Site-specific proteolysis of the transcriptional coactivator HCF-1 can regulate its interaction with protein cofactors

Jodi L. Vogel and Thomas M. Kristie*

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 4-131, 4 Center Drive, Bethesda, MD 20892

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Limited proteolytic processing is an important transcriptional regulatory mechanism. In various contexts, proteolysis controls the cytoplasmic-to-nuclear transport of important transcription factors or removes domains to produce factors with altered activities. The transcriptional coactivator host cell factor-1 (HCF-1) is proteolytically processed within a unique domain consisting of 20-aa reiterations. Site-specific cleavage within one or more repeats generates a family of amino- and carboxyl-terminal subunits that remain tightly associated. However, the consequences of HCF-1 processing have been undefined. In this study, it was determined that the HCF-1-processing domain interacts with several proteins including the transcriptional coactivator/corepressor four-and-a-half LIM domain-2 (FHL2). Analysis of this interaction has uncovered specificity with both sequence and context determinants within the reiterations of this processing domain. In cells, FHL2 interacts exclusively with the nonprocessed coactivator and costimulates transcription of an HCF-1-dependent target gene. The functional interaction of HCF-1 with FHL2 supports a model in which site-specific proteolysis regulates the interaction of HCF-1 with protein partners and thus can modulate the activity of this coactivator. This paradigm expands the biological significance of limited proteolytic processing as a regulatory mechanism in gene transcription.

FHL2 | transcription | herpes simplex virus | protein interactions

Site-specific or limited proteolytic processing has emerged as an important mechanism contributing to the regulation of basic cellular processes such as gene transcription, cell cycle progression, apoptosis, signal transduction, and differentiation. As a control mechanism for transcription of RNAPII-dependent genes, limited proteolysis has been shown to determine the nuclear transport of cytoplasmic or membrane-bound transcription factors such as SREBP, CREB3, ATF6, Cubitus interruptus, Tisp40, and Notch (1–8). In these cases, processing provides a mechanism to release sequestered factors to promote nuclear localization and affect the transcription of target genes. In other cases, exemplified by IRF2, C/EBPβ, and Stats 3, 5, and 6, processing removes domains required for transcriptional activation, thus producing negative regulatory factors (9–11). Although many of these processing events are means by which the cell may respond to stimuli, proteolysis of factors such as p53 and CDP/Cut contribute to a program that modulates cell-cycle progression (12, 13).

The transcriptional coactivator host cell factor-1 (HCF-1) undergoes a unique site-specific proteolytic processing (14–16). The protein was originally identified as a component of the herpes simple virus (HSV) immediate-early (IE) gene enhancer complex (17), where it mediates the combinatorial transcriptional regulation of the viral IE genes (18). It has since been defined as a transcriptional coactivator for cellular factors such as GABP, Sp1, E2F4, Krox20, CREB3, and Zhangfei (18–22). In addition, HCF-1 interactions with chromatin modification components (Set1/Ash2 and PDCD2) (23, 24), other coactivators (PGC) (25), and mRNA splicing machinery (26) as well as gene expression profiling studies (27) have indicated that HCF-1 is a control component of cellular functions such as general transcription, DNA replication-repair, mRNA processing, and signal transduction. HCF-1 is also essential for multiple stages of cell-cycle progression (28, 29), and this requirement may reflect the protein's broad transcriptional functions.

Proteolytic processing of HCF-1 is unique. The central region of the protein, the proteolytic processing domain (PPD), contains a series of 20-aa reiterations (Fig. 1*A*). Autocatalytic cleavage of the 220-kDa precursor occurs within one or more of these repeats to generate a family of 100- to 180-kDa amino- and carboxyl-terminal polypeptides (15). In contrast to the processing of other transcription factors such as SREBP and ATF6, processing of HCF-1 appears to occur primarily in the nucleus and can proceed such that a given HCF-1 molecule may be progressively cleaved at multiple reiterations (30). However, despite this processing, the resulting family of amino- and carboxyl-terminal cleavage products remain tightly associated (31). Thus, the multisite nuclear processing and the association of the resulting subunits suggest that the cleavage may play a role in the regulation of this coactivator. Recently it has been demonstrated that processing may segregate the functions of HCF-1 in cell-cycle progression because HCF-1 amino-terminal subunits promote the G_0-to-G_1 transition, whereas carboxyl-terminal subunits promote cytokinesis (29). However, the biological consequences of the processing remain elusive.

In this study, it was determined that the PPD is not solely a target for processing but is also a domain involved in multiple protein–protein interactions. One PPD-binding partner, FHL2, is a member of the four-and-a-half LIM domain (FHL) family, which functions as a transcriptional coactivator/corepressor (32–40). The interaction of the HCF-1 PPD with FHL2 reveals that this unique domain contains distinct specificities for protein binding and that the HCF-1 reiterations are not equivalent. FHL2 selectively interacts with the uncleaved HCF-1 form and coactivates an HCF-1-dependent promoter, elucidating a mechanism in which proteolytic processing can control specific HCF-1 protein interactions and thus modulate the transcriptional potential of the coactivator.

