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Species Selectivity of Mixed-Lineage Leukemia/Trithorax and HCF Proteolytic Maturation Pathways

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Site-specific proteolytic processing plays important roles in the regulation of cellular activities. The histone modification activity of the human trithorax group mixed-lineage leukemia (MLL) protein and the cell cycle regulatory activity of the cell proliferation factor herpes simplex virus host cell factor 1 (HCF-1) are stimulated by cleavage of precursors that generates stable heterodimeric complexes. MLL is processed by a protease called taspase 1, whereas the precise mechanisms of HCF-1 maturation are unclear, although they are known to depend on a series of sequence repeats called HCF-1PRO repeats. We demonstrate here that the Drosophila homologs of MLL and HCF-1, called Trithorax and dHCF, are both cleaved by Drosophila taspase 1. Although highly related, the human and Drosophila taspase 1 proteins display cognate species specificity. Thus, human taspase 1 preferentially cleaves MLL and Drosophila taspase 1 preferentially cleaves Trithorax, consistent with coevolution of taspase 1 and MLL/Trithorax proteins. HCF proteins display even greater species-specific divergence in processing: whereas dHCF is cleaved by the Drosophila taspase 1, human and mouse HCF-1 maturation is taspase 1 independent. Instead, human and Xenopus HCF-1PRO repeats are cleaved in vitro by a human proteolytic activity with novel properties. Thus, from insects to humans, HCF proteins have conserved proteolytic maturation but evolved different mechanisms.

Site-specific proteolysis has emerged as an important mechanism contributing to the regulation of basic cellular processes such as development (24), metabolism (3), cell cycle progression (7), and apoptosis (22). Site-specific proteolysis regulates both physiological and disease processes, often by activating latent functions.

In human cells, a number of nuclear proteins, such as the trithorax group mixed-lineage leukemia (MLL) protein (11, 21, 35) and herpes simplex virus (HSV) host cell factor 1 (HCF-1) (15, 31, 32), are synthesized as precursors that undergo proteolytic maturation to generate stable, noncovalently associating heterodimeric complexes. The importance of these proteolytic maturation processes is underscored by the finding that a human proteolytic activity with novel properties. Thus, from insects to humans, HCF proteins have conserved proteolytic maturation but evolved different mechanisms.

Of these four proteins, MLL is the largest, consisting of 3,969 amino acids. It was originally discovered because the gene encoding MLL is the site of chromosomal translocations in human childhood leukemias (1, 5, 8). MLL possesses histone H3 lysine 4 (H3K4) methyltransferase activity (20, 21) and is required for maintaining proper Hox and Cyclin gene expression (26, 37). MLL is cleaved by taspase 1 (10) at two sites to generate the associated amino-terminal (MLLα) and carboxy-terminal (MLLc) subunits (11, 21, 35). This proteolysis enhances the H3K4 methyltransferase activity of the MLLc subunit, which promotes Cyclin gene expression and cell cycle progression (26).

The enzyme responsible for Trx cleavage is not known, but it has been hypothesized that it is a homolog of taspase 1 because the region of cleavage (16) contains a putative taspase recognition site and abrogates Trx protein maturation (16). Nevertheless, the cellular mechanism of proteolytic maturation is completely known only for human MLL.

MLL is cleaved by a novel endopeptidase called taspase 1 that utilizes an N-terminal threonine generated by autoproteolysis as the nucleophile for polypeptide cleavage (10). For HCF-1, autocatalytic processing of bacterially synthesized HCF-1 has been observed (28), but the mechanisms of HCF-1 maturation in human cells remain to be clarified. Curiously, in addition to sharing proteolytic maturation processes, MLL and HCF-1 bind each other (36), and both play important roles in the regulation of the cell division cycle (6, 23, 26, 30). These relationships encouraged us to compare their proteolytic maturation pathways along with those of the Drosophila Trx and dHCF homologs.

Species-selective proteolysis is important for the regulation of cellular activities. The histone modification activity of the human trithorax group mixed-lineage leukemia (MLL) protein and the cell cycle regulatory activity of the cell proliferation factor herpes simplex virus host cell factor 1 (HCF-1) are stimulated by cleavage of precursors that generate stable heterodimeric complexes. MLL is processed by a protease called taspase 1, whereas the precise mechanisms of HCF-1 maturation are unclear, although they are known to depend on a series of sequence repeats called HCF-1PRO repeats. We demonstrate here that the Drosophila homologs of MLL and HCF-1, called Trithorax and dHCF, are both cleaved by Drosophila taspase 1. Although highly related, the human and Drosophila taspase 1 proteins display cognate species specificity. Thus, human taspase 1 preferentially cleaves MLL and Drosophila taspase 1 prefers cleaves Trithorax, consistent with coevolution of taspase 1 and MLL/Trithorax proteins. HCF proteins display even greater species-specific divergence in processing; whereas dHCF is cleaved by the Drosophila taspase 1, human and mouse HCF-1 maturation is taspase 1 independent. Instead, human and Xenopus HCF-1PRO repeats are cleaved in vitro by a human proteolytic activity with novel properties. Thus, from insects to humans, HCF proteins have conserved proteolytic maturation but evolved different mechanisms.

