Phosphorylation Directly Regulates the Intrinsic DNA Cytidine Deaminase Activity of Activation-induced Deaminase and APOBEC3G Protein*  

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The beneficial effects of DNA cytidine deamination by activation-induced deaminase (AID; antibody gene diversification) and APOBEC3G (retrovirus restriction) are tempered by multiple regulatory mechanisms that combine to minimize this detrimental outcome. Here, we provide evidence in support of a new role for threonine-serine phosphorylation in regulating AID and A3G function (MDM2 (25), replication protein A (26), heat shock protein 90 (HSP90) (27), germinal center-associating nuclear protein (GANP) (28), calcium and integrin-binding protein 1 (CIB1) (29), beta-catenin-like protein 1 (CTNNBL1) (30)), with protein kinase A (PKA) (31–34) being most relevant to this work. PKA phosphorylates AID at threonine 27 and serine 38, with serine 38 phosphorylation promoting interactions with replication protein A and facilitating class-switch recombination (CSR) and somatic hypermutation (31–33, 35). Phosphorylation of the homologous residue in A3G, threonine 32, also has functional consequences by rendering the protein less susceptible to HIV-1 Vif-induced ubiquitylation and degradation (34). Here, we provide evidence in support of a new role for threonine-serine phosphorylation in directly suppressing the intrinsic DNA deaminase activity of AID and A3G. Extensive conservation of this particular residue suggests that phospho-regulation may extend to most other DNA deaminase superfamily members.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—pEGFP-N3-A3G and pEGFP-N3-AID have been described (36, 37). Mutants of AID and A3G were made by QuikChange site-directed mutagenesis (Stratagene). The retroviral vector pMX-EGFP was constructed by subcloning EGFP from plasmid pEGFP-N3 (Clontech) into pMX-P1E (a gift from V. Barreto) using EcoRI/NotI. pMX-AID-IRES-EGFP was generated by first amplifying untagged AID from pEGFP-N3-AID by PCR using primers 5'-GCT AGC GCC ACC ATG GAC AGC CT and 5'-CCT GCA GGT CAA AGT CCC AAA GTA. The insert was cut with Nhel/SfI and ligated into pCSII-
IRES-EGFP (a gift from N. Smita). The AID-IRES-EGFP insert was then PCR-amplified using primers 5′-GAA TTC ATG GAC AGC TTC TTG ATG AAC and 5′-CCA CAT AGC GTA AAA GGA GCA AC, cut using EcoRI/NorI, and ligated into pMX-PHE. The MLV amphotropic envelope vector pRKS-10A1 and the HIV-1 accessory vector ΔNRF were generous gifts from N. Smita. The MLV accessory vector pMD-OGP was provided by F. Randrow. The Vif-deficient HIV-1Δvif provirus has been used previously (38, 39). The Escherichia coli expression constructs pTrc99a-AID-GST and pTrc99a-A3G-GST were generated by subcloning AID from pEGFP-N3-AID or A3G from pEGFP-N3-A3G into pTrc99a-GST using Ncol/Sall. The untagged bacterial expression plasmids pTrc99a-hAID and pTrc99a-A3G have been described (37).

In Vitro Peptide Kinase Assays—CaMKII (New England Biolabs) or PKA (New England Biolabs or gift from L. Masterson) was recombinantly expressed and purified from human cells as above were used for AID DNA binding reactions. 25 pmol of purified protein was diluted serially 1:2, and each dilution was mixed with 1 pmol fluorescein-labeled oligo (5′-ATT ATT ATT ATT CCA ATG GAT TTA TTT ATT TWR CTA TTT ATT T) in binding buffer (10 mM HEPES (pH 7.6), 1% glycerol, 100 mM KCl, 10 mM MgCl2, 100 μM EDTA, 500 μM DTT). Reactions were incubated for 30 min at 37 °C before separation on a 5% Tris borate-EDTA acrylamide gel. The gel was dried and imaged by phosphorimager (Storm, Molecular Dynamics).

