Asparaginyl Hydroxylation of the Notch Ankyrin Repeat Domain by Factor Inhibiting Hypoxia-inducible Factor*

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The stability and activity of hypoxia-inducible factor (HIF) are regulated by the post-translational hydroxylation of specific prolyl and asparaginyl residues. We show that the HIF asparaginyl hydroxylase, factor inhibiting HIF (FIH), also catalyzes hydroxylation of highly conserved asparaginyl residues within ankyrin repeat (AR) domains (ARDs) of endogenous Notch receptors. AR hydroxylation decreases the extent of ARD binding to FIH while not affecting signaling through the canonical Notch pathway. ARD proteins were found to efficiently compete with HIF for FIH-dependent hydroxylation. Crystallographic analyses of the hydroxylated Notch ARD (2.35 Å) and of Notch peptides bound to FIH (2.4–2.6 Å) reveal the stereochemistry of hydroxylation on the AR and imply that significant conformational changes are required in the ARD fold in order to enable hydroxylation at the FIH active site. We propose that ARD proteins function as natural inhibitors of FIH and that the hydroxylation status of these proteins provides another oxygen-dependent interface that modulates HIF signaling.

Although the post-translational hydroxylation of extracellular proteins such as collagen is well characterized, examples of similarly hydroxylated intracellular proteins are very limited (1). Recently, prolyl and asparaginyl hydroxylation of HIFα7 has been shown to act in an unprecedented mode of intracellular signaling that links oxygen availability to the HIF transcriptional response (2). Three oxygenases (prolyl hydroxylase domain 1–3) hydroxylate two conserved HIFα prolines, signaling for proteasomal degradation (3). FIH catalyzes hydroxylation of the HIFα C-terminal transactivation domain (CAD) at Asn-803, resulting in blockage of the HIF-α300/CBP interaction and inhibition of HIF-mediated transcription (4, 5).

Since the discovery of the HIF hydroxylases, a fundamental question has been whether these enzymes have other substrates. To address this question, we have carried out studies aimed at identifying proteins that interact with FIH. Several proteins co-immunoprecipitating with FIH, including Notch3, contained an ARD. Notch is a transmembrane receptor that regulates cell fate decisions, differentiation, and proliferation (6). Mammals express four Notch proteins (N1–N4) that, upon binding of ligands such as Delta-1, stimulate cytoplasmic release of the Notch intracellular domain (ICD) (7). The notch ICD is recruited to target genes (e.g. hes-1) by the transcription factor CSL (CBF-1/suppressor of hairless/Lag-1), where, through its ARD, it coordinates the assembly of a nuclear transcriptional activation complex involving Mastermind-like (MAML) proteins and Ski-interacting protein (SKIP) (7, 8). It has been reported that Notch signaling is sensitive to oxygen tension (9), and multiple direct and indirect interactions between the Notch and HIF signaling pathways have recently been described (9–12).

Here we report that the Notch ARD is hydroxylated by FIH at conserved Asn residues. Taken together with the recent identification of ARD-containing FIH substrates in the NF-κB system (13), these results imply that post-translational hydroxylation of ARDs is common. ARDs are found in over 200 proteins encoded by the human genome (SMART data base) (14) and generally comprise a variable number of 33 residue repeats folded into paired antiparallel α-helices linked by “β-hairpin” type loops. The repeats typically stack to form an L-shaped profile with the concave face acting as a protein-protein interaction surface (15). Numerous structural studies have characterized the ARD fold, but none have used Asn-hydroxylated material (15). Our findings therefore raised important questions about the effect of hydroxylation on the ankyrin fold, how ARDs and HIF bind to the FIH active site, and whether ARD hydroxylation interacts with HIF signaling.

Crystallographic and kinetic studies demonstrated that binding of FIH requires major conformational changes to the ARD...
and that ARD hydroxylation is competitive with that of HIFα-CAD. The combined functional and biochemical studies imply that the hydroxylation status of cellular ARDs governs the availability of FIH for the hydroxylation of HIF, thus providing a novel oxygen dependent means of modulating HIF signaling.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse anti-FIH was used to detect human FIH, as described (16). Polyclonal rabbit anti-FIH was raised against full-length recombinant FIH and used to detect mouse FIH. Other antibody sources were as follows: anti-PK (Serotec), anti-HA (Roche Applied Science), anti-β-tubulin (Sigma), anti-Myc (9E10 from CRUK), Rabbit anti-Hes-1 (gift from Toray Pharmaceuticals, Inc.), and mouse anti-His (Sigma).

