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FLASH interacts with p160 coactivator subtypes and differentially suppresses transcriptional activity of steroid hormone receptors

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Abstract

We previously reported that tumor necrosis factor α receptor- and Fas-associated FLASH interacts with one of the p160 nuclear receptor coactivators, glucocorticoid receptor-interacting protein (GRIP) 1, at its nuclear receptor-binding (NRB) domain, and that inhibits the transcriptional activity of the glucocorticoid receptor (GR) by interfering with association of GR and GRIP1. Here, we further examined the specificity of FLASH suppressive effect and the physical/functional interactions between this protein and two other p160 family subtypes. The suppressive effect of FLASH on GR transactivation was observed in several cell lines and on the chromatin-integrated mouse mammary tumor virus (MMTV) promoter. FLASH strongly interacted with the NRB domain of the thyroid hormone receptor activator molecule (TRAM) 1, a member of the steroid hormone receptor coactivator (SRC) 3/nuclear receptor coactivator (N-CoA) 3 subtypes, as well as with SRC2/N-CoA2 p160 coactivator GRIP1, while its interaction with SRC1a, one of the SRC1/N-CoA1 proteins, was faint in yeast two-hybrid assays. Accordingly, FLASH strongly suppressed TRAM1- and GRIP1-induced enhancement of GR-stimulated transactivation of the MMTV promoter in HCT116 cells, while it did not affect SRC1a-induced potentiation of transcription. Furthermore, FLASH suppressed androgen- and progesterone receptor-induced transcriptional activity, but did not influence estrogen receptor-induced transactivation, possibly due to their preferential use of p160 coactivators in HCT116 and HeLa cells. Thus, FLASH differentially suppresses steroid hormone receptor-induced transcriptional activity by interfering with their association with SRC2/N-CoA2 and SRC3/N-CoA3 but not with SRC1/N-CoA1.
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Keywords: FLASH; p160 coactivators; Nuclear receptor-binding domain; LXXLL motif; Steroid hormone receptors

1. Introduction

Steroid hormones have major roles in numerous biologic activities [1]. For example, glucocorticoids are crucial for the integrity of central nervous system (CNS) function and for maintenance of cardiovascular, metabolic and immune homeostasis [2]. Sex steroids, such as androgen, estrogen and progesterone, are critical in sexual differentiation, gonadal function, fertility and procreation [3]. Their actions are mediated by their respective intracellular receptors, the steroid hormone receptors (SRs), e.g. the glucocorticoid (GR), androgen (AR), estrogen (ER) and progesterone receptors (PR), which function as hormone-activated transcription factors that regulate the expression of target genes of the respective hormones [4]. The transcriptional activity of the ligand-activated SR is exerted by interaction with molecular components of the transcription machinery [5]. Of particular importance is a family of proteins, the nuclear receptor coactivators, which bridge promoter-bound SR and the transcription initiation complex. Coactivators also possess histone acetyltransferase activity, through which they help unwind DNA from nucleosomes, increasing the accessibility of promoter DNA to nuclear receptors, other transcription factors, and components of the transcription machinery [6].

p160 type nuclear receptor coactivators play an essential role in SR-induced transcriptional activation [7]. The promoter-bound SR helps tethering them to the promoter regions of steroid hormone-responsive genes. Other coactivators, such as p300 and its homologous protein

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cAMP-responsive element-binding (CREB) protein-binding protein (CBP), as well as the p300/CBP-associated protein (p/CAF), are then attracted to the promoters and form receptor–coactivator complexes [6]. There are three subclasses of p160 proteins, steroid receptor coactivator (SRC) 1/nuclear receptor coactivator (N-CoA) 1; TIF-II or glucocorticoid receptor-interacting protein (GRIP) 1, also called SRC2/N-CoA2; and, p300/CBP/co-integrator-associated protein (p/CIP), ACTR, TRAM1, RAC3, also called SRC3/N-CoA3 [6,7]. All three exhibit high similarity in their amino acid sequences and contain three copies of the coactivator signature motif sequence LXXLL in their nuclear receptor-binding (NRB) domain at the middle region of the molecule. Through these motifs, p160 coactivators specifically interact with the activation function (AF) 2 surface formed in the ligand-binding domain (LBD) of the GR after ligand activation [6,8]. It is known that each SR accumulates a different set of p160 coactivator subtypes on their responsive promoters to stimulate transcription depending on their preference, as well as availability of p160 subtypes [9,10].