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centrally located 26-amino-acid repeats called HCF-1 proteolytic (HCF-1pro) repeats, generating the associated amino-terminal (HCF-1N) and carboxy-terminal (HCF-1C) subunits (15, 31, 32). The HCF-1N and HCF-1C subunits play separate roles in two key phases of the cell cycle: the HCF-1N subunit promotes passage through the G1 phase, and the HCF-1C subunit is necessary for proper mitosis and cytokinesis during the M phase (14). Proteolytic processing is necessary to ensure proper HCF-1 function, as HCF-1C subunit functions are inhibited if the HCF-1 precursor cannot be processed (14).

Consistent with the importance of HCF-1 proteolytic maturation, the 1,500-amino-acid dHCF protein, although lacking HCF-1pro repeats, also undergoes proteolytic maturation to generate associated dHCFN and dHCFc subunits (18). The dHCFN and dHCFc subunits display considerable structural similarity to the human HCF-1N and HCF-1C subunits (Fig. 1) as well as functional conservation. Thus, as for the HCF-1N subunit, the dHCFN subunit can associate with the HSF transcription activator VP16 and stabilize the VP16-induced transcriptional regulatory complex (18), as well as associate with the Drosophila cell cycle regulators dE2F1 (the homolog of human E2F1) and dE2F2 (the homolog of human E2F4) (27).

Following our interest in HCF protein proteolytic maturation and in the conservation of HCF proteins in animals, we found that dHCF, albeit not human HCF-1, contains a potential taspase 1 cleavage site within the region predicted by Mahajan et al. (18) for cleavage. This observation has led us to find that the dHCF and Trx proteins are both cleaved by the Drosophila taspase 1 homolog, whereas human HCF-1 proteolytic cleavage is independent of taspase 1. Thus, the Drosophila and human trithorax group proteins share proteolytic maturation pathways, while the Drosophila and human HCF proteins, although conserving proteolysis as a maturation process, mature via different proteolytic mechanisms.

**MATERIALS AND METHODS**

**Plasmid constructions and DNA template preparations.** A full-length Drosophila taspase 1 cDNA (LD00507) was obtained from the Drosophila Genomics Resource Center, Bloomington, IN, and cloned into the pET-28a (+) His-tagged bacterial expression vector (Novagen). The His-tagged full-length human taspase 1 cDNA bacterial expression vector and the vector for in vitro transcription/translation containing wild-type and CS1-2 mutated MLL amino acids 2400 to 2900 have been described previously (10). The full-length dHCF cDNA cloned in a T7 epitope-tagged Drosophila expression vector (pACXT-dHCF) was a kind gift of Angus Wilson (New York University). For in vitro transcription/translation of the precursor, a PCR fragment encoding dHCF amino acids 733 to 1212 was amplified from pACXT-dHCF and inserted in the pNCITE vector (30). For the Trx precursor, a PCR fragment encoding Trx amino acids 1973 to 2458 was reversed transcribed to cDNA, cloned into the pACXT-Trx expression vector (30).

**Taspase 1 protein expression.** His-tagged human taspase 1 and Drosophila taspase 1 were expressed in Escherichia coli BL21(DES) cells and purified by Ni affinity chromatography (QIAGEN) as recommended by the manufacturer. Proteins eluted from the resin were dialyzed against phosphate-buffered saline, 25% glycerol.

**Cell culture and extracts.** HeLa cells were grown at 37°C in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS). Drosophila SL2 cells were grown at 25°C in Schneider’s medium (GIBCO) with 10% heat-inactivated FBS. Nuclear and cytosolic extracts from HeLa and SL2 cells were prepared as previously described (4). Wild-type or taspase 1 -/- mouse embryonic fibroblasts (MEFs) from day 12.5 embryos were grown as described previously (26). Extract was prepared by lysis in radioimmunoprecipitation assay buffer with Complete protease inhibitor cocktail (Roche) on ice for 30 min and clarified by centrifugation at 20,000 × g for 20 min.

**In vitro cleavage assays.** Taspase 1 in vitro cleavage assays were performed as previously described (10). For comparative experiments, recombinant human taspase 1 and Drosophila taspase 1 activities were titrated on the respective cognate MLL and Trx substrates and used in the amount required to cleave 50% of the substrate (corresponding to 10 ng of human taspase 1 and 50 ng of Drosophila taspase 1). For the xHCF-1 in vitro cleavage assay, 1 µg of human taspase 1 and Drosophila taspase 1 were used. Cell extract in vitro cleavage assays were performed using 22 µl of the indicated cell extract in a 30-µl reaction mixture and incubating reaction mixtures at 30°C for 2 h or the indicated time period. HeLa cell extract heat treatment was at 65°C for 20 min, and protease inhibitors were utilized at the final concentration of 0.5 mg/ml Pefabloc (Roche) and 1× Complete protease inhibitor cocktail (Roche). The reaction mixtures were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the resulting radioactive signals were visualized with a Typhoon Trio+ imaging system and quantified with ImageQuant (Amersham Biosciences).