Plasmids—Mouse N1 ARD-(1898–2105), N3 ARD-(1821–2026), and N4 ARD-(1619–1819) were amplified by PCR and cloned into pGEX-4T-1 or pEF6-VSVG (gift from R. Marais, Institute of Cancer Research, London, UK). ΔN1 ICD (residues 1751–2191 mouse N1) and N1 ICD (residues 1751–2531 mouse N1) with PK epitopes at the C terminus were generated by PCR and cloned into pCDNA3 or pEF6. pCMV Myc-Deltex1 was a gift from S. Artavanis-Tsakonas (Yale University, New Haven, CT). pCMV2 FLAG-MAML1 and pCDNA3 Myc-CSL were generously provided by J.C. Aster (Harvard Medical School, Boston, MA). pGEX-KG ΔMAML1 (residues 7–254) was generated by Ncol/Sacl digest of pCMV2 FLAG-MAML1. pEF6 (gift from R. Marais, Institute of Cancer Research, London, UK) FIH and FIH D201A were generated by PCR using pCDNA3 FIH and pCDNA3 FIH D201A as template (16). pCGSP HA-SKIP was kindly provided by M. Hayman (Stony Brook University, New York). pGAL4DBD-HIF1α-(652–826), pGAL4DBD-VP16, and pUAS-luc have been described previously (17). E2002K, N1945A, N2012G, and N1945A/N2012G mutants were generated using standard techniques. The integrity of all constructs was verified by sequencing.

Transfection—RNA interference used Oligofectamine (Invitrogen). Human FIH and control Drosophila HIF siRNA oligonucleotides were as reported (16). Mouse FIH siRNA oligonucleotides were from Dharmacon (sense, 5′-GGCAAUUUCCAGAACUUUAdTdT-3′). Plasmid transfections used Fugene (Roche Applied Science), except for P19 cells, which used Lipofectamine Plus (Invitrogen).

Immunoprecipitation and Pull-downs—Cells were lysed in IP + buffer (13). PK-tagged proteins were precipitated with conjugated agarose beads (Sigma). FLAG-FIH-inducible U2OS cells used for Notch interaction assays have been described (16); Notch receptors were immunoprecipitated with either rabbit anti-N2 (Chemicon), Rabbit anti-N3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or goat anti-N1 antibody (Santa Cruz Biotechnology). Rabbit anti-N2 and -N3 immunocomplexes were then Western blotted with the corresponding Notch antibody, followed by a TrueBlot™ horseradish peroxidase-conjugated secondary antibody (eBioscience). Goat anti-N1 immunocomplexes were Western blotted with mouse anti-N1 (Sigma), followed by standard horseradish peroxidase-conjugated secondary antibody. Endogenous N1-FIH complexes were immunopurified using a goat anti-N1 antibody (Santa Cruz Biotechnology) and either probed as described above for N1 or with anti-FLAG-horseradish peroxidase antibody (Sigma). GST-pull-down assays were performed with 293T cell extracts overexpressing the interaction partner of interest. Precleared extracts were incubated with glutathione beads coated with 5 μg of GST or GST-bait. For purification of endogenous human N1, 20 × 15-cm2 plates of 293T cells were lysed in IP+ and incubated with mouse anti-N1 antibody (Sigma) coupled to protein G-agarose. Beads were then washed and eluted in glyrine, pH 2.5. PK-ΔN1 ICD-His-FIH pull-downs were undertaken by purifying PK-ΔN1 ICD from 293T cells or cells overexpressing FIH. Anti-PK-conjugated agarose beads were washed before adding His-FIH. PK-ΔN1 ICD-His-FIH complexes were washed and eluted in SDS sample buffer.

Mass Spectrometry—Liquid chromatography (LC)-MS used an an Agilent 1100 LC system (Jupiter C4 column) and a Waters QTof micromass spectrometer in positive ion ESI mode. For in vitro competition assays, Fourier transform MS was used; 2-μl samples were injected onto a Nano PepMap C18 column (75 μm x 15 cm; Dionex) using a Dionex UltiMate 3000 LC system linked to a Bruker Apollo NanoElectrospray ion source (capillary voltage: 1400 V, dry temperature = 150 °C) and a Bruker APEX-Qe quadrupole hexapole-Fourier transform mass spectrometer (7T). MS/MS analyses of endogenous N1 hydroxylation and non-denaturing electrospray ionization MS methodology is described in the supplemental materials.

Reporter Assays—Luciferase activity was assayed 24 h after transfection with CSL-luc, β-galactosidase and N1 ICD (13). In vivo FIH activity was assayed using a UAS-luc reporter and GAL4DBD-HIF1αCAD (residues 652–826). HeLa cells were treated with 10 nm FIH siRNA as a positive control for inhibition of HIF1αCAD Asn-803 hydroxylation. Cells were then transfected with β-galactosidase, UAS-luc, and GAL4DBD-HIF1αCAD plus or minus Notch ARDs or N1 ICDs. 48 h after transfection, samples were assayed for β-galactosidase and luciferase activity. UAS-luc/GAL4DBD-VP16 assays were performed in parallel.

Recombinant Protein Preparation and in Vitro Protein Hydroxylation—pGEX-4T1 ARDs were transformed into Escherichia coli BL21 (DE3) and induced with isopropyl β-d-1-thio-galactopyranoside at 28 °C. GST-tagged ARDs were purified using glutathione-Sepharose chromatography. Fractions containing pure protein were concentrated before removing glutathione with a PD-10 column (Amersham Biosciences). GST-ARDS were >95% pure by SDS-PAGE. GST-MAML1-(7–254) was purified (>90% by SDS-PAGE) as for Notch ARDs, except isopropyl β-d-1-thio-galactopyranoside induction was at 37 °C. Recombinant Notch ARDs were incubated with purified FIH for time course studies and 2-oxoglutarate (2OG) decarboxylation assays as described (18). His-HIF1αCAD purification is described in the supplemental materials.