Protein samples purified from human cells as above were used for A3G deaminase activity reactions. Starting with 1.2 pmol purified protein, 2-fold serial dilutions were made and mixed with 1 pmol of substrate oligo (5′-ATT ATT ATT ATT CCA ATG GAT TTA TTT ATT TAT TTA TTT ATT T fluorescein), 1.0 μg/μl RNase A (Qiagen), and 0.001 units/μl uracil DNA glycosylase (NEB). The reactions were incubated at 37 °C for 2 h, and then NaOH was added to 100 μM before incubating at 90 °C for another 30 min. The reactions were separated on a 16% Tris/urea-acrylamide gel and visualized by fluorescence imaging (FLA-5000, Fuji).

Fluorescence Microscopy Studies—For steady-state AID and AID localization, pEGFP-N3-A3G, pEGFP-N3-AID, or mutants thereof were transfected into HeLa cells grown in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone). 48 h later, the cellular localization was determined by fluorescence microscopy (Deltavision). For AID import activity, leptomycin-B (20 ng/ml) was added 2 h prior to imaging as above.

HIV Restriction Assays—CEM-SS and CEM-GFP (courtesy of M. Malim) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Hyclone). Stable cell lines expressing pEGFP-N3, pEGFP-N3-A3G, or mutants were generated in the permissive cell line CEM-SS by electroporating linearized DNA and selecting for stable integrants with 1 mg/ml G418 (Mediathek) as described (38). Clones were confirmed to have similar expression levels by Western blot analysis using an antibody against A3G (rabbit polyclonal raised against a C-terminal peptide). Virus was produced by transfecting HIV-1 provirus using TransIT-LT1 (Mirus) HEK293T cells maintained in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone). 48 h after transfection, virus-containing supernatants were filtered with a 0.45-μm filter. Viruses were then titered using the CEM-GFP reporter cell line as described (38). Spreading infections were initiated by adding virus to CEM-SS stable cell lines at a multiplicity of infection of 0.05. Supernatants from infected cultures were collected at 2- to 4-day intervals and added to CEM-GFP. After 48-hours, the cells were fixed in 4% paraformaldehyde.
and analyzed for GFP expression by flow cytometry (Quanta SC MPL, Beckman Coulter). Procedures for the detection of A3G in producer cells and viral particles have been described (44).

Class-switch Recombination Assays—All experiments were conducted in accordance with the University of Minnesota Animal Care and Use Committee guidelines. The C57BL/6 AID/H11002/H11002 mice have been described (45). Ex vivo CSR assays were conducted by purifying resting B-cells from spleen by magnetic sorting (130-090-862, Miltenyi Biotec). Isolated B-cells were then cultured in RPMI supplemented with 10% FBS, 50 ng/ml IL-4, and 50 μg/ml LPS. After 48 h, the media were replaced with transducing viral supernatant supplemented with 20 mM HEPES and 16 μg/ml polybrene and centrifuged (600 g, 2 h, 30 °C). The cells were then resuspended into fresh media containing IL-4 and LPS and cultured for 4 days. Efficiency of switching to IgG1 was determined by staining with anti-IgG1-PE (BD Biosciences) and analyzed by flow cytometry (FACSCanto II, BD Biosciences).

RESULTS

AID-Thr-27, A3G-Thr-32, and A3G-Thr-218 Are Homologous and Located within a Region of High Sequence and Structural Conservation—Prior studies demonstrated phosphorylation of AID-Thr-27 in vivo and in vitro by mass spectrometry and radiolabeling(31, 33, 35) and A3G-Thr-32 by immunoblotting (34). We noted that these two threonines are homologous to A3G-Thr-218, whose high-resolution structures have shown to be located within a solvent-accessible loop (4–7) (Fig. 1A). This threonine anchors a conserved motif that matches a consensus PKA phosphorylation site (R-H/R-X-T) (46) (Fig. 1B). Notably, nearly all AID/A3 family members have homologous threonine or serine residues at this exact position (Fig. 1C). Rare exceptions are only apparent in specific mammalian lineages (carnivores and rodents) or in redundant or inactive domains (most alleles of human A3H are unstable) (47). In the catalytic domain of A3G, the first arginine in this motif (Arg-215) is located adjacent to the catalytic glutamate, and it has been implicated in binding substrate single-stranded DNA (5–7). Taken together, these observations, and particularly the high level of conservation and the structural positioning next to the active site, suggest that phosphorylation and dephosphorylation may serve as a posttranslational switch that helps control the DNA deaminase activity of these mutagenic enzymes.