**RNAi and cell transfection.** A 183-bp double-stranded RNA (dsRNA) (RNAi) and a 495-bp dsRNA (RNA2) against the Drosophila taspase 1 gene sequence as well as a nonspecific firefly luciferase dsRNA were used for RNA interference (RNAi). dsRNAs were synthesized with a MEGAscript T7 kit (Ambion), using the following primers: PCR products amplified with primer pairs (RNA1: 5’-CAGCTGGTCTGTGTCGGCTA and TCTAGGAGCTGCAGGAAGGTG (RNA: 1) and TGGGTGTCCTCAATGCTTGGC and TCCAGCGTACAGATCTCGG each containing an additional 5’ T7 promoter sequence. For dsRNA treatment, 1 × 10⁵ SL2 cells were seeded in six-well plates in 1 ml of serum-free Schneider’s medium and
Similarly, as shown in Fig. 2C, 3 and 4). These results suggest that, like its human homolog, the precursor carrying the mutation in the CS2-like site (lanes 1) can cleave the wild-type Trx precursor (lanes 1 and 2) but not the CS1-like mutant precursor (lanes 3 and 4). These results indicate that dHCF is cleaved at a single taspase 1 recognition site.

We also analyzed proteolytic maturation of endogenous dHCF by generating an antibody directed to the N terminus of the protein (see Materials and Methods). Immunoblot analysis of the same SL2 extracts shown in Fig. 2D revealed that endogenous dHCFN comigrates with the processed rdHCFN (compare lanes 1 and 2 in Fig. 2D and E). With this antibody, we could not detect any endogenous full-length dHCF protein, but we could detect the ectopic CS1-like mutant rdHCFNL (compare lane 3 with lane 1). The lack of endogenous full-length dHCF indicates, as previously shown using a dHCFC antibody (9), that dHCF is efficiently processed in Drosophila cells.

To determine whether Drosophila taspase 1 is the sole protease responsible for dHCF processing in vivo, we depleted Drosophila taspase 1 from Drosophila cells by RNAi. As shown in Fig. 2F, independent treatment of SL2 cells with two different taspase 1 dsRNAs (RNAi 1 and RNAi 2) led in each case to the detection of dHCFFl (lanes 3 and 4) compared to untreated (lane 1) or mock RNAi-treated (luciferase, lane 2) cells. The levels of dHCFFl were limited, however, possibly owing to high levels of stable dHCFN remaining from before the RNAi treatment. In contrast, we observed prominent inhibition of dHCF cleavage when the RNAi-induced Drosophila taspase 1 depletion was associated with concomitant synthesis of dHCF by simultaneous transfection of the epitope-tagged dHCF expression vector. Thus, as shown in Fig. 2G, in taspase 1 RNAi-treated cells, dHCF processing is significantly reduced (lanes 3 and 4) compared to that in untreated (lane 1) or mock RNAi-treated (luciferase, lane 2) cells. In summary, our in vitro and in vivo results indicate that Drosophila taspase 1 is responsible for dHCF processing at a single site and indeed may be the sole protease responsible for dHCF maturation. Thus, in flies, where dHCF lacks HCF-1PRO repeats, HCF protein maturation is apparently not autocatalytic as observed for an HCF-1PRO repeat region of human HCF-1 synthesized in E. coli (28).

dHCF is not an effective substrate for human taspase 1. We were surprised to find that dHCF is a substrate of Drosophila taspase 1, because Izeta et al. (12) have shown that dHCF is not processed in hamster cells and yet such cells would be expected to possess taspase 1. To explore this apparent discrepancy, we compared directly the abilities of human and Drosophila cell extracts to cleave the dHCF precursor. Indeed, as the results of Izeta et al. (12) would suggest, the dHCF precursor, albeit cleaved by a Drosophila SL2 extract, was not effectively cleaved by a human HeLa cell extract, as shown in Fig. 3A (compare lanes 2 and 3). This dissimilarity is likely the result of intrinsic differences in the human taspase 1 and Drosophila taspase 1 enzymes, because the same species-specific activity was also observed with purified recombinant human taspase 1 and Drosophila taspase 1 (compare lanes 5 and 6) (see Materials and Methods for enzymatic activity normalization). These results explain why Izeta et al. (12) did not observe dHCF processing by using mammalian cells and emphasize the
importance of using a homologous system to assay HCF protein processing (18).

The human and Drosophila taspase 1 enzymes have co-evolved with their specific MLL/Trx substrates. The dHCF Drosophila taspase 1 and human taspase 1 cleavage results suggest that taspase 1 activity has changed during evolution. To study this evolution further, we compared the human taspase 1 and Drosophila taspase 1 enzymes on homologous substrates: precursors from the human MLL and Drosophila Trx proteins (Fig. 3B). As shown in Fig. 3C, a human HeLa cell and a Drosophila SL2 cell extract display enhanced activity on the cognate MLL and Trx precursor, respectively (compare lanes 2 and 3 with lanes 5 and 6) (in this assay only MLL-CS2 cleavage is indicated). Furthermore, purified recombinant human taspase 1 and Drosophila taspase 1 display the same cognate substrate preference (Fig. 3D, compare lanes 2 and 3 with lanes 5 and 6). These results suggest that the taspase 1 enzymes have coevolved with their substrates MLL and Trx.