Results

The HCF-1 PPD: Processing and Protein Interactions. As shown in Fig. 1*A*, the domains of the coactivator HCF-1 include (*i*) a kelch domain that interacts with transcription factors (VP16, E2F4, Krox20, CREB3, and Zhangfei) (20, 21, 41–43) and transcriptional coactivators (PGC) (25); (*ii*) a basic region that interacts

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Abbreviations: HCF-1, host cell factor-1; HCF-1nc, noncleavable HCF-1; HSV, herpes simplex virus; IE, immediate-early; FHL, four-and-a-half LIM domain; PPD, proteolytic processing domain.

*To whom correspondence should be addressed. E-mail: tkristie@nih.gov.

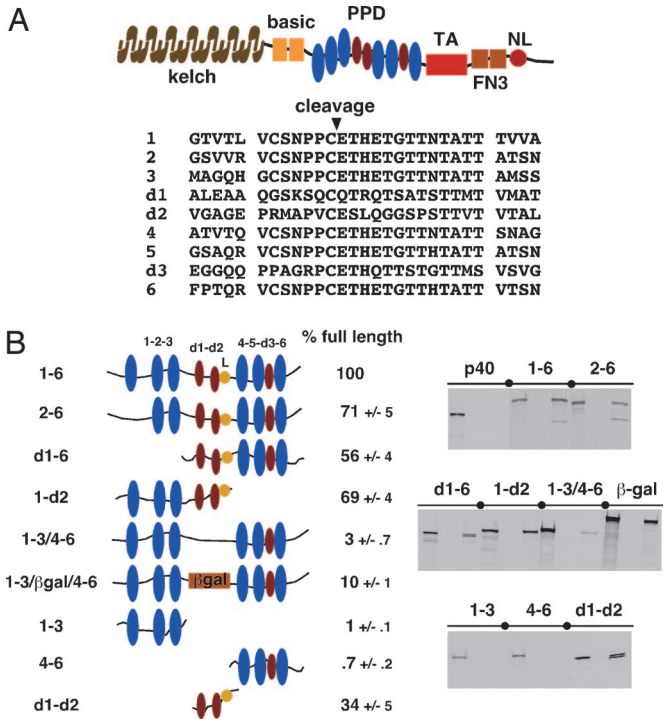

Fig. 1. HCF-1–FHL2 interaction mediated by the central region of the PPD. (*A*) The HCF-1 PPD is shown relative to the amino-terminal (kelch and basic) and the carboxyl-terminal (TA, transactivation; FN3, fibronectin type III; NL, nuclear localization signal) domains. An alignment of the 20 amino acid consensus repeats (blue ovals) and divergent repeats (red ovals) is shown. (*B*) The HCF-1 PPD contains consensus repeats (blue ovals, 1–2–3, 4–5, and 6), divergent repeats (red ovals, d1–d2 and d3), and an LXXLL motif (yellow circles, L). GST or GST-FHL2 fusion proteins (3 μg) were incubated with the illustrated PPD proteins (10 fmol). The amount of protein bound is expressed as a percent of the amount of bound full-length PPD (repeats 1–6). The results shown are the averages of several independent experiments. Each set of lanes represents input (10% of the total PPD protein in each reaction), GST elutate, and GST-FHL2 elutate. p40, nucleolar protein p40/EBP2.

with the stimulatory factors GABP and Sp1 (22, 44); (*iii*) the PPD; (*iv*) a transactivation domain that is required to mediate the transcriptional potential of factors such as VP16, CREB3, and Krox20 (21, 45); (*v*) fibronectin III repeats that, in part, mediate the association of the amino- and carboxyl-terminal HCF-1 subunits (31); and (*vi*) a nuclear localization signal (46).

The PPD consists of a series of conserved 20-aa reiterations that include six consensus and three divergent repeats. In addition, a coactivator LXXLL motif is present immediately adjacent to the divergent repeat 2. Autocatalytic processing of HCF-1 occurs through site-specific cleavage within the consensus repeats at one or more reiterations.

In the course of identifying HCF-1-protein interactions in yeast two-hybrid screens, several cellular proteins were isolated that interacted with a bait containing repeats 2 and 3 of the HCF-1 PPD. One of these proteins, FHL2, is an FHL family member involved in transcriptional coactivation/corepression. To determine the specificity of this interaction, FHL family members (FHL1, FHL2, and FHL3) and other LIM domain proteins (LM01 and MLP) were tested in a two-hybrid assay for their ability to interact with the HCF-1 PPD. Quantitative β-gal reporter expression indicated that there was a strong interaction of the HCF-1 PPD (1–6) with FHL2, a significantly weaker interaction with FHL3, and no detectable interaction with FHL1, LMO1, or MLP (see Fig. 6, which is published as supporting information on the PNAS web site). The results show that the interaction of the HCF-1 PPD with FHL2 was specific, even to the point of discriminating between FHL2 and the highly related protein FHL3. Furthermore, because the protein was originally isolated using the HCF-1 PPD repeats 2 and 3, it was likely that the interaction was, at least in part, mediated by the HCF-1 repeats.

