7B2 or d7B2 was successfully secreted (Fig. 4a). Physical interaction between mPC2 and d7B2 was confirmed by co-immunoprecipitation and SDS-PAGE on 15% gels (data not shown).

We then examined the level of intracellular expression of dPC2 in HEK-293 cells by performing immunoprecipitation of labeled cells under denaturing conditions after transfection with plasmids encoding dPC2, mPC2, d7B2, and r27 kDa 7B2. Drosophila PC2 transfected together with either d7B2 or r27 kDa 7B2 was found to be almost as well expressed as mPC2 in HEK-293 cells (Fig. 4b). We assayed these cell extracts for PC2 activity using an immunopurification procedure (Fig. 4c). Following immunopurification of all forms of PC2 from HEK-293
cells were labeled with [35S]Met/Cys and chased for 2 h. The chase control vector (pCEP4), r27 kDa, r21 kDa 7B2, or d7B2. Transfected transiently transfected into HEK-293 cells together with either the cannot be secreted in HEK-293 cells. dPC2 and mPC2 constructs were enzymatically inactive when expressed in HEK-293 cells—secreted and is enzymatically inactive when expressed in HEK-293 cells. Drosophila PC2 is enzymatically active and is secreted only as mature dPC2 in Drosophila S2 cells. a, enzymatic activity assay of dPC2. Plasmids encoding Drosophila PC2 and 7B2 were transfected into S2 cells. Data depict either PC2 alone (closed triangles), 7B2 alone (open inverted triangles), or dPC2 and d7B2 together (open circles). After transfection, the overnight medium was tested for PC2 activity under standard assay conditions. Values are the means ± S.D. (n = 3; error bars smaller than the symbols are not shown). b, Western blot analysis of the conditioned medium from S2 cells. 40 μl of each overnight conditioned medium were subjected to SDS-PAGE (8.8% gel), transferred to nitrocellulose, and blotted with antiserum against the C-terminal region of dPC2.

To investigate the possibility that Drosophila proPC2 might require particular host cell maturation conditions that cannot be supplied by mammalian cells.

Drosophila PC2 Is Successfully Activated by d7B2 and Secreted in Drosophila S2 Cells—To investigate the possibility that dPC2 might require species-specific factors for production of an activable zymogen, we subcloned dPC2 and d7B2 into insect expression vectors and transiently transfected either plasmids encoding dPC2 and d7B2 alone, or dPC2 and d7B2 together, into Drosophila S2 cells. We assayed the overnight conditioned medium for PC2 activity in the presence or absence of 1 μM hCT peptide 1–31. As shown in Fig. 5a, medium from S2 cells cotransfected with dPC2 and 7B2 cDNAs exhibited much higher enzymatic activity compared with medium obtained from cells transfected with only dPC2 or only d7B2. Drosophila PC2 activity could not be inhibited by hCT peptide at 1 μM, a concentration that results in total inhibition of mPC2 (data not obtained from coexpressions with either r27 kDa 7B2 or d7B2 were enzymatically active (Fig. 4c).

The complete lack of activity of dPC2 expressed in HEK-293 cells together with d7B2 was unexpected given the previously demonstrated ability of d7B2 to function properly with mPC2. We considered the possibility that the lack of activity of dPC2 might result from suboptimal assay conditions for this enzyme. To assess this possibility, we performed PC2 assays of immunopurified dPC2 at several different pHs (pH 5, 5.5, 6, 6.5, and 7), but dPC2 activity was not detected (data not shown). The lack of dPC2 activity in HEK-293 and CHO/r7B2 cells was therefore considered most likely to be due to improper intracellular conditions that resulted in a lack of proper folding (and therefore secretion) of dPC2 in these mammalian cell types. We hypothesize that Drosophila proPC2 might require particular host cell maturation conditions that cannot be supplied by mammalian cells.
After 36 h, cells were labeled with [35S]Met/Cys for 20 min and chased either d7B2 or r27 kDa 7B2 were transiently transfected into S2 cells. The absolute requirement of dPC2 for 7B2 for secretion could represent a property of S2 cells or could be specific to the dPC2 sequence. To distinguish between these possibilities, mPC2 and mouse 7B2s were also transiently transfected with r21 kDa 7B2 into S2 cells, labeled under the same conditions as for dPC2 labeling, and chased for 2 h. The chase medium as well as the cell extracts were then immunoprecipitated using antisera against the C terminus of mPC2 (Fig. 6b). As shown in Fig. 6b, mPC2 was secreted into the medium, independent of the presence of 7B2, and was secreted as a mixture of proPC2 and the cleaved form.