Zhou et al. (38) have defined a human taspase 1 recognition heptapeptide sequence. The MLL CS2 and Trx CS2-like sites differ by a single amino acid (from QLDGVDD in MLL CS2 to QMDGVDD in Trx). To examine the determinants responsible for the cognate enzyme substrate preference of Drosophila taspase 1 on Trx, we converted the Trx CS2-like taspase 1 site to the MLL CS2 sequence via a single amino acid change (Fig. 3B). As shown in Fig. 3E, the humanized Trx precursor [Trx(MLLCS2)] is still a better substrate for Drosophila taspase 1 than human taspase 1, indicating that the species-specific taspase 1 selectivity observed here results from sequence differences that lie outside the so-far-identified heptapeptide recognition sequence.

Human HCF-1 is not a taspase 1 substrate. HCF-1 is cleaved at any one of the six HCF-1 PRO repeats (15, 31), and when the region containing all six sites is deleted (14, 32) or the six sites are all individually inactivated by amino acid substitution (29), the protein is no longer cleaved. To study HCF-1

![Image](https://example.com/image1)

**FIG. 2.** Trx and dHCF are cleaved by Drosophila taspase 1. (A) Schematic of Trx and dHCF cleavage precursors. The lines labeled Pre indicate the region contained within each precursor. Wild-type (wt) and mutant (mt) versions of the putative taspase 1 cleavage sites are shown below. Note that the two diagrams are not drawn to the same scale. (B) Drosophila taspase 1 (dTaspase1) proteolytic activity on the Trx precursor. 35S-labeled wild-type (lanes 1 and 2) or CS2-like mutant (lanes 3 and 4) Trx precursors were incubated for 2 h at 37°C with (lanes 2 and 4) or without (lanes 1 and 3) purified recombinant Drosophila taspase 1. Products were resolved by SDS-PAGE and revealed by autoradiography. •, N-terminal cleavage product. A smaller C-terminal fragment is not visible owing to the reduced specific activity of this product. (C) Drosophila taspase 1 proteolytic activity on the dHCF precursor. 35S-labeled wild-type (lanes 1 and 2) or CS1-like mutant (lanes 3 and 4) dHCF precursors were incubated with (lanes 2 and 4) or without (lanes 1 and 3) purified recombinant Drosophila taspase 1. Products were resolved by SDS-PAGE and revealed by autoradiography. •, location of larger N-terminal and smaller C-terminal cleavage products. (D) dHCF cleavage at the Drosophila taspase 1 cleavage site in vivo. SL2 cells were mock transfected (lane 1) or transfected with wild-type (lane 2) or CS1-like mutant (lane 3) T7-tagged full-length dHCF expression vector. Proteolysis by endogenous protease was assessed by anti-T7 tag (αT7) immunoblotting. rdHCFN, full-length rdHCF; rdHCFN N, rdHCF N-terminal subunit. Molecular mass markers are listed on the left. (E) Anti-dHCFN antibody (αdHCFN) reveals processing of endogenous dHCF. Endogenous dHCF proteolysis of the samples shown in panel D was revealed by immunoblot analysis with affinity-purified anti-dHCFN antibody. (F) RNAi depletion of Drosophila taspase 1 impairs endogenous dHCF processing. SL2 cells were mock transfected (lane 1) or transfected with wild-type (lane 2) or CS1-like mutant (lane 3) T7-tagged full-length dHCF expression vector. Proteolysis by endogenous protease was assessed by anti-T7 tag (αT7) immunoblotting. rdHCFN, full-length rdHCF; rdHCFN N, rdHCF N-terminal subunit. Molecular mass markers are listed on the left. (G) RNAi depletion of Drosophila taspase 1 impairs dHCF processing. SL2 cells were treated with the indicated dsRNAs as in panel F for 48 h before transfection of the T7-tagged full-length dHCF expression vector. dHCF cleavage was analyzed 48 h after the transfection by anti-T7 immunoblotting.
proteolytic cleavage, we first asked whether the HCF-1\textsubscript{PRO} repeat is a taspase 1 substrate. Therefore, we prepared an in vitro HCF-1 protease substrate containing three of the six HCF-1\textsubscript{PRO} repeats (HCF-1\textsubscript{PRO} repeats 1, 2, and 3, called HCF-1rep123) (Fig. 4A) and incubated it with recombinant human taspase 1. Figure 4B shows that human taspase 1 had no effect on this HCF-1 precursor (compare lanes 1 and 2) at a concentration that effectively cleaved the MLL precursor (compare lanes 5 and 6), suggesting that taspase 1 is not an HCF-1\textsubscript{PRO} repeat protease.