Inherent Specificity for Protein Interactions Encoded Within the HCF-1 PPD. The HCF-1 PPD consists of six conserved reiterations (rpt 1–6), three interspersed divergent repeats (d1, d2, and d3), and a putative LXXLL coactivator motif (Fig. 1*B*). To define the interaction determinants within this reiterated domain, constructs representing various configurations of the intact or proteolytically processed products were tested for interaction with the GST-FHL2 fusion protein (Fig. 1*B*). Deletion of either repeat 1, the amino-terminal consensus repeats (1–3), or the carboxyl-terminal consensus repeats (4–6) moderately impaired the interaction (2–6, 76%; d1–6, 52%; 1–d2, 73%), although deletion of the amino-terminal reiterations consistently impacted the interaction more severely than deletion of repeats 4–6. In contrast, deletion of the HCF-1 PPD region containing the divergent repeats d1–d2 and the LXXLL motif nearly abrogated the interaction (1–3/4–6, 3%). Because deletion of this central region might alter the relative spacing of the remaining consensus reiterations, this region was replaced with an equivalent-sized protein segment derived from β-gal (1–2/β-gal/4–6). This replacement resulted in some recovery of the HCF-1–FHL2 interaction (11%), suggesting that the configuration of the intact HCF-1 PPD was a consideration. However, alteration of the central repeat region (d1–d2–LXXLL) still significantly impacted the HCF-1 PPD–FHL2 interaction. Consistent with the suggestion that the central divergent repeats represent the primary determinants for the FHL2–HCF-1 interaction, deletion of d1–d2–LXXLL from constructs containing either amino- or carboxyl-terminal repeats abrogated the interaction (1–3, 1%; 4–6, 0.5%). Conversely, the divergent repeat region alone (d1–d2) retained significant binding (39%).

To further define these determinants, a series of mutations were constructed targeting the residues in the d1–d2 repeats and the adjacent LXXLL motif (Fig. 2*A*). Interestingly, mutations at equivalent positions in the carboxy-terminus of either d1 or d2 affected the binding of FHL2 (Fig. 2*B*, mutations 5, 6, and 14). In addition, alteration of the LXXLL motif also significantly affected the interaction. However, because FHL2 was originally isolated by interaction with the consensus repeats, it remained likely that some determinants contained within these consensus repeats would also contribute. Therefore, residues across each consensus repeat 1, 2, or 3 were mutated within the context of the intact PPD (Fig. 2*C*). Strikingly, only mutations within repeat 1 affected FHL2 binding (Fig. 2*D*, r1-m7 and r1-m8), in contrast to the equivalent mutations in repeat 2 or 3. Repeats 1, 2, and 3 are identical with the single exception of the initial amino acid residue of repeat 3, and all contain the identical (TATT) residues altered in mutant 7. However, only mutations at these residues in repeat 1 were significant, indicating that an inherent specificity exists even within the consensus reiterations and that context or conformation of the domain is a significant consideration.

Preferential Interaction of FHL2 with Nonprocessed Precursor HCF-1. The *in vitro* analysis suggests that HCF-1 processing could regulate the interaction of FHL2 with the HCF-1 PPD. Therefore, the cleavage site in each of the six consensus repeats was altered to produce an HCF-1 protein that did not undergo processing (Fig. 3*A*, HCF-1nc). As shown, transfection of CV-1 cells with a construct expressing a V5-tagged WT protein results in the expected family of HCF-1 polypeptides (100–220 kDa). In contrast, transfection with a construct expressing the V5-tagged cleavage mutant results in detection of only the full-length