PKA and CaMKII Phosphorylate A3G-Thr-218 in Vitro—AID-Thr-27 and A3G-Thr-32 can be phosphorylated by PKA (31, 33–35). To determine whether these observations extend to A3G-Thr-218, whose high-resolution structures have shown to be located within a solvent-accessible loop (4–7) (Fig. 1A), we asked whether recombinant PKA could phosphorylate a peptide representing the soluble loop in which this residue resides, VRGRHET218YLCYE. We found that PKA and CaMKII can phosphorylate A3G-Thr-218 in vitro (34).
Interestingly, the A3G phospho-null variant T218A showed considerably lower levels of catalytic activity. Similar CaMKII, which also phosphorylates R-X-X-T motifs (46), was able to phosphorylate the A3G-Thr-218 peptide but not the alanine mutant derivative. Both enzymes were also able to phosphorylate a serine in a control peptide (Kemptide). These data demonstrate that A3G-Thr-218 is a suitable substrate for at least two kinases, PKA and CaMKII.

**Phospho-mimetic Mutations Inhibit DNA Cytidine Deaminase Activity**—To address whether phosphorylation is capable of attenuating the DNA cytidine deaminase activity of AID and A3G, phospho-null and phospho-mimetic derivatives of these proteins were tested in an *E. coli*-based activity assay. The rifampicin-resistance (RifR) mutation assay has been used extensively to assess intrinsic DNA cytidine deaminase activity (41, 42). Consistent with prior reports, AID and A3G triggered 3- and 4-fold increases in the median RifR mutation frequency compared with catalytically inactive controls, AID-E58Q and A3G-E259Q (Fig. 2, A and B). In comparison, phospho-mimetic AID-T27E and A3G-T218E had nearly identical ssDNA binding abilities, which were indistinguishable from the wild-type enzyme (Fig. 3, B, D, and E).

As a control to demonstrate the specificity of AID for ssDNA, an AID-R24E mutant was analyzed in parallel and shown to be defective in DNA binding. This arginine is conserved and homologous to A3G-Arg-215, which NMR chemical shift perturbation and mutagenesis experiments have implicated strongly in DNA binding (5–7). Additional EMSA data can be found in supplemental Fig. S2. Overall, these EMSA results clearly show that phospho-mimetic substitutions in A3G and AID do not cause visible decreases in the ability of each protein to bind ssDNA.

**Mutants of AID and A3G Localize Normally within Living Cells**—The subcellular localization of AID/APOBEC family members has been well studied (16–20). A3G is predominantly cytoplasmic. AID is also mostly cytoplasmic, but it is imported into the nuclear compartment by an importin-α pathway and exported back to the cytoplasm by a CRM1 pathway. To ask whether our phospho-null or phospho-mimetic mutants retain normal, steady-state subcellular distributions, we performed a series of AID/A3G-GFP localization studies in living HeLa cells. No detectable alteration in the steady-state cytoplasmic distribution of A3G-EGFP, AID-EGFP, or their mutant derivatives was detected (Fig. 4, A and B). Moreover, experiments done in the presence and absence of the CRM1 inhibitor leptomycin B indicated that the nuclear import and export activities were also intact for all AID-EGFP constructs. These data therefore indicate that A3G, AID, and their mutant derivatives are capable of interacting with the cellular factors responsible for localization and, furthermore, that AID is able to enter the nucleus, where it will have the opportunity to access the immunoglobin locus, its physiologic DNA deamination target.

**AID-T27E Is Defective for Class-switch Recombination**—One of the physiological functions of AID is catalyzing cytidine-to-uridine deamination in the immunoglobin loci of human B cells. Therefore, we would expect the phospho-mimetic AID-T27E to be defective in this activity. Indeed, Fig. 2A shows that the phospho-mimetic AID-T27E, as well as the phospho-null AID-T27A, showed significantly elevated levels of catalytic activity consistent with a proportion of the wild-type protein being already phosphorylated (and thereby inactivated) in HEK293T cells. Taken together, the *E. coli* and the purified protein activity data indicate that phosphorylation of the conserved threonine, AID-Thr-27 or A3G-Thr-218, may serve to attenuate the intrinsic DNA deaminase activity of these proteins (supported further by HEK293T cell extract data in supplemental Fig. S1).