To further exclude the requirement of taspase 1 in HCF-1 maturation, we asked whether HCF-1 processing is affected by the absence of taspase 1. Takada et al. (26) have shown using MEFs that MLL processing is disrupted in \textit{taspase 1}/H11002\textsubscript{−/−} knock-out mice. In contrast, as shown in Fig. 4C, human taspase 1 and \textit{Drosophila} taspase 1 activities on MLL and Trx cleavage. MLL (lanes 1 to 3) and Trx (lanes 4 to 6) precursors were incubated without extract (lanes 1 and 4) or with HeLa (lanes 2 and 5) or SL2 (lanes 3 and 6) cytosolic extracts. (D) Human taspase 1 and \textit{Drosophila} taspase 1 activities on MLL and Trx cleavage. MLL (lanes 1 to 3) and Trx (lanes 4 to 6) precursors were incubated without taspase 1 (lanes 1 and 4) or with recombinant human taspase 1 (lanes 2 and 5) or \textit{Drosophila} taspase 1 (lanes 3 and 6). (E) Human taspase 1 and \textit{Drosophila} taspase 1 activities on humanized Trx\textsubscript{MLLCS2} precursor. The Trx\textsubscript{MLLCS2} precursor was incubated without taspase 1 (lane 1) or with recombinant human taspase 1 (lane 2) or \textit{Drosophila} taspase 1 (lane 3). For all cleavage products, black dots indicate the N-terminal cleavage products; smaller, lower-specific-activity C-terminal fragments are not visible.

Together, these results indicate that HCF-1\textsubscript{PRO} repeat is a taspase 1 substrate. Therefore, we prepared an in vitro HCF-1 protease substrate containing three of the six HCF-1\textsubscript{PRO} repeats (HCF-1\textsubscript{PRO} repeats 1, 2, and 3, called HCF-1rep123) (Fig. 4A) and incubated it with recombinant human taspase 1. Figure 4B shows that human taspase 1 had no effect on this HCF-1 precursor (compare lanes 1 and 2) at a concentration that effectively cleaved the MLL precursor (compare lanes 5 and 6), suggesting that taspase 1 is not an HCF-1\textsubscript{PRO} repeat protease.

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A human cell activity cleaves HCF-1\textsubscript{PRO} repeats. To identify an activity responsible for HCF-1\textsubscript{PRO} repeat processing, we used a wild-type HCF-1rep123 substrate or substrates with HCF-1\textsubscript{PRO} repeats 1 and 2 mutated either individually (HCF-1repX23 and HCF-1rep1X3) or together (HCF-1repXX3) as illustrated in Fig. 5A. As shown in Fig. 5B, in the absence of cell extract, none of the precursors was effectively cleaved (lanes 1, 3, 5, and 7). In the presence of HeLa cell extract, however, each precursor was cleaved at the wild-type but not mutated HCF-1\textsubscript{PRO} repeats (compare lanes 2, 4, 6, and 8), displaying a cleavage pattern consistent with HCF-1\textsubscript{PRO} repeat specific cleavage. These results suggest that HeLa cell extracts possess an activity that can specifically cleave an HCF-1\textsubscript{PRO} repeat precursor.

To establish the nature of this HCF-1\textsubscript{PRO} repeat enzymatic activity, we performed a time course experiment using the HCF-1rep123 precursor. As shown in Fig. 5C, under the same assay conditions used for Fig. 5B, the HCF-1\textsubscript{PRO} repeat cleavage product increased with incubation time for approximately 8 h. Accumulation of the product resulting from cleavage at the HCF-1\textsubscript{PRO} repeat 1 was quantified as shown in Fig. 5D. These results suggest that in cell extracts, the HCF-1\textsubscript{PRO} repeat proteolytic cleavage is relatively slow but stable for 8 h.

The HeLa cell HCF-1\textsubscript{PRO} repeat proteolytic activity is heat sensitive but protease inhibitor resistant. To characterize the HCF-1\textsubscript{PRO} repeat protease further, we assayed the sensitivity of the HeLa cell extract activity to heat and protease inhibitor treatment as shown in Fig. 5E. The HeLa cell extract was sensitive to heat treatment (compare lanes 1 to 3) but resistant
to Pefabloc, a serine protease inhibitor (compare lanes 3 and 4) (an assay with thrombin and a substrate with a thrombin cleavage site showed that the serine protease inhibitor was active [data not shown]), and Complete, a serine and cysteine protease inhibitor cocktail (compare lanes 3 and 5). Thus, the HCF-1PRO repeat protease displays both sensitivity (heat) and resistance (serine and cysteine protease inhibitors) to various treatments. We note that the resistance of the HCF-1PRO repeat protease to a serine protease inhibitor differentiates it from the autocatalytic activity described by Vogel and Kristie, which was serine protease inhibitor sensitive (28).

HCF protein maturation mechanism has changed during evolution. The identification of taspase 1 as the protease responsible for dHCF maturation and the observation that human HCF-1 is cleaved by a different activity at the HCF-1PRO repeats indicate that, from fly to human, HCF proteins have conserved proteolytic maturation but evolved different mechanisms. These differences between flies and humans appear to be generally specific to insects or vertebrates, as illustrated in Fig. 6. Thus, the Apis mellifera (honeybee) HCF protein possesses a consensus taspase 1 cleavage site at the same relative location as in the dHCF protein (Fig. 6A), suggesting that taspase 1 cleavage of HCF proteins may be generally conserved in insects. In parallel, a comparison of human, frog, and fish HCF-1 proteins shows that the position (Fig. 6B) and sequence (Fig. 6C) of the HCF-1PRO repeats have been very highly conserved in these three distantly related vertebrate species. Thus, between insects and vertebrates, there appears to have been an evolutionary switch in HCF protein processing mechanism that has been highly conserved within each group.