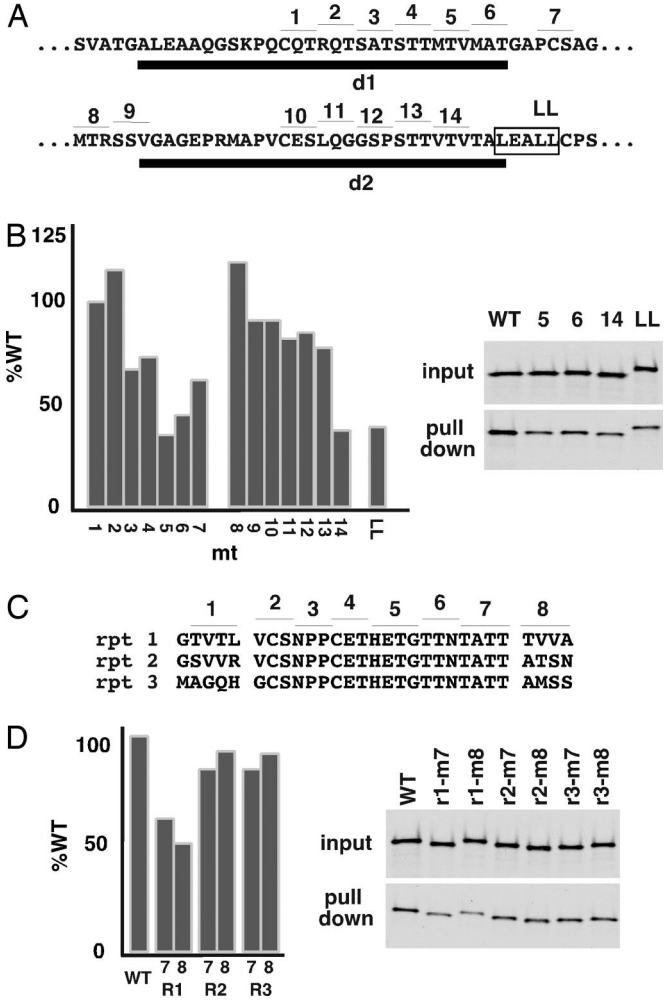

Fig. 2. Interaction specificity encoded within the HCF-1 PPD consensus repeats. GST or GST-FHL2 fusion proteins were incubated with WT or mutant PPD proteins. (*A*) The numbers above the sequence of d1 and d2 divergent repeats denote the cluster of amino acids that were changed to alanine to generate the mutant PPD proteins. LL indicates the two amino acids in the LXXLL motif (boxed) that were changed to alanine. (*B*) The amount of bound protein is graphically represented relative to the amount of WT protein bound (100%) and is representative of several independent experiments. Input was 10% of the total input of PPD protein in each reaction. The autoradiogram shows the results of the mutant PPD proteins (5, 6, 14, and LL) that were impaired in the interaction with FHL2 relative to the WT protein. (*C*) The consensus repeats 1, 2, and 3 are aligned, and the numbers denote the clusters of amino acids that were changed. (*D*) The graph and gel show the results of those PPD proteins having mutations in equivalent positions (7 and 8) of repeat 1, 2, or 3.

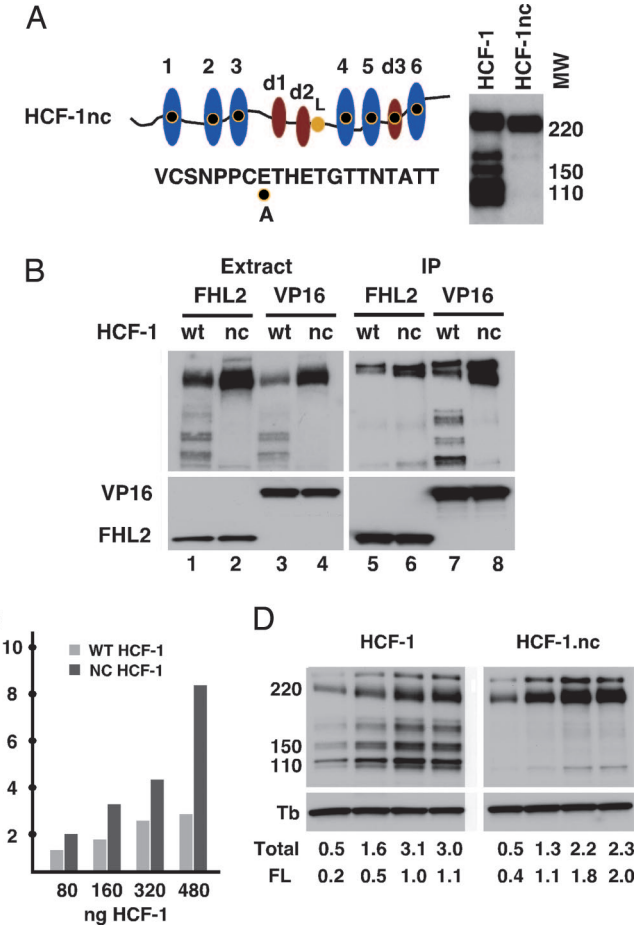

Fig. 3. Preferential interaction of FHL2 with the 220-kDa HCF-1 precursor. (*A*) The noncleavable HCF-1 PPD (HCF-1nc) is illustrated with the cleavage site in each repeat (E) mutated to (A). CV-1 cells were transfected with V5-tagged WT or HCF-1nc proteins. Cell lysates were probed using anti-V5 antiserum. (*B*) CV-1 cells were cotransfected with FLAG-tagged FHL2 or VP16 and the V5-tagged WT HCF-1 or HCF-1nc proteins. FHL2 or VP16 were immunoprecipitated using anti-FLAG serum and probed with anti-V5 and anti-FLAG seras. wt, wild-type; nc, noncleavable; Extract, cell lysate; IP, immunoprecipitate. (*C*) CV-1 cells were cotransfected with Gal4DB-FHL2 fusion, a Gal4-luciferase reporter, and increasing amounts (80–480 ng) of the WT HCF-1, HCF-1nc, or the control vector. The fold activation is the luciferase activity of the cotransfected cells divided by the basal level and is representative of several independent experiments. (*D*) Equivalent amounts of extracts of CV-1 cells cotransfected with increasing amounts of WT or HCF-1nc were probed using anti-V5 antisera and anti-β-tubulin (Tb) sera. The amount of full-length HCF-1 protein (FL) and HCF-1 subunit forms were quantitated by using a Kodak Image Station 4400 and normalized to the amount of the β-tubulin internal control.