Inflammatory and apoptotic stimuli during both physiologic and pathologic responses strongly influence SR activities [11–14]. Autoimmune/inflammatory/allergic diseases are frequently associated with reduced sensitivity of tissues to glucocorticoids [11]. Growth and development of the breast and the cyclic growth/development and degradation of the uterine endometrium and the ovarian corpus luteum are regulated by steroid hormones and apoptotic mediators [13]. Previously, we demonstrated that the “Fas-associated huge” protein FLASH specifically interacts with the NRB domain of one of the p160 protein, GRIP1, and suppresses transcriptional activity of GR by interfering with its association with this coactivator [15]. FLASH plays a critical role in inflammatory and apoptotic responses by forming complexes with the cytoplasmic portions of Fas and the tumor necrosis factor α (TNFα) receptor and transducing their signals into the cytoplasm [16,17].

In this report, we further examined the interaction specificity of FLASH to subtypes of p160 coactivators and its suppressive effect on their enhancement of GR-induced transactivation. In addition, we tested its suppressive effect on the transcriptional activity of other steroid hormone receptors.

2. Materials and methods

2.1. Plasmids

pRShGRα, p5HBhAR-A, pSVLPRA and HE0, which express the human glucocorticoid receptor α, androgen receptor, progesterone receptor-A and estrogen receptor α, respectively, are kind gifts from Drs. R.M. Evans (Salk Institute, La Jolla, CA), E.M. Wilson (University of North Carolina, Chapel Hill, NC), S.S. Simons Jr. (National Institutes of Health, Bethesda, MD), and P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). pME18S-FLAG-FLASH, which expresses the full-length human FLASH is a generous gift from Dr. S. Yonehara (University of Kyoto, Kyoto, Japan). pMMTV-Luc and ERE-tk-luc, which contain the full-length mouse mammary tumor virus promoter and synthetic vitellogenin A2 ERE sequence from −336 to −310, are kind gifts from Drs G.L. Hager (National Cancer Institute, Bethesda, MD) and J.H. Segars (National Institutes of Health, Bethesda, MD), respectively. TAT3-Luc contains the luciferase gene under the control of three synthetic GREs from the rat tyrosine aminotransferase promoter, and was a generous gift from Dr. J.N. Miner (Liang Pharmaceuticals, San Diego, CA). pMAM-neo-Luc, which has the MMTV promoter that drives the luciferase gene, along with the neomycin-resistant cassette, was purchased from Clontech (Palo Alto, CA). pSG5-GRIP1, pSG5-SRC1a and pBK/CMV-TRAM1, which express mouse GRIP1, and human SRC1a and TRAM1, are generous gifts from Dr. M.G. Stallcup (University of Southern California, Los Angels, CA), M.G. Parker (Imperial College London, London, UK) and A. Takeshita (Tranomon Hospital, Tokyo, Japan), respectively. pLexA-SRC1a(598–771) and pLexA-TRAM1(595–765), which express the LexA-DBD-fused indicated portions of SRC1a and TRAM1 that contain their NRB domains, were constructed by inserting the corresponding cDNAs into pLexA (Clontech). pLexA-GRIP1(596–774) and pB42AD-FLASH(1709–1982) were reported previously [15]. p8op-LacZ and pSV40-β-Gal were purchased from Clontech or Promega (Madison, WI), respectively.