Interestingly, in some fish (e.g., F. rubripes) and frog (e.g., X. tropicalis) species, in addition to the perfectly conserved HCF-1PRO repeats there is a partially conserved taspase 1 cleavage site (six-of-seven match to a heptapeptide consensus taspase 1 cleavage site) (38) located just C terminal of the last HCF-1PRO repeat (Fig. 6B). This partially conserved taspase 1 cleavage site could represent an active site, indicating that in some species HCF-1 could be processed by both taspase 1 and an HCF-1PRO repeat protease. Alternatively, it could be inactive and instead represent the vestige of an active taspase 1 cleavage site in an ancestral HCF-1 protein. To distinguish between these two possibilities, we asked whether an xHCF-1 cleavage precursor containing the partially conserved taspase 1 cleavage site and the adjacent two HCF-1PRO repeats (xHCF-1rep89 [Fig. 6D]) could be a substrate for either taspase 1 and/or the HCF-1PRO repeat protease. As shown in Fig. 6D, the xHCF-1rep89 substrate is not cleaved by either Drosophila taspase 1 or human taspase 1 at concentrations that can effectively cleave the dHCF precursor (compare lanes 1 to 3 and 4 to 6). Instead, as for human HCF-1 (lanes 8 and 9), the xHCF-1rep89 precursor is cleaved by the HeLa cell extract at positions corresponding to the HCF-1PRO repeats (compare lanes 4 and 7). These data suggest that, like mammalian HCF-1 proteins, xHCF-1 is not cleaved by taspase 1 but is instead processed at the HCF-1PRO repeats by the same activity responsible for human HCF-1 maturation. The ability of the human cell extract to cleave the Xenopus HCF-1PRO repeats provides functional evidence of the high degree of conservation of the HCF-1PRO repeat proteolytic process in vertebrates.

**DISCUSSION**

In this study, we have compared the proteolytic maturation processes of two pairs of evolutionarily conserved proteins: the
trithorax group proteins human MLL and Drosophila Trx and the HCF proteins human HCF-1 and dHCF. We have shown that, like MLL, the Drosophila Trx is a substrate for the taspase 1 protease. Interestingly, the enzyme-substrate specificity, i.e., human taspase 1 with MLL and Drosophila taspase 1 with Trx, has coevolved. In contrast, with respect to HCF proteins, very different proteolytic pathways have evolved. Thus, the dHCF protein is cleaved by Drosophila taspase 1 but its human (and Xenopus) HCF-1 counterparts are cleaved by what is likely an unusual proteolytic activity.

Evolution of taspase 1 enzyme-substrate specificity. Hsieh et al. (10) noted that sequences encoding the taspase 1 enzymes have been conserved in vertebrates and insects but not in the worm Caenorhabditis elegans and that, correspondingly, the MLL/Trx developmental regulators in vertebrates and insects but not in worms have taspase 1 recognition sites. Consistent with this observation, Trx is indeed a taspase 1 substrate at its CS2-like taspase 1 cleavage site. Examination of the activities of the human and Drosophila taspase 1 enzymes on the MLL and Trx proteins also revealed a more refined coevolution of enzyme and substrate, as human taspase 1 is more active on its cognate MLL substrate and Drosophila taspase 1 is more active on its cognate Trx substrate. This observation suggests that the presence of the taspase 1 enzyme and MLL/Trx protein maturation have been conserved because the cleavage is critically important for proper development (16, 19, 26). Nevertheless, there is considerable flexibility in the interaction between protease and substrate, and for such enzyme-substrate coevolutionary flexibility to be possible, it is likely that taspase 1 does not possess a large number of essential targets.

We note with interest that vertebrates and insects display common longitudinal body segmentation, which is lacking in C. elegans, and that proper MLL and Trx maturation have been conserved because the cleavage is critically important for proper development (16, 19, 26). Nevertheless, there is considerable flexibility in the interaction between protease and substrate, and for such enzyme-substrate coevolutionary flexibility to be possible, it is likely that taspase 1 does not possess a large number of essential targets.