220-kDa precursor protein in cell extracts [Fig. 3*A*, noncleavable HCF-1 (HCF-1nc)]. In both cases, the expressed HCF-1 protein was localized in the nucleus as detected by immunofluorescence using anti-V5 antisera (data not shown).

To determine the preferential interaction of FHL2 with the family of HCF-1 proteins, constructs expressing FLAG-FHL2 or FLAG-VP16 were cotransfected with either the V5-WT- or HCF-1nc-expressing constructs. The appropriate cotransfected cell extracts were immunoprecipitated with anti-FLAG, and the elutates were probed for HCF-1 (anti-V5). As shown in Fig. 3*B*, VP16 coimmunoprecipitated multiple forms of the processed WT HCF-1 as well as full-length protein (lanes 7 and 8). In this case, no preferential interaction with any particular HCF-1 products was detected. In contrast, FHL2 coimmunoprecipitated primarily the 220-kDa full-length protein from either WT or noncleavable extract (lanes 5 and 6). Because the relative ratio of full-length to processed HCF-1 forms was nearly equivalent in both FHL2- and VP16-containing extracts (Fig. 3*B* and data not shown) and the expression of FHL2 did not inhibit the processing of WT HCF-1 (see Fig. 7, which is published as supporting information on the PNAS web site), the results indicate that FHL2 preferentially interacts with full-length HCF-1 protein. It should be noted that although FHL2 may bind other HCF-1 subunit forms, these forms are in relatively low abundance in the cell type and context in which these experiments were performed (as detailed in *Discussion*).

The preferential interaction of FHL2 with full-length HCF-1 suggests that the HCF-1nc would more efficiently stimulate

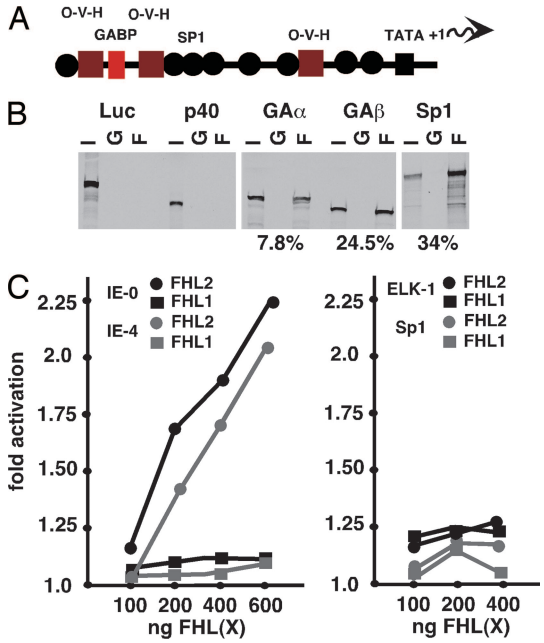

Fig. 4. FHL2 interacts with the HSV IE accessory factors and costimulates IE gene expression. (*A*) A typical HSV IE gene (IE-4) is illustrated showing the binding sites for the enhancer core (O-V-H), GABP, and Sp1 that are present in each of the IE gene promoters. (*B*) GST or GST-FHL2 fusion protein was incubated with 2 fmol of GABPα, GABPβ, Sp1, or the control proteins luciferase (Luc) or p40. Bound proteins were quantitated relative to the amount of protein added to each reaction. Input lanes contained 10% of the labeled protein in each reaction. I, input; G, Gst; F, Gst-FHL2. (*C*) CV-1 cells were cotransfected with reporters containing the promoters of HSV IE-4, IE-0, EKL-1, or Sp1 (150 ng) and increasing amounts of FHL2, FHL1, or the control vector as indicated. The fold activations represent the luciferase activities of the cotransfected cells divided by the basal levels. The fold activation averages from several independent experiments are graphed (SD $\leq$ 0.1-fold).

FHL2-dependent transcription in a mammalian two-hybrid assay. As shown in Fig. 3*C*, cells were cotransfected with a construct expressing an FHL2-Gal4 DNA-binding domain fusion, Gal4-luciferase reporter, and increasing amounts of either the WT HCF-1 or HCF-1nc. In parallel, transfections were probed for HCF-1 (anti-V5), and the total amounts of full-length and HCF-1 forms were quantitated (Fig. 3*D*). As shown (Fig. 3*C*), the HCF-1nc significantly stimulated FHL2-dependent transcription (8.5-fold), whereas the WT protein was clearly less effective (3-fold), even though the expressed levels of WT HCF-1 were generally greater than those of the HCF-1nc protein (Fig. 3*D*). Given the preferential interaction of FHL2 with the nonprocessed HCF-1 protein, it is likely that the level of stimulation mediated by the WT HCF-1 is due to the percent of precursor protein present (31–40% of total HCF-1 protein; Fig. 3*D*).