2.2. Cell transfections and reporter assays

Human colon carcinoma HCT116, cervical carcinoma HeLa, rhabdomyosarcoma A204 and African green monkey kidney COS7 cells, all purchased from the American Type Culture Collection (Rockville, MD), were maintained in MacCoy’s 5A, Dulbecco’s Modified Eagle (DMEM) or DMEM/F-12 media supplemented with 10% fetal bovine serum, 50 U of penicillin and 50 μg/ml of streptomycin. The cells were transfected using Lipofectin (Life Technologies, Gaithersburg, MD) or $CaPO_4$, as described previously [18]. Aliquots of 0.5–2.0 μg/well of pME18S-FLASH, and/or 1.0–2.0 μg/well of pSG5-GRIP1 fl were used with 1.0 μg/well of pMMTV-Luc or ERE-tk-Luc. In HCT116 and COS7 cells, aliquots of 0.5 μg/well of steroid hormone receptor-expressing plasmids were cotransfected in all experiments, since these cells do not contain functional steroid hormone receptors. In HeLa and A204 cells, the endogenous GRα was employed in reporter assays, while plasmids expressing AR, PR or ER were added to the transfection media in the case of HeLa cells. An aliquot of 0.5 μg/well of pSV40-β-Gal was also included to normalize luciferase activity in all transfections. Empty vectors were used to maintain the same amount of transfected DNA. COS7/MMTV cells, which has the chromatin-integrated lucifrease gene under the control of the MMTV promoter, was developed by selecting a stably transformed cell clone by pMAM-neo-Luc in the presence