**DNA Binding Is Unaffected by Phospho-mimetic Substitutions**—To ask whether the diminished catalytic activity of the phospho-mimetic substitution mutants is due to defective ssDNA binding, we tested the ssDNA binding ability of AID and A3G in electrophoretic mobility shift assays. A3G- myc–his used in the deaminase reactions above was used for ssDNA binding experiments. Purified protein was diluted serially, incubated with a fluorescently labeled oligo, and fractionated on a native polyacrylamide gel. As expected, A3G and the catalytic mutant E259Q bound ssDNA in a dose-dependent manner (Fig. 3, B, D, and E) (48). Likewise, A3G-T218A and A3G-T218E had nearly identical ssDNA binding abilities, which were indistinguishable from the wild-type enzyme (Fig. 3, B, D, and E).

Similar EMSA experiments were done with wild-type AID and mutant derivatives, but the sensitivity of the assay had to be increased by using a radiolabeled ssDNA oligo substrate. Again, the wild-type and the phospho-null and phospho-mimetic variants produced near identical mobility shifts (Fig. 3, F, G, and H).

As a control to demonstrate the specificity of AID for ssDNA, an AID-R24E mutant was analyzed in parallel and shown to be defective in DNA binding. This arginine is conserved and homologous to A3G-Arg-215, which NMR chemical shift perturbation and mutagenesis experiments have implicated strongly in DNA binding (5–7). Additional EMSA data can be found in supplemental Fig. S2. Overall, these EMSA results clearly show that phospho-mimetic substitutions in A3G and AID do not cause visible decreases in the ability of each protein to bind ssDNA.

![FIGURE 2. Intrinsic DNA cytidine deaminase activity of AID and A3G constructs. A and B, results from E. coli-based RifR mutation assays, with each X representing data from an independent culture. Median mutation frequencies are indicated by horizontal bars and numbers. Also shown are Western blot analyses of AID or A3G constructs from representative cultures with a nonspecific band (NSB) as a loading control.](image-url)
uridine deamination events in immunoglobulin heavy chain gene switch region DNA and thereby triggering additional DNA repair processes that ultimately manifest as antibody isotype switch recombination (1, 45). Therefore, as a functional test of AID activity, we assayed the phospho-null and phospho-mimetic mutants in an ex vivo B-cell CSR system (30–33).

FIGURE 3. A3G-T218E has diminished deaminase activity but retains DNA binding ability. A, representative images from titrated A3G oligo deaminase assays. The upper band is the intact oligo, and the lower band is the product of deamination, uracil excision, and strand cleavage. B, representative images from A3G ssDNA binding assays. Free oligo and protein-bound complexes are labeled. C, quantification of A3G deaminase activity in A and replicas not shown. Data are plotted as the mean ± S.D. of three independent experiments. D, quantification of A3G EMSA data in B. Data are plotted as the mean ± S.D. of three independent experiments. E, Coomassie-stained gel illustrating the purity of A3G enzymes used in these experiments. F, representative images from AID ssDNA binding assays. G, quantification of AID EMSA data in F. Data are plotted as the mean ± S.D. of three independent experiments. H, Coomassie-stained gel illustrating the purity of AID enzymes used in the ssDNA binding assays. The identity of the AID bands was confirmed by immunoblotting (not shown).
AID and A3G Phosphorylation

