Tumor Necrosis Factor α Receptor- and Fas-associated FLASH Inhibit Transcriptional Activity of the Glucocorticoid Receptor by Binding to and Interfering with Its Interaction with p160 Type Nuclear Receptor Coactivators*

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Tumor necrosis factor α (TNFα) and its downstream transcription factor nuclear factor-κB (NF-κB) suppress glucocorticoid action, contributing to tissue resistance to glucocorticoids in several pathologic inflammatory states. p160 nuclear receptor coactivators on the other hand, contribute to the transcriptional signal of the glucocorticoid receptor (GR) through interaction with it via LXXLL motifs in their nuclear receptor-binding (NRB) domain. To discover TNFα-induced factors that regulate GR activity at the coactivator level, we performed yeast two-hybrid screening using the NRB domain of the glucocorticoid receptor-interacting protein 1 (GRIP1) as bait. We found that FLICE-associated huge protein (FLASH), which transduces TNFα and Fas ligand signals, bound the NRB domain of GRIP1 at a region between the second and third LXXLL motifs. FLASH suppressed both GR transactivation and GRIP1 enhancement of the glucocorticoid signal and inhibited the physical interaction between GR and the GRIP1 NRB domain. Transfected green fluorescent protein-fused FLASH was located in both the cytoplasm and nucleus, while endogenous FLASH shifted its subcellular localization from the cytoplasm into the nucleus in response to TNFα. FLASH antisense and super-repressor IκBα inhibited the action of TNFα independently of each other and additively. These findings indicate that FLASH participates in TNFα-induced blockade of GR transactivation at the nuclear receptor coactivator level, upstream and independently of NF-κB.

Glucocorticoids exert profound influences on many physiologic functions by virtue of their diverse roles in growth, development, and maintenance of homeostasis (1, 2). The presence of glucocorticoids is crucial for the integrity of central nervous system function and for maintenance of cardiovascular, metabolic, and immune homeostasis (3). Their actions are mediated by the glucocorticoid receptor (GR),† which functions as a hormone-activated transcription factor that regulates the expression of glucocorticoid target genes (4). The transcriptional activity of the ligand-activated GR is exerted by interaction with molecular components of the transcription machinery. Of particular importance is a family of proteins, the nuclear receptor coactivators, which bridge promoter-bound GR and the transcription initiation complex. Coactivators also possess 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 (5).

p160 type nuclear receptor coactivators play an essential role in GR-induced transcriptional activation (6). These large proteins are mainly localized in the nucleus. The promoter-bound GR helps tethering them to the promoter regions of glucocorticoid-responsive genes. Other coactivators, such as p300 and its homologous protein cAMP-responsive-element-binding protein (CREB)-binding protein (CBP), as well as the p300/CBP-associated protein (p/CAP), are then attracted to the promoters forming receptor-coactivator complexes (7–11). There are three subclasses of p160 proteins: steroid receptor coactivator 1 (SRC1); TIF-II or glucocorticoid receptor-interacting polypeptide 1 (GRIP1), also called SRC2; and, p300/CBP/co-integrator-associated protein (p/CIP), also called ACTR, TRAM-1, RAC3, or SRC3 (5, 6). All three exhibit high similarity in the 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 (12, 13).

Since glucocorticoids have a broad array of life-sustaining functions and play an important role in the therapy of many human diseases, changes of tissue sensitivity to glucocorticoids may be associated with and influence the natural history of and response to glucocorticoid treatment of many of these states (14, 15). Several autoimmune and allergic/inflammatory states, such as rheumatoid arthritis, osteoarthritis, Crohn’s disease, ulcerative colitis, and asthma, are often associated with resist-

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‡ The abbreviations used are: GR, glucocorticoid receptor; TNFα, tumor necrosis factor α; TRAF, TNFα receptor-associated factors; TNF-α; TNFα receptor; FLASH, FLICE-associated huge protein; NF-κB, nuclear factor-κB; SR, super-repressor; FasL, Fas ligand; DRD, death-effector domain-recruiting domain; RIP, receptor-interacting protein; DISC, death-inducing signaling complex; GRIP1, glucocorticoid receptor-interacting protein 1; TIF-II, transcription intermediary factor II; SRC, steroid receptor coactivator; pCIP, p300/CBP-integrator-associated protein; NRB, nuclear receptor binding; CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; p/CAP, p300/CBP-associated factor; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; GRE, glucocorticoid-responsive element; GFP, green fluorescence protein; GST, glutathione-S-transferase; AF, activation function.
ance of the inflamed tissues to glucocorticoids (14, 16, 17). In addition, the acute respiratory distress syndrome and septic shock have been associated with systemic glucocorticoid resistance (18). The mechanism(s) underlying such inflammation-related glucocorticoid resistance is not well understood, but several proinflammatory cytokines and their downstream signaling cascades may be involved in the process (1).