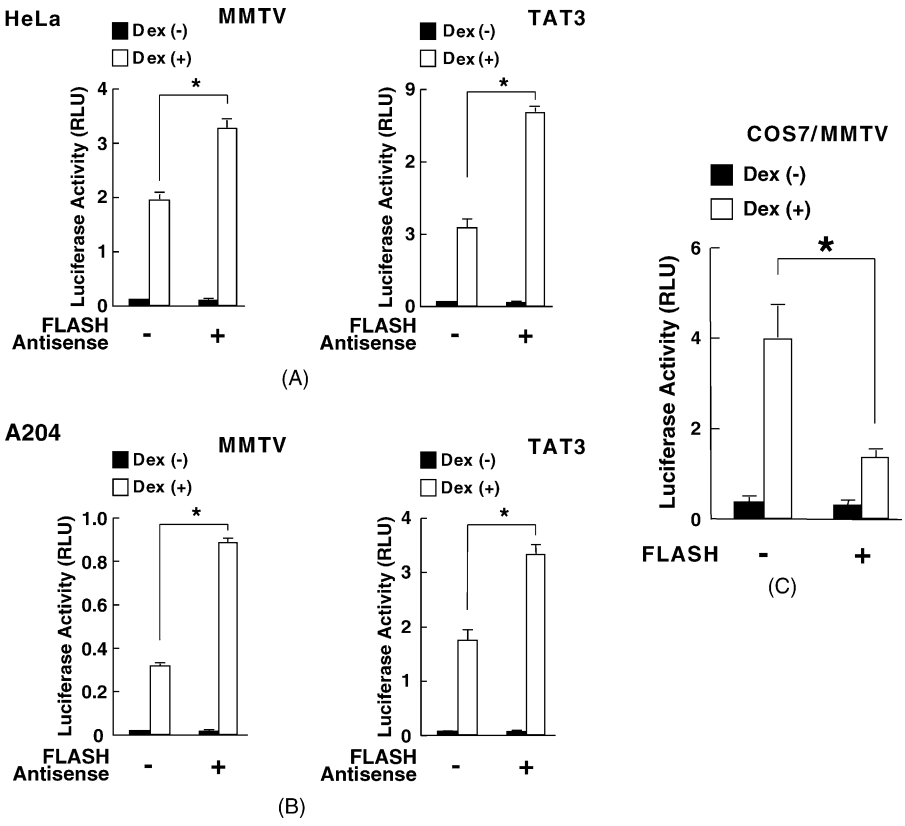

Fig. 1. FLASH suppresses transcriptional activity of GR in HeLa (A) and A204 (B) cells and on the chromatin-integrated MMTV promoter (C). (A and B) FLASH suppresses GR-induced transcriptional activity of the MMTV (left panels) and TAT3 (right panels) promoters in HeLa (A) and A204 (B) cells. HeLa (A) or A204 (B) cells were treated with an antisense for FLASH (+) or a control morpholino oligonucleotide (−), and were subsequently transfected with pMMTV-Luc or TAT3-Luc, and pSV40-β-Gal. (C) FLASH suppresses GR-induced transactivation of the MMTV promoter, which is stably integrated in the host cells' chromatin in COS7/MMTV cells. COS7/MMTV cells were transfected with pRShGRα and pSV40-β-Gal together with or without the FLASH-expressing plasmid. Bars represent mean ± S.E. values of the luciferase activity normalized for β-galactosidase activity in the absence or presence of 10^{-6} M of dexamethasone. $^{*}P < 0.01$, compared to the baseline.

of neomycin and by subsequent limiting dilution and clonally expansion. A 10^{-6} M of dexamethasone, progesterone, 10^{-7} M of dihydrotestosterone or 10^{-8} M of estradiol were added 24 h after transfection. The cells were harvested after an additional 24 h for luciferase and β-galactosidase assays.

The morpholino antisense oligonucleotide for FLASH expression, encoding 5′-CACCATTGTCATCATCTGCTGCCAT-3′, which targets the first 25 bases of the FLASH coding sequence, and the control antisense that contains the sense sequence of FLASH were described previously [15]. To introduce the antisense oligonucleotide, we employed the ethoxylated polyethylenimine (EPEI)-based special delivery protocol prepared by the company. Twenty-four hours after the treatment, HeLa or A204 cells were transfected with 1.5 μg/well of pMMTV-Luc, 0.5 μg/well of pSV40-β-Gal, 10^{-6} M of dexamethasone were added to the medium 24 h later and the cell lysates were harvested in 48 h for lucifrease and β-galactosidase assays as described previously [15].

2.3. Yeast two-hybrid assay

Yeast strain EGY48 (Clontech) was transformed with the *lacZ* reporter plasmid p8op-LacZ, and the indicated pLexA-derived plasmids and pB42AD-FLASH(1709–1982). The cells were grown in a selective medium to the early stationary phase, permeabilized by $CHCl_3$-SDS treatment, and β-galactosidase activity was measured in the cell suspension using Galactolight^{TM} PLUS (Tropix, Bedford, MA). β-Galactosidase activity was normalized for OD value at 600 nm. Fold induction was calculated by the ratio of adjusted β-galactosidase values of cells cultured in the presence of galactose versus glucose.

2.4. Statistical analyses

Statistical analysis was carried out by ANOVA, followed by Student's *t*-test with Bonferroni correction for multiple comparisons.

3. Results

We previously reported that Fas- and TNFα receptor-associated FLASH interacts with the NRB domain of one of the p160 nuclear receptor coactivators, GRIP1, and

suppresses GR-stimulated transactivation by interfering with the interaction between GR and GRIP1. Since we tested FLASH only in HCT116 cells in the previous report, we examined whether FLASH also suppresses GR transactivation in other cell types. We also used the TAT3 promoter, which contains synthetic GREs from the rat tyrosine aminotransferase, in addition to the MMTV promoter. Since human cervical carcinoma-derived HeLa and rhabdomyosarcoma-derived A204 cells express endogenous FLASH (data not shown), we treated these cells with an antisense morpholino oligonucleotide for FLASH, which can reduce the expression levels of FLASH in HCT116 cells [15], and examined transcriptional activity of GR (Fig. 1A and B). Treatment with the FLASH antisense significantly increased the transcriptional activity of both the MMTV and the TAT3 promoters in these cell lines in a dexamethasone-dependent fashion, indicating that endogenous FLASH acts as a negative regulator of the GR-induced transactivation of two different glucocorticoid-responsive genes in these cells in addition to HCT116 cells. To examine FLASH effect on a chromatin-integrated glucocorticoid-responsive promoter, we developed COS7MMTV cells, which have the luciferase gene under the control of the chromatin-integrated MMTV promoter that is fully responsive to glucocorticoids via its four GREs. In these cells, exogenously expressed FLASH strongly suppressed the transcriptional activity of this promoter in a dexamethasone-dependent fashion, clearly indicating that FLASH is also functional on the glucocorticoid-responsive promoters integrated in the native chromatin (Fig. 1C).