Although HCF proteins are not known to be involved in

FIG. 5. HCF-1PRO repeats are faithfully processed by human cell extracts. (A) Schematic of HCF-1 cleavage precursors. The line labeled Pre indicates the region contained within each precursor. These HCF-1 precursors contain the first three HCF-1PRO repeats in a wild-type version (HCF-1rep123) or with mutated repeat 1 (HCF-1repX23), mutated repeat 2 (HCF-1rep1X3), or mutated repeats 1 and 2 (HCF-1repXX3). Products corresponding to full-length precursor (Pre) and N-terminal cleavage products for HCF-1PRO repeats 3, 2, and 1 are shown below. (B) HeLa cell extract cleavage of HCF-1PRO repeats. 35S-labeled HCF-1 precursors from panel A were incubated without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, and 8) nuclear HeLa cell extract. Numbers on the right of each panel indicate the N-terminal cleavage product derived from the indicated HCF-1PRO repeat; x indicates the missing cleavage product corresponding to the mutated HCF-1PRO repeat; C-terminal fragments are not visible owing to their reduced specific activity. (C) Time course of the HeLa cell extract proteolytic activity. 35S-labeled HCF-1rep123 precursor was incubated with nuclear HeLa cell extracts for the indicated periods of time. (D) Quantification of time course data. The relative accumulation of HCF-1PRO repeat 1 cleavage product from panel C was quantified to represent HCF-1PRO repeat cleavage activity over time. (E) Characterization of the HeLa cell proteolytic activity. 35S-labeled HCF-1rep123 precursor was incubated without extract (lane 1) or with nuclear HeLa cell extract (lanes 2 to 5) that was heat treated (lane 2), untreated (lane 3), or treated with Pefabloc (lane 4) or Complete protease inhibitor (PI) mixture (lane 5).
FIG. 6. Evolution of HCF protein maturation. (A) HCF protein maturation in insects. Top, schematic representation of dHCF. The taspase 1 cleavage site is indicated by the arrowhead. Bottom, charge profiles of the fly Drosophila melanogaster and the honeybee Apis mellifera HCF proteins. Peaks above zero indicate basic regions, and peaks below zero indicate acidic regions; basic and acidic regions are shown in blue and red, respectively. For each protein, sequences of taspase 1 cleavage sites are indicated above arrowheads. (B) HCF protein maturation in vertebrates. Top, schematic representation of human HCF-1. Segments corresponding to the basic and acidic regions and to the HCF-1PRO repeats are indicated above the schematic. Bottom, charge profiles of the human Homo sapiens, frog Xenopus tropicalis, and fish Fugu rubripes HCF proteins as in panel A. For each protein, the region corresponding to the HCF-1PRO repeats is overlined. Sequences corresponding to partially conserved taspase 1 cleavage sites are indicated above arrowheads; in red is indicated the residue that does not match the taspase 1 site consensus. (C) Sequence conservation of the HCF-1PRO repeats in vertebrates. An alignment of fish (top), frog (center), and human (bottom) HCF-1PRO repeats is shown. The human HCF-1PRO repeats are numbered as in reference 15. Residues matching a consensus based on the most frequent residue at each position are shaded. Positions in the consensus sequences for which a conserved residue cannot be defined are indicated by dashes. Underlined positions indicate sequence conservation among HCF-1PRO repeats of all three species. ●, residues important for human HCF-1PRO repeat cleavage in vivo (32). (D) In vitro cleavage of xHCF-1. Top, schematic representation of xHCF-1. The line labeled Pre indicates the region contained in the cleavage precursor. The dashed line indicates the imperfect taspase 1 cleavage site. Bottom, xHCF-1 precursor (xHCF-1rep89) was incubated without additions (lane 4) or with either Drosophila taspase 1 (dTaspase1) (lane 5), human taspase 1 (hTaspase1) (lane 6), or HeLa cell extract (lane 7). dHCF (lanes 1 to 3) and human HCF-1 (hHCF-1rep123; lanes 8 and 9) precursors are shown as positive controls for taspase 1 (dHCF) and HCF-1PRO repeat (hHCF-1rep123) cleavage. ●, HCF protein cleavage products.
regulating segmentation, invertebrate HCF proteins display species-specific patterns of proteolytic maturation that follow the Trx proteins. Thus, in *Drosophila*, dHCF and Trx are both cleaved by *Drosophila* taspase 1, and in the honeybee the HCF and Trx homologs possess taspase 1 recognition sites. In contrast, as with the Trx homologs, the sea urchin and worm HCF proteins do not possess evident taspase 1 recognition sites (data not shown); indeed, worm HCF does not undergo proteolytic maturation (34). We suggest that in invertebrates, the HCF and Trx proteins coevolved taspase 1-induced maturation. In contrast, in vertebrates, HCF-1 proteins took on a very different proteolytic maturation mechanism. Nevertheless, the parallels between MLL/Trx and HCF protein maturation suggest that in addition to regulation of the cell cycle, HCF-1 may also play a role in regulation of genes involved in segment determination.

**HCF-1 is likely cleaved by an unusual protease.** We have described an enzymatic activity in HeLa cell extracts that specifically cleaves the HCF-1PRO repeat. We do not know at this time the precise relationship between the HeLa cell extract described here and the HCF-1 autocatalytic activity described previously (28), but we note some differences between the two activities. The autocatalytic activity, which depends on sequences within the HCF-1c region, results in cleavage at not only HCF-1PRO repeats but also an additional site, called 102, which lacks evident HCF-1PRO repeat sequence similarity and is located just C terminal of the sixth HCF-1PRO repeat (15, 28). This autocatalytic site is, however, not used in vivo when the HCF-1PRO repeats are inactivated by mutation or deletion (14, 29, 32), and we have not observed 102 site cleavage using a precursor substrate spanning this site in the HeLa cell extract assay (F. Capotosti and W. Herr, unpublished results). Thus, in both HeLa cells and HeLa cell extracts, HCF-1 proteolytic maturation appears to be HCF-1PRO repeat specific. We also note that, unlike the autocatalytic 102 site activity, which is sensitive to the serine protease inhibitor Pefabloc (28), the HeLa cell HCF-1PRO repeat activity is resistant to this protease inhibitor (Fig. 5E). Clarifying the relationship between the HeLa cell activity described here and the HCF-1 autocatalytic activity will require further investigation.