Crystallography—Hydroxylated N1 ARD was produced by resuspending GST-N1 ARD and His-FIH cell pellets in a 1:1 ratio in 40 mM HEPES, 0.2 mM NaCl, pH 7.4, and 1 mg of DNase I. 10 mM 2OG and 40 μM Fe(II) were added prior to sonication, followed by two further treatments with 2OG and Fe(II). GST-N1 ARD was purified as described above. Thrombin-cleaved GST-N1 was loaded onto a glutathione affinity column.
Notch Asparaginyl Hydroxylation by FIH

FIGURE 1. Notch receptors are FIH substrates. A, N1–N3 interact with FIH. FIH-inducible U2OS cells were treated with 1 μg/ml doxycycline for 24 h prior to Notch receptor immunoprecipitation (IP) and anti-FLAG or anti-Notch immunoblotting. Treatment with 1 μM dimethyloxalylglycine (DMOG) for 16 h stabilized Notch-FIH complexes (lanes 4 and 5). B, N1 and N3 ARD promote 2OG decarboxylation. Reactions contained the indicated GST fusions plus recombinant FIH. C, FIH activity is dependent on N1 Asn-1945 and Asn-2012 hydroxylation. Recombinant FIH and mouse N1 and N3 ARD promote 2OG decarboxylation. Reactions contained the indicated GST fusions plus recombinant FIH. 2OG decarboxylation assays were repeated twice in duplicate. Results varied ±5%. D, N1 ICD Asn-1945 and Asn-2012 hydroxylation in vivo. Transfection with PK-ΔN1 ICD (with or without FIH, FIH D201A, or empty vector (EV) control) for 24 h prior to immunoprecipitation and MS. For FIH siRNA, cells were treated with 10 nM siRNA prior to N1 ICD transfection.

to remove GST, and the eluant was subjected to gel filtration on a Superdex-75 column. Hydroxylated N1 ARD was >95% pure; MS analysis showed >95% Asn-1945 and <10% Asn-2012 hydroxylation. Recombinant FIH and mouse N1-(1930–1949) (RSDAAKRLLEASADANIQDN) or N1-(1997–2016) (LEGMLEDLINSHAVNAVDD) peptides were co-crystallized using conditions reported for FIH-HIF1αCAD complex (19). Detailed crystallization, structural determination, and modeling methods used for the hydroxylated N1 ARD and FIH-N1 complexes are provided in the supplemental materials.

Statistical Analysis—Where possible, data are presented as the mean ± S.D. Differences between means were considered significant when p was <0.05 using Student’s t test.

RESULTS

Notch Receptors Are Potential FIH Substrates—We identified four ARD-containing proteins (N3, Rabankyrin, Tankyrase, RNase L) in a screen for FIH-interacting proteins by immunoprecipitation of FLAG-tagged FIH from extracts of U2OS cells overexpressing this protein, followed by MS analyses.® Given the known links between Notch signaling and hypoxia (9), we focused studies on the Notch-FIH interaction. Initially, we tested whether other Notch receptors interact with FIH by immunoprecipitation (Fig. 1A). N1, N2, and N3 all bound to

® M. Cockman, unpublished data.

FIH, and interactions were enhanced by a cell-permeable FIH inhibitor (dimethyloxalylglycine), raising the possibility that Notch ICDs are FIH substrates.

Comparison of human N1–N4 sequences with HIF1αCAD revealed the presence of two sites in the ARDs that together with HIF1αCAD form a (D/E)-2X-1N consensus motif (supplemental Fig. S1). Both of the putative hydroxylation sites were present in the “β-hairpin” type loops connecting individual ARs. The N1 N-terminal site (“Site 1”) is positioned in the second AR, whereas the C-terminal site (“Site 2”) is in the fourth. In contrast to N1–N3, N4 lacks the (D/E)XN motif; the acidic residue at the –2-position is absent from N4 Site 1, whereas Site 2 lacks the Asn residue itself, suggesting that N4 is not an FIH substrate.

FIH Hydroxylates N1 Asn-1945 and -2012—To determine whether Notch ARDs are indeed FIH substrates, we tested GST fusions of mouse Notch ARDs in assays based on 2OG (co-substrate) decarboxylation (18). GST-N1 ARD and GST-N3 ARD were similarly active, whereas GST-N4 ARD was not (Fig. 1B). Consistent with this, the ARDs of N1 and N3 (but not N4) were sufficient to interact with FIH in cells (supplemental Fig. S2). Thus, N1 and N3 ARD are FIH substrates in vitro, and the presence of a (D/E)XN motif may indicate whether an AR is an FIH substrate.