In our earlier study, we tested the interaction of FLASH with only one of the p160 coactivators GRIP1 and effect of both on only GR transactivation [15]. Since p160 coactivators have three subgroups, namely SRC or N-CoA1, N-CoA2 and N-CoA3, which may have overlapping and/or specific roles in the regulation of steroid hormone receptor activity [7], we examined the specificity of FLASH interactions with other p160 coactivators. We found that FLASH bound TRAM1 strongly, similarly to GRIP1, while its association with SRC1a was very weak in a yeast two-hybrid assay (Fig. 2A).

To address the functional interaction of FLASH and these p160 molecules, we next examined the suppressive effect of FLASH on their enhancement of GR-induced transactivation in HCT116 cells (Fig. 2 B). GRIP1 enhanced GR-induced transactivation by 5-fold, while SRC1a and TRAM1 did by 2.5- and 2-fold, respectively. FLASH suppressed GR-induced transactivation by about 60%, possibly antagonizing the activity of endogenous p160 coactivators. FLASH further suppressed GRIP1- or TRAM1-induced enhancement of GR transactivation by about 80% and 70%, respectively, while it did not affect SRC1a-induced enhancement of GR transactivation, consistent with the binding results observed in the yeast two-hybrid assay.

To examine the specificity of FLASH-induced suppression on other SRs, we cotransfected FLASH with AR, PR and ER along with their responsive promoters in addition to GR in HCT116 and HeLa cells. FLASH suppressed AR- and PR-induced transactivation of the MMTV promoter in addition to GR, while it did not affect ER-induced transcriptional activity on its responsive promoter (Fig. 3A).

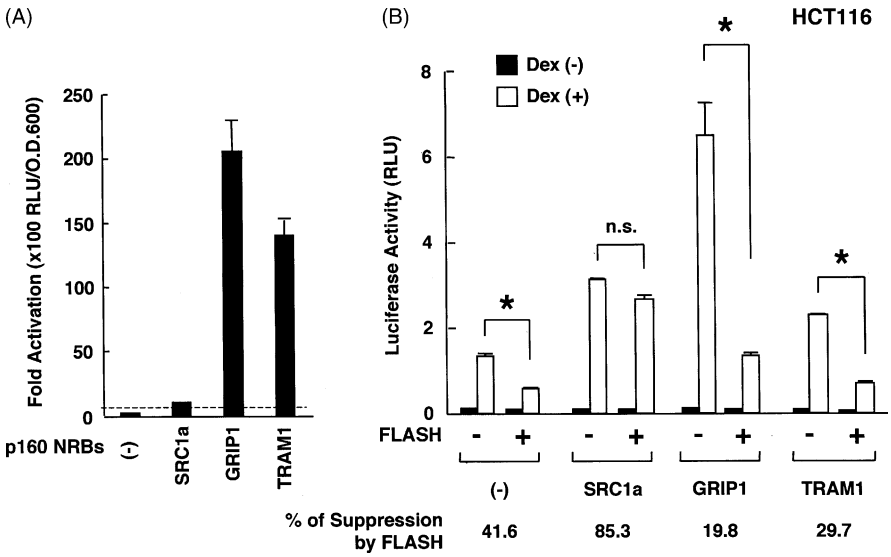

Fig. 2. FLASH differently interacts with the NRB domain of three p160 coactivators and suppresses their enhancement of GR-induced transactivation. (A) FLASH interacts strongly with GRIP1 and TRAM1, and weakly with SRC1a in a yeast two-hybrid assay. EGY48 yeast cells were transformed with pLexA, pLexA-SRC1a(598–771), pLexA-GRIP1(596–774) or pLexA-TRAM1(595–765) together with p8op-LacZ and pB42AD-FLASH(1709–1982) and were cultured in selective media containing galactose or glucose. β-Galactosidase activity was determined and expressed as fold induction of the activity obtained in cells cultured in galactose-containing media to that in glucose-containing media. (B) FLASH suppresses GRIP1- and TRAM1-, but not SRC1a-induced enhancement of GR transactivation of the MMTV promoter in HCT116 cells. HCT116 cells were transfected with the indicated p160 protein-expressing plasmids, pMMTV-Luc and pSV40-β-Gal, together with and without FLASH-expressing plasmids. Bars represent mean ± S.E. values of the luciferase activity normalized for β-galactosidase activity in the absence or presence of 10^{-6} M of dexamethasone. $^{*}P < 0.01$, compared to the baseline.