Figure 6. Conversion and secretion of Drosophila proPC2 in S2 cells requires 7B2. a, pulse-chase metabolic labeling of S2 cells after transient transfection. Plasmids encoding dPC2 alone or dPC2 with either d7B2 or r27 kDa 7B2 were transiently transfected into S2 cells. After 36 h, cells were labeled with [35S]Met/Cys for 20 min and chased for 0 or 1 h in Met/Cys-containing medium. dPC2s from cell extracts and media were immunoprecipitated under denaturing conditions. Note the absence of secreted PC2 in the absence of 7B2, and the complete intracellular cleavage of Drosophila proPC2 to PC2. Both d7B2 and r7B2 facilitated dPC2 in an identical manner. T1 represents 0 h chase time; T2, 1 h chase time. b, a similar experiment was performed using mPC2 except that chase times were 0 or 2 h (T0 or T2). The asterisk represents the intermediate form of proPC2s. C represents cell extracts; M represents medium.

We used Western blotting to confirm the presence of secreted dPC2 forms in the medium. In accordance with the observation of proPC2 activity, media obtained from cells cotransfected with dPC2 and d7B2 contained high levels of secreted dPC2 protein (Fig. 5b). Interestingly, Drosophila proPC2 found in the medium consisted entirely of the mature lower molecular mass form, indicating complete propeptide cleavage prior to secretion. Medium obtained from S2 cells transfected only with dPC2 in the absence of d7B2 exhibited neither secreted PC2 protein nor enzymatic activity. Western blot analysis of dPC2 from the latter cell extracts showed high intracellular expression of proPC2 (data not shown). It therefore appears that coexpression with d7B2 is absolutely required for secretion of dPC2 and, further, that in the presence of 7B2, S2 cells are able to carry out intracellular conversion of Drosophila proPC2. These data point to significant differences in the cell biology of Drosophila proPC2 as compared with mouse proPC2, which in constitutive cell lines is only poorly converted prior to secretion and has no requirement for 7B2 for secretion (32, 43, 57).

We confirmed these results using pulse-chase metabolic labeling of S2 cells transiently transfected with dPC2/d7B2 and mPC2/r7B2 (Fig. 6). 90% of newly synthesized dPC2 was secreted into the medium during a 1-h chase, and immunoreactive secreted PC2 consisted entirely of the lower molecular mass form (Fig. 6a). Drosophila PC2 from S2 cells transfected with dPC2 in the absence of 7B2 remained intracellular during the chase period. We also could not detect secreted dPC2 when transfected without 7B2 in the 2-h chase medium (data not shown). In contrast, mPC2 was secreted into the medium in a 7B2-independent fashion during a 2-h chase (see below). Drosophila PC2 transfected together with r27 kDa 7B2 behaved identically to Drosophila PC2 transfected together with Drosophila 7B2 (Fig. 6a), indicating that the Drosophila and mouse 7B2s were interchangeable.

The absolute requirement of dPC2 for 7B2 for secretion could represent a property of S2 cells or could be specific to the dPC2 sequence. To distinguish between these possibilities, mPC2 was also transiently transfected with r21 kDa 7B2 into S2 cells, labeled under the same conditions as for dPC2 labeling, and chased for 2 h. The chase medium as well as the cell extracts were then immunoprecipitated using antisera against the C terminus of mPC2 (Fig. 6b). As shown in Fig. 6b, mPC2 was secreted into the medium, independent of the presence of 7B2, and was secreted as a mixture of proPC2 and the cleaved form. Mouse PC2 cotransfected with r27 kDa 7B2 also yielded the same result as in Fig. 6b (data not shown). These data indicating that dPC2, but not mPC2, absolutely requires 7B2 for secretion are unique because inactive mPC2 forms, both proPC2 as well as processed forms, can be well secreted from CHO cells in the absence of 7B2 (43).²

We conclude that although both dPC2 and mPC2 share the absolute requirement for 7B2 for expression of enzymatic activity, the maturation of dPC2 differs substantially from that of mPC2 in three respects: (1) the ability of the zymogen to mature intracellularly in constitutive cells; (2) the absolute requirement for 7B2 for secretion of dPC2; and (3) the secretion of only mature forms.

### DISCUSSION

In this paper we present the characterization of an important prohormone converting enzyme in Drosophila, dPC2 (amontillado). Drosophila PC2 RNA is found in a stereotyped pattern in the embryonic CNS (53) and is necessary for the production of the movements that allow hatching. Drosophila PC2 may contribute to the processing of various neuropeptide precursors (e.g. those resulting in FMRFamide (58), dromyosuppressin (59), and corazonin (60)). The mature peptides then act as neurotransmitters and hormones in the regulation of physiology (61) and the organization of behavior (62, 63). Like its mammalian counterpart, Drosophila proPC2 requires the aid of the helper protein 7B2 for expression of enzymatic activity; however, as detailed below, the insect enzyme exhibits significant differences in its cell biology from the mammalian homologs.