FHL2 Functions as a Costimulator of the HSV IE Genes. The HSV IE genes are well characterized targets of HCF-1-dependent regulation. The substantial basal-level expression of these genes is mediated by cellular transcription factors such as GABP and Sp1, whereas the viral-induced expression is mediated by the enhancer core complex consisting of Oct-1, the viral transactivator VP16, and HCF-1 (Fig. 4*A*). For the IE genes, HCF-1 has been shown to be the essential component required to mediate both the expression via GABP/Sp1 and the transcriptional induction via the enhancer core complex. Given the defined roles of FHL2 in transcriptional regulation, the potential impact of FHL2 on the HCF-1-dependent IE gene regulation was investigated. As shown in Fig. 4*B*, FHL2 specifically interacts with the HCF-1 partners GABP and Sp1, as illustrated by the coprecipitation of GABPα (7.8% of input), GABPβ (24.5% of input), and Sp1 (34% of input) in a GST-FHL2 pull-down assay. In contrast, no interaction is detected with GST alone or with the control GST-luciferase and GST-p40 fusion proteins. Identical results were also obtained in a GST-FHL2 pull-down of the endogenous proteins from cell lysates (data not shown).

Because GABP and Sp1 contribute to the basal-level expression of the HSV IE genes in an HCF-1-dependent manner, the effect of FHL2 on the expression of IE gene reporters was determined. As shown in Fig. 4*C*, IE reporter constructs containing either the HSV IE-4 or IE-0 regulatory-promoter domains were specifically stimulated by FHL2 in a dose-dependent manner. In contrast, reporter constructs containing the promoters derived from the ELK-1 and Sp1 transcription units were unaffected. In addition, the LIM domain protein FHL1, which failed to interact with HCF-1, also failed to stimulate the IE reporter genes. The results indicate that FHL2 can costimulate the expression of an HCF-1-dependent gene, likely via concerted interactions with HCF-1 and HCF-1-dependent transcription factors.

Discussion

Site-specific proteolytic processing is a critical regulatory mechanism involved in basic cellular processes such as cell-cycle progression, gene expression, apoptosis, and signal transduction. In contrast to degradative processing, the regulation of gene expression by limited proteolytic processing has been defined for factors such as SREBP, CREB3, ATF6, NFκB, p53, C/EBPβ, Stat(s), Notch, IRF2, Tisp40, Cubitus interruptus, and CDP/Cut. In cases exemplified by SREBP, sequestered factors are released by intramembrane cleavage that allows transport of the factor to the nucleus. Sequestered by a transmembrane tether in the endoplasmic reticulum, SREBP is specifically cleaved to release an amino-terminal polypeptide upon sterol deprivation. The released cleavage product migrates to the nucleus and regulates the expression of genes involved in sterol metabolism. Other proteolytic regulatory mechanisms involve the removal of critical functional domains, leading to the production of repressor proteins.

Site-specific proteolytic processing of the transcriptional coactivator HCF-1 is unique. The protein is cleaved at a series of internal reiterations to generate a family of amino- and carboxyl-terminal subunits that remain tightly associated. The processing of HCF-1 occurs predominantly in the nucleus (30) and is mediated autocatalytically via domains located in the carboxyl-terminal subunit of the protein (15). Studies using a heterologous chimeric protein containing the HCF-1 reiterations have also suggested that processing is processive, leading to multiple cleavages within a given series of HCF-1 reiterations (30). However, the biochemical implications of processing remained elusive.

As demonstrated here, the 450-aa processing domain also serves as a protein interaction domain, indicating that processing can regulate the interaction and, therefore, transcriptional potential of the coactivator. The interaction of the HCF-1 PPD with FHL2 supports this model and illustrates the intrinsic specificity encoded within the HCF-1 reiterations (Fig. 5). Here, determinants contributing to the binding of FHL2 are contained within the central divergent repeats (d1–d2), the adjacent coactivator motif LXXLL, and the first consensus repeat. Most interestingly, equivalent mutations within the consensus repeats 1, 2, and 3 do not have equivalent effects on the HCF-1–FHL2 interaction even though the amino acid sequences of the three repeats are nearly identical. The discrimination between these repeats by FHL2 indicates the specificity within the consensus reiterations and suggests that the