To look for the mechanism(s) underlying the specificity of FLASH effect on these SRs, we performed the same examination as in Fig. 2B for the other SRs in HCT116 cells (Fig. 3B). GRIP1 and TRAM1 differentially enhanced the AR- and PR-induced transcriptional activity of the MMTV promoter, while SRC1a did this with relatively weak potency. FLASH expression strongly suppressed GRIP1- and TRAM1-induced enhancement of transcriptional activity of these receptors but not that of SRC1a, similarly to their effects on GR. In contrast, SRC1a strongly enhanced ER-induced transcriptional activity, while GRIP1 and TRAM1 moderately potentiated it. FLASH suppressed GRIP1 and TRAM1-induced enhancement, whereas it did not have an obvious effect on the SRC1a-induced potentiation. These results may indicate that transcriptional activity of AR and PR is more responsive to, and possibly, more dependent on GRIP1 and TRAM1 than on SRC1a, similarly to GR, while ER has the opposite phenotype in HCT116 cells. The above-identified preference of coactivator use by each SR may explain the different suppressive effect of FLASH on their transcriptional activity.

4. Discussion

Endogenous FLASH acted as a negative regulator of GR transcriptional activity in several different cell lines as well as two different glucocorticoid-responsive promoters, while overexpression of FLASH suppressed GR-induced transactivation of the MMTV promoter, which was integrated in the host cell chromatin. FLASH interacted with the NRB domain of all subtypes of p160 coactivators in a yeast two-hybrid assay. FLASH, however, was associated strongly with TRAM1, one of the SRC3/N-CoA3 coactivators, as well as an SRC2/N-CoA2 subtype, GRIP1, while its interaction with SRC1a was 20 times less than the other two p160 proteins. These results are consistent with the amino acid sequence homology of the NRB domains of these molecules. Indeed, GRIP1, which was used as a bait in the original screening [15], has 98% similarity with its human homologue TIF-II at their NRB domain. These portions of GRIP1 and TIF-II have 70–80% homology with that of human SRC3/N-CoA3 subtype TRAM1, while they are quite different from the human SRC1a NRB. In agreement with its specificity of interaction to p160 coactivator subtypes in the yeast two-hybrid assay, FLASH significantly suppressed TRAM1-induced enhancement of GR-stimulated transactivation of the MMTV promoter similarly to GRIP1, while it did not affect SRC1a-induced enhancement of GR transactivation. These results indicate that FLASH may inhibit GR-stimulated transcriptional activity of its responsive promoter by interfering with the interaction of ligand-activated GR with SRC2/N-