**Drosophila 7B2 Is Interchangeable between Mammalian and Insect Cells**—The neuroendocrine-specific protein 7B2 possesses a signature hexapeptide present in all species cloned thus far, PPNPCP (51). We used this sequence to identify d7B2 and r7B2 from Drosophila melanogaster and rat 7B2s can confer activity, albeit reduced, upon mPC2 and dPC2, respectively. Note that the 36-residue segment (NH2-PPNPCP-COOH) represents the intermediate form of proPC2 as well as processed forms, can be well secreted from CHO cells in the absence of 7B2 (43).²

² I. Lindberg, unpublished results.
activity (36). Cross-species inhibition has been previously observed; even though the two *Lymnaea* CT peptide sequences have several substitutions as compared with the rodent CT peptide, segments of the *Lymnaea* CT peptides, LCT1 and LCT2, inhibit mPC2 activity weakly (IC₅₀ values of 154 and 36 μM, respectively; Ref. 49). These *Lymnaea* peptides were, however, completely unable to inhibit dPC2 activity (results not shown). Taken together, the CT inhibition data suggest that the nonconserved CT peptide sequences flanking the conserved heptapeptide and the KK pair may confer species-specific inhibition, most likely by binding to nonconserved sites within the various PC2s. To confirm this hypothesis, the inhibition of dPC2 and mPC2 activity by d7B2 CT-related peptides would need to be tested.

Some controversy exists in the identification of regions required for the 7B2-PC2 interaction. Benjannet et al. (32) have demonstrated that the pentabasic furin cleavage site in 7B2 is important for binding to PC2. However, we have shown that binding of the 21-kDa form of 7B2, which lacks the pentabasic sequence, is sufficient to effect proPC2 activation (35). Like *Lymnaea* 7B2, d7B2 has no furin cleavage site and is nonetheless active in facilitating the activation of proPC2. Thus the presence of a furin cleavage site may not be critical for the role of 7B2 in proPC2 activation. *Drosophila* 7B2 has a dibasic amino acid site (Lys²²³-Lys²²⁴) near the beginning of the CT peptide that could conceivably substitute for the furin site; however, another enzyme rather than furin must necessarily perform this cleavage because furin cannot cleave at paired lysines (Ref. 64; reviewed in Ref. 65).

*Drosophila* proPC2 Requires an Insect Cell Host for Activation—In the present study, we show that *Drosophila* PC2 cannot be synthesized in an activable form in mammalian cells even in the presence of its cognate 7B2, whereas this insect enzyme is both secreted and proteolytically processed in insect cells. Unlike immunopurified mouse proPC2, *Drosophila* proPC2 immunopurified from HEK-293 cells coexpressing 7B2 was unable to undergo conversion to active PC2. Therefore, *Drosophila* proPC2 may not fold properly in HEK-293 cells. Although the relatively low expression level of dPC2 observed in HEK-293 cells is a concern (potentially because of degradation of unfolded protein), our data support the idea that this insect protease cannot be made in active form in mammalian cells. By contrast, a constitutively secreting insect cell line, S2, was able to support the expression of enzymatically active dPC2, if cotransfected with 7B2.

*Drosophila* PC2 has an Absolute Requirement for 7B2 for Secretion—In the present study, we show that dPC2 secretion is dependent on the coexpression of 7B2. This is quite dissimilar to mammalian PC2s, which can be secreted in the absence of 7B2 in constitutively secreting mammalian cells such as CHO (43), COS-7 (57), and BSC-40 (66) cells, although under these circumstances the secreted PC2 forms are enzymatically inactive and incompetent for activation (30, 43). Overexpression of 7B2 is not required for secretion of proPC2/PC2 from neuroendocrine cells such as AtT-20 (30), rMTC 6–23 (67), and SK-N-MCIXC (68), although overexpression of 7B2 has been observed to facilitate the secretion of PC2 from neuroendocrine cells (30). The absolute requirement of dPC2 for 7B2 for secretion is thus unprecedented and suggests a curious additional role for 7B2 in the secretion of dPC2 in S2 cells.