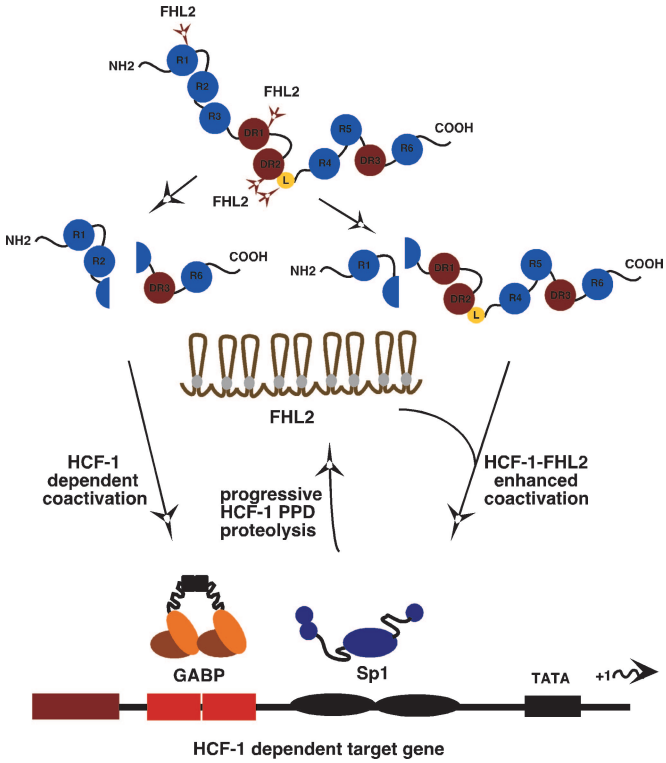

Fig. 5. Proteolytic regulation of HCF-1 interactions and coactivation potential. Shown is a model in which processing at HCF-1 reiterations determines the ability of HCF-1 to interact with protein partners (FHL2), thus modulating the HCF-1 coactivation potential. The determinants involved in the interaction of FHL2 with the HCF-1 PPD are indicated (arrows). On the right side of the figure, site-specific cleavage generates an HCF-1 molecule that retains the high-affinity determinants for binding FHL2. The product of this cleavage may recruit FHL2, resulting in an enhanced HCF-1-dependent transcriptional coactivation of a target gene via factors such as GABP and Sp1. Progressive processing in the cell nucleus may ultimately result in destabilization of the HCF-1–FHL2 complex and down-regulation of the coactivation potential. Conversely, the processing shown on the left side of the figure generates an HCF-1 molecule that would have a low affinity for FHL2, resulting in a reduced level of HCF-1-dependent transcriptional coactivation of the target gene.

context of the individual repeats or the overall conformation of the domain may be a significant determinant.

Processing of any given HCF-1 molecule may occur at one or more of the consensus reiterations. Therefore, it would be expected that some cleaved HCF-1 molecules would retain the critical interaction determinants for binding FHL2. However, FHL2 primarily coimmunoprecipitates the full-length HCF-1 precursor protein from cell extracts. This is likely due to the low abundance of appropriate cleavage products (derived from cleavage at repeats 4, 5, or 6 that would generate amino-terminal subunits containing repeats 1–d2, 1–4, or 1–d3, respectively). Additionally, processing may significantly alter the conformation of the PPD, thus affecting the affinity of the interaction.

FHL2 is a transcriptional coactivator/corepressor for factors such as AR, Fos, Jun, β-catenin, ELK-1, PLZF, SRF, Hand 1, and FOXO1 (32–40, 47). In many cases, transcriptional regulation is achieved by mediating protein–protein interactions or modifications as shown by: (*i*) stimulation of the acetylation of β-catenin, leading to regulation of Wnt-signaled target genes (34); (*ii*) repression of ELK-1 activation by preventing nuclear accumulation of activated ERK (47); (*iii*) enhancement of the FOX01 and SIRT1 interaction, leading to deacetylation of FOX01 (40); and (*iv*) corepression of PLZF by recruitment of corepressors forming the histone deacetylase complex (35).

Coactivation of the HSV IE genes represents a new target for FHL2 regulation. The expression of these genes is determined at the level of the coactivator HCF-1, which is essential for mediating the induced transcriptional potential of the core (Oct-1 and VP16) and the activities of the ancillary factors (GABP and Sp1). The interaction of FHL2 with both GABP and Sp1 and costimulation of the HSV IE genes indicate that FHL2 functions in concert with HCF-1 to promote a cooperative interaction of these components in promoter contexts such as the HSV IE genes. The significance of FHL2 costimulation may lie in distinct cell contexts where other stimulatory components are limiting such as in sensory neurons during the reactivation of HSV from latency. In these cells, HCF-1 is specifically sequestered in the cytoplasm and is transported to the nucleus in response to stress stimuli that results in viral reactivation (i.e., UV irradiation, tissue damage, growth factor withdrawal). Because the viral transactivator VP16 is not expressed under these conditions, the model states that HCF-1 functions in concert with other cellular factors such as GABP to promote the expression of the viral IE genes during the reactivation process. Interestingly, in some cell types, FHL2 is regulated and transported by stress stimuli (36, 48), and the ability to function with HCF-1 to costimulate the transcription of the HSV IE or other target genes may be important in this context.