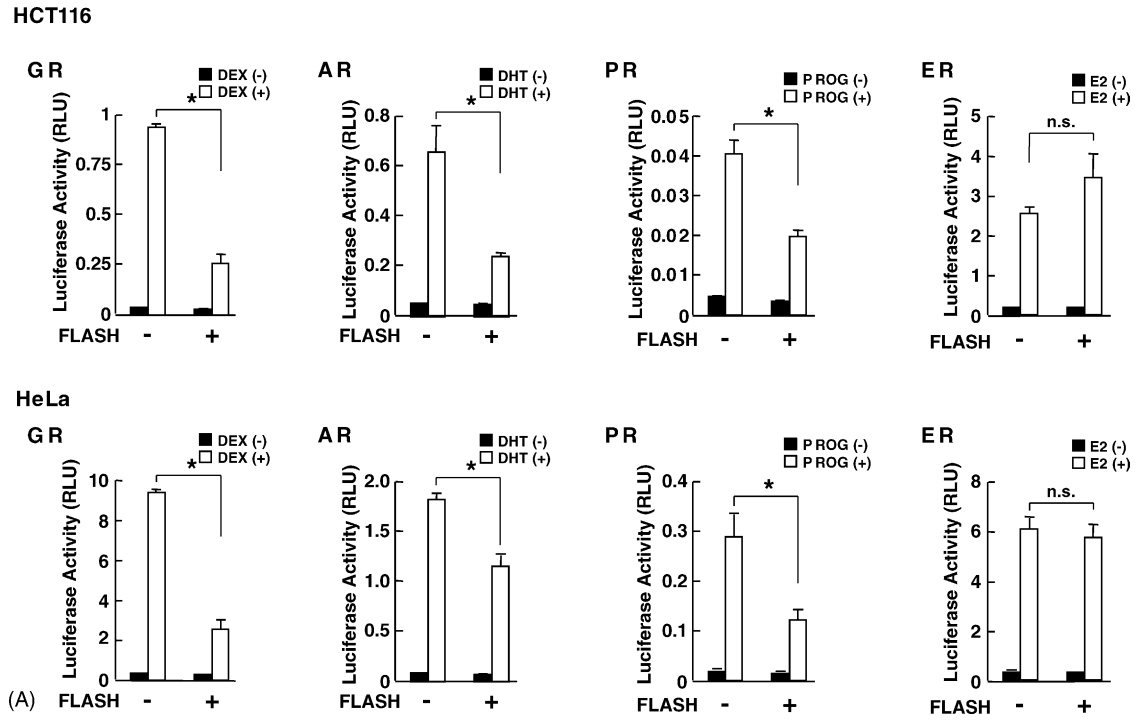

Fig. 3. Differential effect of FLASH on several steroid hormone receptor-induced transcriptional activities. (A) FLASH suppresses GR-, AR- and PR-, but not ER-induced transactivation of their responsive promoters in HCT116 (upper panels) and HeLa (lower panels) cells. HCT116 or HeLa cells were transfected with GRα- (only for HCT116 cells), AR-, PR-A- and ERα-expressing plasmids together with pMMTV-Luc (for GR, AR and PR-A) or ERE-tk-luc (for ERα), and pSV40-β-Gal. Bars represent mean ± S.E. values of the luciferase activity normalized for β-galactosidase activity in the absence or presence of indicated ligands. $^{*}P < 0.01$ compared to the baseline. (B) Three p160 coactivators differently enhance the transcriptional activity of SRs, and FLASH selectively suppresses their enhancing effect in HCT116 cells. HCT116 cells were transfected with the indicated p160 protein-expressing plasmids, pMMTV-Luc (for AR, PR), ERE-tk-Luc (for ER), respective receptor-expression plasmids and pSV40-β-Gal, together with and without FLASH-expressing plasmids. Bars represent mean ± S.E. values of the luciferase activity normalized for β-galactosidase activity in the absence or presence of 10^{-6} M of dexamethasone. $^{*}P < 0.01$, compared to the baseline.