*Drosophila* proPC2 Is Fully Processed Prior to Secretion—Interestingly, *Drosophila* proPC2 apparently cannot be secreted as such but is completely cleaved to the mature form prior to secretion. In contrast, a mixture of proPC2 and mature PC2 is secreted from mammalian cells, both non-neuroendocrine and neuroendocrine cell lines, with non-neuroendocrine cells secreting predominantly proPC2 (32, 42, 43, 57, 68, 69).²

Because r7B2 also exhibits the same facilitatory effect on *Drosophila* proPC2 cleavage, these results cannot be due to the specific interaction of d7B2 with dPC2 but represent a property of S2 cells and/or the *Drosophila* proPC2 sequence. Considering that S2 cells represent a constitutive cell line (70) lacking the regulated secretory compartment known to be required for productive intracellular maturation of vertebrate proPC2s (24, 56, 71), *Drosophila* proPC2 conversion in S2 cells is likely to take place within late secretory compartments, such as the trans-Golgi network. However, the S2 trans-Golgi network compartment apparently does not offer a comparably hospitable environment for the complete maturation of mouse proPC2, which is secreted from S2 cells in a manner similar to HEK-293 cells, i.e. predominantly, although not entirely, uncleaved. The S2 host cell environment cannot therefore be solely responsible for the differences in maturation of the mammalian versus the insect zymogens; sequence differences between the two types of PC2s must play a major role.

An inspection of the primary sequence of all known PC2s reveals that invertebrate PC2s exhibit glycosylation site patterns that differ radically from those of vertebrates. Fig. 7 shows a schematic comparison of the sequences encoding PC2s from *Drosophila*, *C. elegans*, and mouse. Compared with mPC2, *Drosophila* PC2 possesses a completely different pattern of putative glycosylation sites (other vertebrate PC2s contain the same glycosylation pattern as mouse). Note that *C. elegans* PC2 (ePC2) has two additional putative glycosylation sites as compared with dPC2, as well as three glycosylation sites represented in dPC2. These different glycosylation patterns among vertebrates and invertebrates might be responsible for the failure of proper folding and of activation of dPC2 in mammalian cell lines such as CHO and HEK-293. This speculation is supported by our finding that we were also unable to detect ePC2 activity upon transfection of ePC2 cDNA into r21 kDa 7B2-expressing CHO cells.³ Others have previously observed that glycosylation is key to proper maturation of proPC2 in neuroendocrine cells; conversion of proPC2 to PC2 apparently occurs so rapidly upon acquisition of endoglycosidase H (endo H) resistance that cells contain no endo H-resistant proPC2 nor endo H-sensitive PC2 (71, 72). We have previously observed that mutation of all three glycosylation sites in the catalytic P domains of mPC2 from Asn to Gln causes the protein to be retained in the endoplasmic reticulum and to be subjected to degradation (42).⁴ A similar result occurs if the glycosylation site in the catalytic domain is spared.⁴ Considering the impor-

³ B. Tu and I. Lindberg, unpublished results.

⁴ J. R. Hwang and I. Lindberg, unpublished results.
tance of correct N-linked glycosylation for mPC2, one explana-
tion for the lack of proper maturation of dPC2 in mammalian
cells might be that mammalian cells fail to properly glycosylate
the Drosophila enzyme. However, we were unable to detect a
difference in molecular mass of the pro form of dPC2 from
HEK-293 and S2 cells as judged by SDS-PAGE (data not
shown), suggesting strongly that the total number of sites used
is the same in both the mammalian and insect cells. Intracellular
proPC2s from both Drosophila and rat were similarly
sensitive to endo H when expressed in HEK-293 cells (data not
shown), which implies that both species of proPC2 undergo
endoplasmic reticulum glycosylation (and also that transloca-
tion into the secretory pathway has successfully occurred). It is
possible that insect cell-specific differences in Golgi modifi-
cations of the N-linked oligosaccharides are critical for activation
and secretion of Drosophila proPC2. Alternatively, Drosophila
proPC2 may require insect cell-specific factors for either folding
in or exit from the endoplasmic reticulum.

It is interesting to note that despite the lack of regulated
secretory vesicles in S2 cells, the cell biology of dPC2 in S2 cells
is actually more similar to mPC2s expressed in neuroendocrine
cells than to mPC2s expressed in constitutively secreting cells;
i.e., proPC2 matures within the cell, and 7B2 enhances secre-
tion of the mature form (30, 67, 68). We hypothesize that the
ability of Drosophila proPC2 to become activated in constitut-
ev cells constitutes a functionally loose expression of activity
that may be important to the production of biologically impor-
tant peptides in Drosophila. Additionally, our results provide
interesting evolutionary variations in the cell biology of this protein
that may be applicable to the maturation of other regulated
secretory proteins.

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