Since N1 is the best characterized family member, it was chosen for further analyses. To address whether N1 Site 1 and/or Site 2 are required for FIH activity, we compared N1 ARD N1945A, N1 ARD N2012G, and N1 ARD N1945A/N2012G with wild-type N1 ARD and HIF1αCAD. 2OG decarboxylation with N1 ARD was much greater than with HIF1αCAD, consistent with hydroxylation at two sites (Fig. 1C). Mutation of either site individually reduced decarboxylation to levels comparable with that observed with HIF1αCAD; mutation of both Asn residues reduced it to control levels. MS analysis of the N1 ARD/FIH reaction products confirmed that Asn-1945 and -2012 were >95% hydroxylated (supplemental Fig. S3). Time course analyses suggest that Asn-1945 is more rapidly and more completely hydroxylated by FIH under nonsaturating conditions however.®

Next we asked whether Notch ARDs are hydroxylated by FIH in vivo. To generate sufficient material with both substrate sites amenable for MS, we expressed a C-terminally truncated N1

® C. Coles, unpublished data.
ICD K2002E mutant (ΔN1 ICD) in 293T cells. PK-tagged ΔN1 ICD was immunopurified; MS identified 35% hydroxylation at Asn-1945 and 3% at Asn-2012 (Fig. 1D). These results support the kinetic data showing Asn-1945 as the preferred site of hydroxylation.9 FIH siRNA prior to overexpression of ΔN1 ICD demonstrated that FIH was required for hydroxylation at both
of mouse N1) was detected by MS/MS.\textsuperscript{11} Two peptides containing Asn-1956 (equivalent to Asn-1945 of mouse N1) were detected, one unhydroxylated (Fig. 2A) and the other hydroxylated at Asn-1956 (Fig. 2B). LC-MS data indicated that 96% of the Asn-1956-containing peptide was hydroxylated in 293T cells (Fig. 3A). Similar analysis identified 30% Asn-1956 hydroxylation in HeLa-S3 cells.\textsuperscript{11} Incubating 293T cells in 0.5% oxygen for 48 h prior to endogenous N1 purification and LC-MS analyses reduced Asn-1956 hydroxylation from 96 to 40% (Fig. 3A). Likewise, siRNA knockdown of FIH reduced Asn-1956 hydroxylation from 84% (control siRNA) to 25% (Fig. 3B).

These results confirm that the hydroxylation status of endogenous ARDs is not only dependent on FIH but also cellular oxygen availability. We next sought to understand the role of Asn hydroxylation and in particular whether it regulates Notch signaling activity.

FIH Does Not Regulate the Notch/CSL Pathway—Gustaffson \textit{et al.} reported that FIH overexpression suppressed N1 ICD activity in mouse embryonic teratocarcinoma P19 cells.\textsuperscript{11} In agreement, we found that FIH overexpression in P19 cells significantly suppressed the ability of N1 ICD to activate a Notch 12XCSL luciferase (CSL-luc) reporter (Fig. 4A, left). However, mutant FIH D201A also inhibited N1 ICD activity as effectively as wild-type FIH, implying that the effect is independent of hydroxylation. Moreover, FIH siRNA did not significantly enhance N1 ICD activity (compared with control siRNA), suggesting that, at least under these conditions, endogenous FIH is not exerting tonic control of N1 ICD activity (Fig. 4A, right). Similar reporter assays in HeLa and 293T cells did not show inhibition of N1 ICD activity by FIH, probably due to lower levels of overexpression (supplemental Fig. S6). Again, FIH siRNA did not affect N1 ICD activity in these cells.\textsuperscript{10}

To test whether effects of FIH might be restricted to chromatinized Notch targets, we went on to assess endogenous Notch activity using a co-culture assay. HeLa cells overexpressing (FIH transfection) or underexpressing (siRNA) FIH were seeded onto mouse L-cells or L-cells overexpressing the Notch ligand Delta-1 (20). After 24 h, to allow Notch cleavage and signal transduction, cell extracts were immunoblotted for
FIH intervention did not significantly affect Delta-mediated Notch activation and Hes-1 induction (Fig. 4B); FIH also did not affect the induction of endogenous Hes-1 by overexpression of N1 ICD in HeLa cells. To test the effect of N1 ARD hydroxylation on the interaction with proteins that bind at or near the ARD (7, 21), GST-ARD or GST-ARD (OH) (100% Asn-1945 and 20% Asn-2012 hydroxylation) was incubated with 293T extracts overexpressing proteins of interest. Fig. 4C shows that GST-ARD and GST-ARD (OH) captured similar levels of MYC-Deltex, HA-SKIP, or MYC-CSL. We used a different approach to study the N1 ARD-MAML1 interaction, since overexpressed MAML1 did not bind GST-ARD. GST or GST-ΔMAML1 (amino acids 7–254 of human MAML1 containing the Notch binding domain) was incubated with extract from PK-ΔN1 ICD-transfected cells overexpressing (FIH transfection) or underexpressing (siRNA) FIH (see Fig. 1D). Complexes were probed with anti-PK to detect ΔN1 ICD.