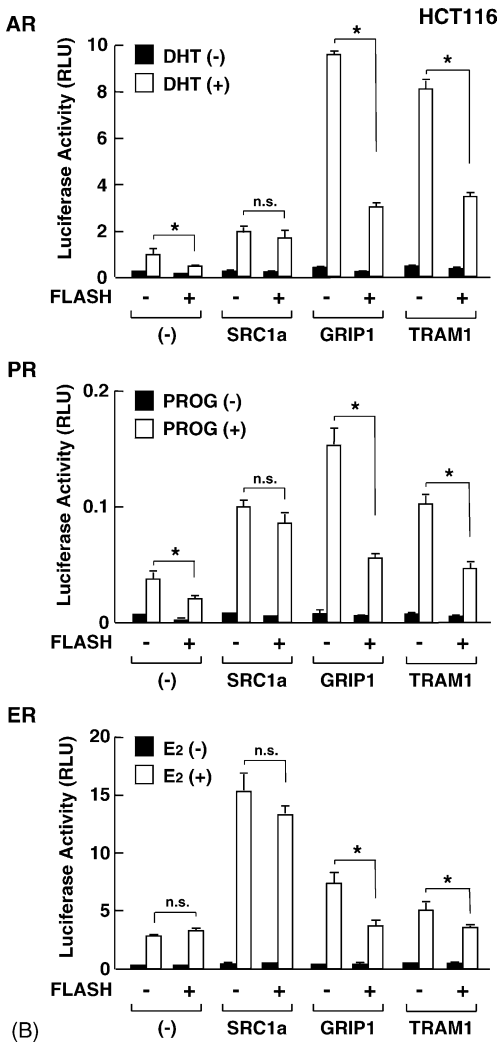

Fig. 3. (*Continued*).

CoA2 and SRC3/N-CoA3 types of p160 coactivators, but may not interfere with SRC1/N-CoA1-mediated activation of GR.

Since p160 coactivators are functional in all steroid hormone receptor-induced transcriptional signaling [6], we also tested FLASH on AR-, PR- and ER-induced transactivation of their responsive promoters. As expected, FLASH expression suppressed AR- and PR-mediated transcription of the MMTV promoter, while it did not influence ER-induced transactivation of the ER-responsive promoter in HCT116 and HeLa cells. Several factors could produce differential suppression of these SR activities. Different SRs can accumulate different sets of p160 coactivator subtypes in responding to ligand stimuli [9]. For example, ligand-activated GR accumulates SRC2/N-CoA2 and SRC3/N-CoA3, while PR does SRC1/N-CoA1 and SRC3/N-CoA3, on the MMTV promoter in T24D cells [9]. To examine the possibility of this mechanism as a potential cause of the FLASH-induced suppression, we examined the effects of SRC1a, GRIP1 and TRAM1 on the transcriptional activity of these SRs in HCT116 cells. In this cell line, GRIP1 and TRAM1 strongly enhanced the transcriptional activity of GR, AR and PR, while SRC1a demonstrated a relatively weak effect. In contrast, the former two p160 coactivators enhanced ER-induced transactivation weakly, whereas the latter coactivator strongly potentiated it. Accordingly, FLASH significantly suppressed GRIP1- and TRAM1-induced enhancement of the transcriptional activity of all tested SRs, while FLASH did not obviously inhibit SRC1a-induced potentiation. These results indicate that GRIP1 and TRAM1, but not SRC1a, may strongly act on GR-, AR- and PR-induced transactivation, while SRC1a plays a stronger role in ER-induced transcriptional activity in HCT116 cells. These pieces of evidence may underlie the differential suppressive effect of FLASH on SR-induced transcriptional activity, such as downregulating the transcriptional activity of the former three receptors but not that of ER.

In addition to the above-indicated activity of FLASH, it is also possible that this protein suppresses the transcriptional activity of these SRs via other, unindentified mechanism(s). Each SR uses different sets of three LXXLL motifs in the NRB domain of p160 coactivators [10,19,20]. Since FLASH binds to a portion of the region of GRIP1 located between the second and the third LXXLL motifs [15], it may differentially influence the interaction of these LXXLL motifs with each different SR. Also, different agonists of a single receptor may also modulate the interaction of the activated receptor to subtypes of p160 coactivators [21]. SRC1a has an additional interface for the interaction with SRs at its very C-terminal end, while the other subtypes of p160 coactivators do not [6]. It is, therefore, possible that FLASH failed to inhibit the interaction of this p160 coactivator with SRs via active interaction through this interface, in addition to the inability of FLASH to associate with this coactivator through its NRB domain. Finally, different cell types exhibit varying patterns of p160 protein subtype relative concentrations [6,7,22]. These factors could additionally influence the suppressive effect of FLASH on these steroid hormone receptors in different tissues and in various developmental or functional states of target cells.

In this report, we further extend the knowledge on the actions of FLASH on the GR-mediated transactivation through different p160 coactivators' subtypes, as well as its suppressive specificity to other steroid hormone receptors. Through these activities, FLASH may regulate sensitivity of tissues to several steroid hormones in both physiologic and pathologic situations that may be related to inflammation and apoptosis.

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