Whatever the relationship, however, the properties of the HCF-1PRO repeat protease are likely to be unusual. As shown in Fig. 6C, there are 16 amino acid residues over 19 positions that are identical among the HCF-1PRO repeat consensus sequence of the fish, frog, and human HCF-1 proteins, an impressive level of sequence conservation for a proteolytic recognition sequence. Furthermore, as described previously (32), alanine substitutions at 12 positions over 18 of the human HCF-1PRO repeat affect HCF-1PRO repeat cleavage in HeLa cells (Fig. 6C), a surprisingly large sequence requirement for proteolytic cleavage. Given this extensive sequence conservation and sequence requirements, we suggest that the functional HCF-1PRO repeat element reflects the assemblage of different recognition sequences for (i) the protease itself and (ii) accessory factors that could regulate proteolysis. Such accessory factors could directly recruit or stimulate the protease or could reflect enzymes that modify the HCF-1PRO repeat (e.g., by phosphorylation or glycosylation) to regulate cleavage. Whatever the reason, the large size of the HCF-1PRO repeat results in considerable specificity, as we have been unable to find a match to the HCF-1PRO repeat in any other protein sequence found in public protein databases, suggesting that in toto the mechanisms of HCF-1PRO repeat cleavage are unique to HCF-1 maturation.

**Alternate mechanisms of HCF protein cleavage result in HCFX and HCFC subunits of similar structure.** The evolutionary comparisons shown in Fig. 6 indicate that although the mechanisms of HCF protein maturation have switched between insects and vertebrates, the end results of proteolytic processing are similar. To illustrate this point, Fig. 6A and B show charge profiles for the two insect (fly and honeybee) and three vertebrate (fish, frog, and human) HCF proteins. These profiles show that the insect and vertebrate HCF proteins all contain basic and acidic regions at corresponding positions. This conservation of amino acid composition is consistent with the important cellular functions these regions have been shown to possess in human HCF-1: G1 phase progression in the case of the basic region (30) and M phase progression (13), transcriptional activation (17), and chromatin association (13) in the case of the acidic region. We note with interest that whether the HCF protein is cleaved by taspase 1, as appears to be the case with the insect HCF proteins, or at the HCF-1PRO repeats, as appears to be the case in the vertebrate proteins, the cleavage site(s) is always positioned between the basic and acidic regions. Thus, although the mechanism for HCF protein proteolysis has changed during evolution, the resulting HCFX and HCFC subunits are very similar in structure.

**How and why might a transition from taspase 1- to HCF-1PRO repeat-dependent HCF-1 proteolysis have evolved?** The unexpected switch in proteolytic processing mechanism between insect and vertebrate HCF proteins leads to the questions of how and why. Concerning how the HCF-1PRO repeats may have arisen, we note with interest that the six HCF-1PRO repeats in fish and human HCF-1 are all encoded by a single large exon of 1,477 bp in human. (The nine repeats in *X. tropicalis* are encoded by two exons [five in the first and four in the second], which may have resulted from a duplication of the six-repeat-containing fish/human exon [Fig. 6C].) We imagine that an HCF-1 progenitor acquired the six HCF-1PRO repeats as a single genetic element by recombination, perhaps transposition, prior to vertebrate evolutionary divergence.

Concerning why a switch may have occurred, one possible explanation is that the region containing the HCF-1PRO repeats has been evolutionarily selected as an additional platform for protein-protein interactions, as Vogel and Kristie (29) have shown that the transcriptional coactivator/corepressor FHL2 interacts with nonprocessed HCF-1, stimulating transcription of an HCF-1 target gene. This interaction between HCF-1PRO repeats and cofactors could allow the modulation of HCF-1 processing and activity. Another possible explanation is that, with an HCF-1PRO repeat protease, HCF-1 protein maturation has become independent from taspase 1 and thus from MLL/Trx maturation. This could result in a more flexible regulation of these two important cell cycle regulators. In any case, however, the importance of HCF-1PRO repeat processing during vertebrate evolution is underscored by the remarkable similarity among the repeats themselves in one species (e.g., human) and between vertebrates as divergent as fish and human (Fig. 6C).

In conclusion, in human and fly, the MLL/Trx and HCF...
proteins have conserved the process of proteolytic maturation, but the MLL/Trx process coevolved with the cognate taspase 1 proteases, whereas HCF-1 and dHCF have apparently evolved very different proteolytic pathways. Whatever the reason for the evolutionary change to HCF-1-pro repeat-dependent processing in vertebrates, it is evident that the HCF-1-pro repeats are unusual cleavage sites, and it is likely that the protease responsible for their cleavage has unusual properties.

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REFERENCES