Hes-1 induction. FIH intervention did not significantly affect Delta-mediated Notch activation and Hes-1 induction (Fig. 4B); FIH also did not affect the induction of endogenous Hes-1 by overexpression of N1 ICD in HeLa cells. To test the effect of N1 ARD hydroxylation on the interaction with proteins that bind at or near the ARD (7, 21), GST-ARD or GST-ARD (OH) (100% Asn-1945 and 20% Asn-2012 hydroxylation) was incubated with 293T extracts overexpressing proteins of interest. Fig. 4C shows that GST-ARD and GST-ARD (OH) captured similar levels of MYC-Deltex, HA-SKIP, or MYC-CSL. We used a different approach to study the N1 ARD-MAML1 interaction, since overexpressed MAML1 did not bind GST-ARD. GST or GST-ΔMAML1 (amino acids 7–254 of human MAML1 containing the Notch binding domain) was incubated with extract from PK-ΔN1 ICD-transfected cells overexpressing (FIH transfection) or underexpressing (siRNA) FIH (see Fig. 1D). Complexes were probed with anti-PK to detect ΔN1 ICD.
proteins, consistent with the lack of effect of FIH-dependent hydroxylation on the Notch/CSL pathway.

Structure of Asn-hydroxylated N1 ARD—FIH-mediated hydroxylation of the Notch ARD is a post-translational modification likely to be common to many other ARDs. Since structural analyses of all ARDs to date have used unhydroxylated material (15), we investigated the structural consequences of FIH-mediated ARD hydroxylation by crystallography. These studies also aimed to define the regio- and stereoselectivity of ARD hydroxylation.

Hydroxylated N1 ARD (N1 ARD (OH)) was produced by co-lysis of bacteria separately producing His-tagged FIH (His-FIH) and GST-N1 ARD with co-factors and co-substrates. Under these conditions, the N1ARD was hydroxylated at 95% at Asn-1945 and 10% at Asn-2012.

Following removal of the GST tag, N1 ARD (OH) was crystallized, and the structure was solved to 2.35 Å resolution by molecular replacement using human N1-(1873–2115) (Protein Data Bank code 1YYH) as a search model (22) (supplemental Table S1). The N1 ARD (OH) structure contains six observed ARs (ARs 2–7) that fit the defined consensus. Like other ARD folds, N1ARD (OH) is curved with a slight twist along its longest axis with neighboring repeats held together by hydrophobic forces that extend throughout the ARD core (Fig. 5A).

A distinguishing feature of the electron density map for the N1 ARD (OH) structure relative to other ARD structures was clear evidence for hydroxylation at the pro-S position of the Asn-1945-β-carbon of Asn-1945 (no such electron density was observed at Asn-2012) (Fig. 5B). This observation supports assignments, based on NMR and synthetic standards, that FIH catalyzes the hydroxylation of HIF1αCAD Asn-803 in an analogous manner (23). It is also the first direct crystallographic evidence for the stereochemistry of post-translational Asn hydroxylation in an intracellular mammalian protein. Interestingly,
the hydroxylation of Asn-1945 positions the newly introduced hydroxyl group to hydrogen-bond to the side chain of Asp-1943 (Fig. 5B) (average distance 2.8 Å), and it is possible that this additional hydrogen bond stabilizes the β-hairpin type loop of the hydroxylated AR.

The conformation of the hydroxylated N1 ARD is very similar to that observed in previously published structures of unhydroxylated N1 ARDs (0.41 Å root mean square deviation based on C-α atoms in the N1 ARD (OH) and human N1-(1873–2115) structures (22)), indicating that hydroxylation does not significantly affect the conformation of the crystalline ARD fold (Fig. 5C). Furthermore, the positions of Asn-2012 and hydroxy-Asn-1945 are distal to any of the protein-protein-DNA interactions in the DNA-bound Notch transcriptional complex (24, 25). These observations are consistent with results showing that hydroxylation does not significantly regulate the Notch/CSL pathway or the interaction of N1 with known ARD binding partners.

Crystal Structures of N1 Site 1 and Site 2 Peptides Bound to FIH—The hydroxylation sites in IxB and Notch ARDs are located within the “β-hairpin” type loops that connect ARs (Fig. 5A and supplemental Fig. S1). In contrast, HIF1α Asn-803 is located in the CAD, which does not have discernible secondary structure when isolated in solution (26). The HIF1αCAD peptide binds to the surface of FIH in an extended groove that runs between the active site and the dimer interface, with the target Asn at the apex of a tight turn (19). Thus, in order for the ARD substrates to bind FIH productively, they must either undergo a considerable and unprecedented structural rearrangement to enable them to adopt a largely extended conformation, or they must bind to FIH in a different manner to HIF1αCAD.

To investigate how AR substrates bind to FIH, we initially endeavored to solve structures of FIH in com-
plex with recombinant full-length N1 ARD. Since this approach has not yet yielded FIH-N1 crystals, we obtained structures of FIH in complex with Fe(II), the cosubstrate 2OG, and the two N1 substrate peptides, N1-(1930–1949) (site 1) to 2.4 Å (Fig. 6A) or N1(1997–2016) (site 2) to 2.6 Å (supplemental Fig. S7), under anaerobic conditions. FIH crystallized as a homodimer with each monomer adopting the double-stranded β-helix fold characteristic of the 2OG oxygenases and binding a single Fe(II), 2OG, and N1 peptide (Fig. 6A and supplemental Fig. S7A; see supplemental Fig. S7 for a detailed analysis of the FIH-N1 peptide structures) (supplemental Table S1). Similar to HIF1αCAD, the target Asn together with adjacent residues forms a tight turn in the FIH active site. In each of the FIH-N1 structures, the target asparagines are buried such that their C-3 prohydrogen projects toward the Fe(II) center (Fig. 6G and supplemental Fig. S7D), consistent with the observed stereo- and regioselectivity of ARD hydroxylation by FIH (Fig. 5B).