The interaction of HCF-1 and FHL2 reveals that the HCF-1 PPD functions both as a target for proteolytic processing and as an interface for protein partners. In this case, the progressive nuclear processing of this protein would have a negative regulatory impact on HCF-1 coactivation, perhaps by destabilization of the activator complexes or promoting alterations in the complex composition as presented in Fig. 5. However, this does not preclude the possibility that in some contexts, processing could alter the ability of repressive regulatory factors to bind HCF-1, resulting in enhancement of the HCF-1 coactivation potential.

This analysis of HCF-1 processing provides a biochemical consequence of this processing and extends the biological significance of proteolysis as an important regulatory mechanism in gene transcription. In a separate study, Julien and Herr (29) have suggested that proteolytic processing may also be important in segregating the distinct cell-cycle functions of the amino- and carboxyl-terminal HCF-1 subunits (29). Therefore, the processing of HCF-1 is an important mechanism for regulating several functions ascribed to HCF-1.

Materials and Methods

Two-Hybrid Screens. HF7c was transformed with pGALrpt23 encoding the HCF-1 PPD repeats 2 and 3 (amino acids 1,057–1,136) and a HeLa Matchmaker library (Clontech). His+ clones were rescreened using an HCF PPD clone encoding repeats 2–6 (amino acids 1,057–1,431, pGALrpt2–6). The FHL2 LIM domains required for mediating the FHL2–HCF-1 interaction are described Fig. 8, which is published as supporting information on the PNAS web site.

***In Vitro* Protein Interaction Assays.** The expression and purification of GST-FHL2 are described in *Supporting Methods*, which is published as supporting information on the PNAS web site. GST pull-down assays were performed as described in ref. 22 using 0.5–3 μg of purified GST or GST fusion protein(s) and 2–10 fmol of *in vitro*-translated [^{35}S]methionine-labeled proteins. Input and elution protein samples were resolved by SDS/PAGE, transferred to nitrocellulose, and quantitated (Typhoon; Molecular Dynamics) before autoradiography. The percent bound of each labeled protein relative to the WT control was calculated as (bound protein/input protein)/(bound WT/input WT) × 100.

HCF-1 and HCF-1 PPD Constructs. DNA encoding HCF-1 was inserted with a carboxyl-terminal V5 epitope tag into pcDNA (pHCF/V5). HCF PPD proteins were produced by assembling clones encoding the following: repeats 1–3 (amino acids 993–1,132), d1–d2 (amino acids 1,133–1,282), repeats 4–6 (1,283–1,450), and β-gal (amino acids 440–596) in pET21. Alanine substitution mutations were made by using the Stratagene QuikChange mutagenesis kit. HCF-1nc was constructed by synthesis of DNA encoding the HCF PPD containing codon changes (E-to-A) at the cleavage position of the six consensus repeats and replacement of the WT coding sequences with the HCF-1nc sequences.

Coimmunoprecipitations and Western Blots. DNAs encoding VP16 and FHL2 were inserted with a FLAG epitope tag into pcDNA. CV-1 cells (4×10^6) were cotransfected with HCF-1 or HCF-1nc constructs (12 μg) and FLAG-VP16 (3 μg) or FLAG-FHL2 (7 μg). Forty-eight hours after transfection, the cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris, pH 7.5/150 mM NaCl/0.5% Nonidet P-40/1 mM NaF/10 mM β-glycerophosphate/0.1 mM Na_3VO_4, complete). Coimmunoprecipitations were performed according to standard protocols using 2.5 mg of protein extract and FLAG-M2 beads (Sigma). Eluted proteins were resolved and probed with anti-FLAG M2 (Sigma) or anti-V5 (Invitrogen) and anti-β-tubulin (H-235; Santa Cruz Biotechnology) antibodies. Blots were developed with Pierce SuperSignal and quantitated by using a Kodak Image Station 4400.

Mammalian Two-Hybrid and Luciferase Reporter Assays. pBD-FHL2 contained the FHL2 coding sequence in pCMV-BD. CV-1 cells (8×10^4) were cotransfected with 4 ng of BD-FHL2, 160 ng of FR-Luc (Stratagene), 4 ng of ph-RL-null (Promega), and increasing amounts of pcDNA, HCF-1, or HCF-1nc plasmids. For FHL2 coactivation assays, luciferase reporters contained the promoter-regulatory domains of IE-0 (−341 to +39), IE-4 (−330 to +33), ELK-1 (−500 to +34), and Sp1 −217 to +44 [gift of C. J. Ciudad (University of Barcelona, Barcelona)]. CV-1 cells ($5 - 10^4$) were cotransfected with 150 ng of luciferase reporter, 5 ng of phRL-null transfection control, and increasing amounts of pCMV-LacZ, pCMV-FHL1, or pCMV-FHL2. The firefly luciferase activity of extracts was measured by using a luminometer (Zylux Corporation, Oak Ridge, TN) and was normalized to the activities of the *Renilla* luciferase.

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