A3G-T218E Lacks HIV-1 Restriction Activity—A3G potently inhibits HIV-1 replication by blocking reverse transcription and daminating viral cDNA cytosines to uracils (2). This antiviral activity is most evident in HIV-1 lacking viral infectivity factor (Vif), a small basic protein that triggers A3G degradation. Thus, a rigorous test of the functional activity of A3G is whether it suppresses the spreading infection of Vif-deficient HIV-1 (38, 39, 49). We therefore created a panel of CEM-SS T cell lines stably expressing wild-type A3G-EGFP, an E259Q catalytic mutant control, a phospho-null T218A construct, or a phospho-mimetic T218E protein. As anticipated from prior studies, wild-type A3G completely suppressed the replication of Vif-deficient HIV-1, and its strong antiviral effect was largely dependent upon the integrity of the catalytic glutamate Glu-259 (50, 51) (Fig. 6A). A3G-T218A showed wild-type levels of restriction consistent with full or elevated levels of enzymatic activity. In contrast, A3G-T218E failed to prevent the replication of Vif-deficient HIV-1. However, this mutant protein did cause reproducible delays in peak viral replication consistent with severely attenuated but not fully defective catalytic activity. As additional controls, N-terminal A3G-T32A or T32E substitutions had no discernable effect, and all cell lines supported similar levels of Vif-proficient HIV-1 spreading infection (Fig. 6A and data not shown). It is notable that, although we were able to confirm A3G-Thr-32 phosphorylation by mass spectrometry, we found no differences in the subcellular localization, HIV restriction capacity, or Vif susceptibility in alanine-or glutamate-substituted derivatives (Fig. 4 and data not shown).

An additional possibility is that A3G-T218E may not restrict HIV-1 because it is not efficiently packaged into budding viri-
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ons. To test and eliminate such a possibility, we harvested virus produced from HEK293T cells expressing A3G and mutants thereof and blotted for the presence of A3G in these viral particles. We found no significant difference in the ability of any of the mutants to get into virions as compared with wild-type A3G (Fig. 6C).

DISCUSSION

The AID/APOBEC family of cytidine deaminases is an important facet of the adaptive and innate immune responses in humans. However, their mutagenic activity must be tightly regulated to prevent potentially detrimental off-target effects. Regulation of these proteins has been described at multiple levels, including transcription, microRNAs, cytoplasmic localization, proteasomal degradation, and phosphorylation (see introduction). Here we describe a novel phosphorylation regulatory mechanism capable of attenuating the intrinsic deaminase activity of AID and A3G. In this study, we demonstrate that phospho-mimetic substitution of a highly conserved threonine renders these proteins inactive in several independent assays. We show that ssDNA binding ability and steady-state subcellular localization (and for AID, also trafficking) are unaffected, indicating that these proteins are structurally intact. In functional assays, this modification prevents AID from facilitating CSR and A3G from restricting HIV-1 Vif replication. It is intriguing that two neighboring phosphorylation sites can have such contrasting effects on the function of AID, with Ser-38 phosphorylation enabling interaction with replication protein A and allowing CSR and somatic hypermutation, and Thr-27 phosphorylation rendering the protein inactive. This begs the question of how PKA is regulated to distinguish between these neighboring residues. Further studies are warranted to better understand these posttranslational regulatory events and investigate the possible involvement of other Ser/Thr kinases that can also recognize PKA consensus motifs, such as CaMKII described here.

The obvious utility of posttranslational regulation by phosphorylation is 2-fold (illustrated by the model in supplemental Fig. S3). First, a threonine- or serine-phosphorylated DNA deaminase would possess a low level of DNA deaminase activity and pose less of a threat to genomic DNA. Genomic DNA integrity is further ensured by the fact that AID, A3G, and many other A3 proteins are predominantly cytoplasmic. Second, signal transduction pathways, which are critical for both adaptive and innate immune responses, could readily switch on DNA deaminase activity by triggering the removal of the phosphate group (phosphatase or phosphotransferase activity). This would ensure an expedited immune response that could be further bolstered by up-regulating AID or A3 expression at the transcriptional and/or translational levels.

We propose that the posttranslational modification of AID and the A3 proteins by phosphorylation provides a means of directly controlling the intrinsic DNA cytidine deaminase activity of these proteins (supplemental Fig. S3). It is likely that this mechanism will be conserved in vertebrates because residues homologous to AID-Thr-27, A3G-Thr-32, or Thr-218 are apparent in almost all other known polynucleotide cytidine deaminase family members (Fig. 1). It is further possible that defects in these signal transduction pathways may manifest as immunodeficiency syndromes (overphosphorylated protein), autoimmune diseases (underphosphorylated protein), and/or carcinogenesis (underphosphorylated protein), especially in combination with other regulatory defects.

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