The structural results reveal that binding of an AR to FIH occurs with induced fit involving conformational changes to both FIH and the ARD. Upon binding of N1 peptides, the FIH Trp-296 side chain moves relative to its position in the FIH-Fe2OG structure; this movement accommodates the hydrophobic side chain of the residue N-terminal to the target N1 Asn. The same Trp-296 movement was observed for N1-(1997–2016) (site 2) to 2.6 Å (supplemental Fig. S7), and the N1 ARD structures (supplemental Table S1). Similar to FIHαCAD, the target Asn together with adjacent residues forms a tight turn in the FIH active site. In each of the FIH-N1 structures, the target asparagines are buried such that their C-3 prohydrogen projects toward the Fe(II) center (Fig. 6G and supplemental Fig. S7D), consistent with the observed stereo- and regioselectivity of ARD hydroxylation by FIH (Fig. 5B).

In silico modeling analyses indicated that in order for the substrate Asn to bind to the FIH active site and for the AR substrate to bind to FIH in the extended conformation described above, there must be a conformational change in the ARD. To detail these structural alterations, we directly compared the conformation of the N1 substrate peptide when bound to FIH with that of the same residues in the ARD fold (Fig. 6F). Aside from the overall extension of the β-hairpin loop, there are other major changes in conformation. Notably, there is significant rotation in the peptide bond between the −1 residue and the target Asn (supplemental Table S2) relative to the conformation in the N1 ARD structure (Fig. 6F and supplemental Fig. S7E). In addition, Leu-1937 and -2004 are located near the center of the H2 helix of the preceding AR, which must unfold in order for the leucinyl side chain to become buried in the hydrophobic pocket of FIH (Fig. 6F and supplemental Fig. S7E).

Asn Hydroxylation Regulates the FIH-N1 ARD Interaction—Reduced binding of hydroxylated product versus substrate is precedent for 2OG oxygenases, such as in the case of the hydroxylation reaction catalyzed by clavaminic acid synthase (27). In the case of Notch, Asn hydroxylation creates a hydrogen bond to the acidic residue at the −2-position of the substrate motif (Fig. 5B) that may also stabilize the β-hairpin loop of the AR and therefore hinder the conformational changes required for productive binding of the AR substrate to FIH. To test these possibilities, we investigated the binding of unhdroxylated or hydroxylated N1 ARD (100% Asn-1945 and 20% Asn-2012) to FIH using electrospray ionization MS under non-denaturing conditions (Fig. 7A and supplemental Fig. S9). In the absence of added Fe(II) and 2OG, $K_v$ values of 4.0 and 48.9 μM, respectively (excluding data for FIH$_2$(N1 ARD)$_2$; supplemental Fig. S9), demonstrated tighter binding of the unhdroxylated N1 ARD. The $K_v$ value of FIH and His-HIF1αCAD (105.9 μM) showed that N1 ARD binds significantly tighter. The analysis also demonstrated that two N1 ARD molecules can bind to dimeric FIH simultaneously under these conditions (supplemental Fig. S9).

To confirm the effects of hydroxylation on FIH binding, we undertook pull-down assays using PK-ΔN1 ICD that was either completely (ΔN1 ICD-OH; FIH overexpression) or incompletely (ΔN1 ICD-H/OH) hydroxylated. The two forms of N1 ICD were incubated with His-FIH, and complex formation was assessed (Fig. 7B); partially hydroxylated ΔN1 ICD captured ~40% of His-FIH, whereas <2% of His-FIH was captured by ΔICD-OH. Together, these results agree with observations 12 M. McDonough, unpublished data.
FIGURE 7. Functional effects of ARD hydroxylation. N1 ARD hydroxylation reduces affinity for FIH. A, mild ionization electrospray MS; $K_d$ plots for binding of the FIH dimer (FIH$_2$) with N1 (including data for FIH$_2$-(N1)$_2$), FIH$_2$ and N1 (excluding data for FIH$_2$-(N1)$_2$), FIH$_2$ and N1 ARD(OH), and FIH$_2$ and His-FI1αCAD. Equimolar FIH plus substrate were mixed and analyzed in the absence of Fe/ZnG. E, enzyme (FIH); S, substrate. B, His-FIH was incubated with PK-N1 ICD-H/OH (47% Asn-1945, 8% Asn-2012 hydroxylation) or PK-ΔN1 ICD-OH (95% Asn-1945, 82% Asn-2012 hydroxylation) before anti-PK pull-down and anti-His immunoblot. C, N1 ARD inhibits HIF1αCAD Asn-803 hydroxylation. FIH and GST-HIF1αCAD were incubated with or without GST-N1 ARD prior to MS quantification of Asn-803 hydroxylation (left). FIH and GST-N1 ARD were incubated with or without GST-HIF1αCAD prior to MS quantification of Asn-1945/2012 hydroxylation (right). D, N1 ICD prevents HIF1αCAD Asn hydroxylation in vivo. 293T cells were transfected with GAL4DBD-HIF1αCAD, ΔN1 ICD, or both in combination, prior to purification and LC-MS analyses to quantify substrate Asn hydroxylation. Note that equivalent amounts of GAL4DBD-HIF1αCAD and ΔN1 ICD were expressed in both competed and uncompleted samples. E, Notch ARDs prevent FIH suppression of HIF1αCAD activity in vivo, as assayed by activity of the UAS-luc/GAL4DBD-HIF1αCAD binary reporter system. FIH siRNA induced maximal luciferase. Notch ARDs that are FIH substrates compete with HIF1αCAD and induce luciferase. Competition of N1 ARD and ICD with HIF1αCAD is dependent on the integrity of Asn-1945/2012. * p < 0.001 versus EV; #, p < 0.001 versus N1 ARD; δ, p < 0.001 versus EV; ψ, p < 0.001 versus N1 ICD.

made with other 2OG oxygenases and may be consistent with a role for the hydrogen bond between the hydroxylated Asn and the −2 acidic residue in hindering the conformational change required for FIH binding.

**Notch ARDs Compete with HIFαCAD**—The structural results reveal that ARs bind to the substrate binding groove of FIH essentially as for HIFαCAD. Given the widespread extent of AR sequences closely related to N1 Site 1 and 2 (15), the structures indicate that many AR sequences may be accommodated within the FIH active site. This, along with the observation that N1 ARD binds FIH tighter than HIFαCAD, raised the possibility that FIH-mediated HIFαCAD hydroxylation may be competitively inhibited by ARD proteins. To test this, we incubated N1 ARD, HIF1αCAD, and FIH in vivo (Fig. 7C, left). HIF1αCAD hydroxylation was dramatically reduced in the presence of an equimolar amount of N1 ARD. Conversely, FIH-mediated N1 Asn-1945 hydroxylation was rapid but relatively unaffected by the addition of HIF1αCAD (Fig. 7C, right). N1 ARD Asn-2012 hydroxylation was minimal over this time course and was also unaffected by HIF1αCAD. To determine whether similar competition was observed in vitro, we overexpressed a GAL4 DNA binding domain (DBD) fusion of HIF1αCAD and PK-ΔN1 ICD either in isolation or in combination, prior to purification and LC-MS analyses of Asn hydroxylation sites. Fig. 7D shows that, in an identical manner to the in vitro data, N1 is a potent inhibitor of HIF1αCAD hydroxylation, whereas N1 Asn hydroxylation is not significantly affected by HIF1αCAD.

To determine whether competition promotes HIF1αCAD activity in vivo, we used a UAS luciferase reporter system that is activated by the unhydroxylated GAL4DBD-HIFαCAD-p300 complex. HeLa cells were transfected with GAL4DBD-HIFαCAD and UAS-luciferase in the presence/absence of FIH siRNA or Notch ARD overexpression (Fig. 7E). As anticipated, FIH siRNA dramatically induced HIFαCAD transcriptional activity and luciferase expression, confirming this system as an assay of FIH activity. Similar to FIH siRNA, N1 ARD overexpression induced significant levels of HIFαCAD transcriptional activity. This effect was dependent on Asn-1945 and Asn-2012, since N1 ARD N1945A/N2012G was much less effective. Similarly, HIF1αCAD activity was significantly induced by wild type N1 ICD overexpression, but not N1 ICD N1945A/N2012G. Overexpression of N3 ARD also induced luciferase, but N4 ARD, which is not an FIH substrate (Fig. 1B), did not (Fig. 7E). The ARD-mediated effects described here were specific, since they were not seen in GAL4DBD-VP16 or CSL-luc transcriptional reporter assays (supplemental Fig. S10).

**DISCUSSION**

Despite the widespread occurrence of post-translational hydroxylation in extracellular proteins, the hydroxylation of...
alkyl amino acid side chains of intracellular proteins has been thought to be rare (1). Together with our recent work on IκB and p105 (13), we have identified the interaction of FIH with a total of eight ARD-containing proteins, including Notch. We show here that selected ARs in the ARDs of Notch receptors are targets for FIH-mediated Asn hydroxylation; two Asn residues in an L^8(D/E)^2X^2-N motif are hydroxylated to different extents both in vitro and in the endogenous protein. Considering the prevalence of this FIH substrate motif in these and the ~200 other human ARD proteins, the results imply that FIH-mediated Asn hydroxylation is a common post-translational modification.

Structural analyses of hydroxylated N1 ARD revealed that although a hydrogen bond was formed between the hydroxy group of Asn-1945 and the carboxylate group of Asp-1943, there was no major change in the crystalline conformation of the ARD compared with reported N1 structures (Fig. 5). Consistent with this, Asn hydroxylation did not affect the interaction of the N1 ARD with components of the Notch transcriptional complex (Fig. 4, C and D). Although it is possible that hydroxylation regulates another Notch activity, such as that in the Deltex-dependent pathway (28), we did not observe any effect of hydroxylation on the interaction of N1 ARD with Deltex (Fig. 4C).

The crystallographic analyses of the hydroxylated Notch ARD and of Notch peptides bound to FIH revealed that the canonical ARD fold must undergo major conformational changes in order to bind the FIH active site. Although work with short Notch substrate peptides may not exactly reflect the changes in order to bind the FIH active site and beyond) to these ARD-containing proteins, considerations of selectivity, FIH-catalyzed ARD Asn hydroxylation is also governed by the sequence immediately surrounding the target Asn. First, there is striking conservation of these residues between FIH substrates (supplemental Fig. S1) (13). Second, N4 site 1 is not hydroxylated (Fig. 1B), probably due to site 1 lacking the acidic residue at the −2-position (supplemental Fig. S1) and the presence of a proline at the −1-position that would be predicted to disrupt the tight turn required for productive binding at the FIH active site (Fig. 6C) (19). Interestingly, it is also probable that the hydrogen bond formed between the hydroxyl group of the target Asn and the acidic residue at the −2-position of the substrate motif might stabilize the β-hairpin type loop and limit the conformational change required for FIH binding, thus contributing to the more than 10-fold reduction in affinity for FIH we observed after N1 Asn-1945 hydroxylation (Fig. 7, A and B). The acidic residue at the −2-position is present in at least one AR of many ARDs. Therefore, this internal hydrogen bond may be a general consequence of FIH-mediated ARD hydroxylation.

Binding studies indicated that the affinity of FIH for the unhydroxylated N1 ARD was more than 20-fold greater than that for the unhydroxylated HIFαCAD. In keeping with this, we have been able to co-immunoprecipitate a variety of ARD-containing proteins from cell extracts under conditions which did not capture HIFα,9 suggesting that this differential affinity is operative in vivo and extends to other ARD family members. We also observed a striking preference for ARD hydroxylation over HIFαCAD hydroxylation in competition assays, with the presence of N1 ARD sequences greatly reducing HIFαCAD hydroxylation but not vice versa. These results were consistently observed both in vitro using purified proteins (Fig. 7C) and in vivo in transfected cells (Fig. 7D). Moreover, co-transfection of N1 and N3 ARDs, but not mutant N1 or the non-FIH substrate N4, reversed FIH-mediated suppression of HIFαCAD activity (Fig. 7E). Taken together with the knowledge that ARD proteins are ubiquitous and in some cases abundant, these findings strongly suggest that ARDs may limit the availability of FIH for suppression of HIFαCAD activity. These findings are consistent with, and potentially provide an explanation for, otherwise puzzling observations of strong HIF transcriptional activity under circumstances when effective FIH suppression might be anticipated. Thus, stabilized HIFα proteins in von Hippel-Lindau-deficient cells (31) and HIFα proteins that escape destruction following overexpression (32) generate strong transcriptional responses despite the presence of FIH, whereas in normoxic cells, modest increases in FIH expression suppress persistent HIF target gene expression (16), again implying that normal levels of FIH are rather surprisingly incompletely active on HIF.

Given the (i) large effects of hydroxylation on the affinity of the N1 ARD for FIH, (ii) tighter binding of unhydroxylated ARDs to FIH than HIFα, and (iii) observation of substantially reduced hydroxylation of endogenous human N1 after exposure of cells to 48-h hypoxia, it is possible that in vivo competition between the ubiquitous ARD proteins and HIFα for FIH is also modulated by the hydroxylation status of cellular ARDs. Thus, due to its relatively high affinity for unhydroxylated ARDs, FIH may be more effectively sequestered in ARD complexes under conditions in which hydroxylation is compromised, such as chronic hypoxia, thereby enhancing the effect of hypoxia on HIF signaling beyond what might be predicted from kinetic studies of FIH-dependent HIFαCAD hydroxylation alone. Under such conditions, it is also possible that FIH affects
the activity of ARD proteins independently of hydroxylation, as observed with N1 ICD following FIH overexpression in P19 cells (Fig. 4A). Interestingly, evidence for physical association of HIF and Notch (11) raises the further possibility of local competition between different FIH binding sites in such complexes.

Previously, the only characterized interfaces between oxygen and HIF regulation were the hydroxylation of HIF α Pro and Asn residues that either enable (HIFα-pVHL) or disable (HIFα-p300) protein-protein interactions important in transcriptional regulation (33). ARD hydroxylation provides a new and more complex interface between HIF and oxygen, which has the potential to amplify the oxygen-dependent regulation of HIF transcriptional activity and contribute to physiological tuning of hypoxia signaling. The proposal that FIH has a range of substrates also has implications for the development of selective therapies that target HIF activation via hydroxylase inhibition.

Acknowledgments—We are grateful to G. Weinmaster (UCLA, Los Angeles, CA) for Delta-1 L-cells, Y.-M. Tian for polyclonal anti-FIH antibody, the Oxford Protein Production Facility (Oxford, UK) for use of the crystallization facility, and L. Kelly, T. Gerken, C. W. Pugh, N. Masson, Yvonne Jones, and all of the members of the Ratcliffe and Schofield laboratories for helpful discussions.

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