Tumor necrosis factor α (TNFα) and its homologues play a central role in the development and maintenance of inflammation in many pathologic inflammatory states (19, 20). TNFα is produced by variety of immune and immune accessory cells, including monococytes, macrophages, and dendritic cells, and exerts diverse effects on cell growth and differentiation, promotes inflammation, and may cause apoptosis (21). TNFα activates nuclear factor κB (NF-κB), a heterodimer transcription factor of the Rel family proteins that plays a major role in inflammation and the immune response. NF-κB is also stimulated by many inflammation-promoting substances, including other proinflammatory cytokines, endotoxins, intracellular proteins liberated from necrotic cells, oxygen radicals, and oxidized molecules (22). NF-κB antagonizes GR action at several different steps, including direct interaction with it and interference with its transcriptional activity (23, 24).

TNFα interacts with its cell surface receptor (TNF-R) and activates IκB and NF-κB phosphorylation, release of NF-κB from a cytoplasmic IκB-NF-κB complex, and nuclear translocation of NF-κB. In parallel, TNFα activates several other intermediate proteins, such as TNFα receptor-associating factors (TRAF), receptor-interacting protein (RIP), and FLICE-associatedhinge protein (FLASH) (21, 25, 26). FLASH is a CED4-homologous protein, involved in apoptosis induced by both TNFα and Fas ligand (FasL) (26, 27). This protein forms the death-inducing signaling complex (DISC) with the cytoplasmic portion of Fas, i.e. the receptor for FasL and caspase-8 in response to FasL. FLASH also participates in the activation of NF-κB through direct interaction with TRAF2. Although FLASH was originally found as a component of a cytoplasmic complex located under the plasma membrane, it contains two presumptive nuclear localization signals and one nuclear export signal, findings that have led to the speculation that it might translocate into the nucleus in response to certain stimuli (27).

To elucidate mechanism(s) that may contribute to glucocorticoid resistance associated with inflammation, we performed yeast two-hybrid screening using the NRB domain of GRIP1 as bait. We found that the C-terminal portion of FLASH specifically interacted with GRIP1 NRB at the region enclosed between the second and the third LXXL motifs. FLASH inhibited both GR transactivation and its enhancement by GRIP1 on a glucocorticoid-responsive promoter by interfering with GR binding to GRIP1. Incubation of cells with TNFα caused translocation of FLASH into the nucleus, blocking ligand-activated GR interaction with GRIP1 and suppressing transactivation.

We suggest that the transcriptional activity of the GR is regulated by TNFα at the level of p160 type coactivators through FLASH, independently of its parallel interaction and interference with transcription factor NF-κB signal transduction pathways.

MATERIALS AND METHODS

Plasmids—pLexA-GRIP1(596–774)–WT and LXXLs-Mut were constructed by inserting the corresponding GRIP1 cDNAs, without or with two leucines replaced by alanines in all three LXXLs, into pLexA (Clontech, Palo Alto, CA). pLexA-GRIP1(600–640), GRIP1(646–899), GRIP1(691–743), and GRIP1(691–774) were constructed by sub-cloning the correponding GRIP1 cDNAs into pLexA. pSG5-GRIP1 fl, which expresses murine full-length GRIP1, is a generous gift from Dr. M. G. Stallcup (University of Southern California, Los Angeles, CA). pME185-FLASH-FLASH encodes human full-length FLASH cDNA and is a kind gift from Dr. S. Yonehara (Kyoto University, Kyoto, Japan). pME185-FLAG-FLASH(1–1696) was produced by re-ligation of pME185-FLAG-FLASH digested with BstXI and NotI. pME185-FLASH-EGFP was constructed by sub-cloning EGFP cDNA into the 3′ end of the FLASH coding region of pME185-FLASH in an in-frame fashion. pB42AD-FLASH(1–1892), FLASH(1–467, 962–999), FLASH(1192–1584), FLASH(1192–1584), FLASH(1709–1982), FLASH(1182–1694), FLASH(929–1584), and FLASH(992–1694) were constructed by inserting the corresponding FLASH cDNA fragments into pB42AD (Clontech). pGEX-4T3-FLASH(1575–1982) was constructed by sub-cloning the cDNA of FLASH(1575–1982) into pGEX-17s (Amersham Biosciences). pGEX-DHFR(956–774) and pGEX-DHFR(956–774) were kind gifts from Dr. R. M. Evans (Salk Institute, La Jolla, CA). pMMTV-Luc was a generous gift from Dr. G. L. Hager (NCI, National Institutes of Health, Bethesda, MD). pGAL4-E1B-Luc, which expresses luciferase under the control of a promoter containing the four GALA-responsive elements connected to the proximal promoter region of adenovirus E1B, was a generous gift from Dr. P. Driggers (Uniformed Services University for the Health Sciences, Bethesda, MD). p8op-LacZ and pSV40-β-Gal were purchased from Clontech or Promega, respectively.

Yeast Two-hybrid Screening and Assay—The yeast two-hybrid screening was performed using GRIP1(596–774) as bait in a human cDNA library with the LexA system (31). A two-hybrid assay, yeast strain EGY48 (Clontech) was transformed with the lacZ reporter plasmid p8op-LacZ, indicating pLexA-GRIP1s and pB42AD-FLASH plasmids. The cells were grown in a selective medium to the early stationary phase and permeabilized by CHCl3-SDS treatment. The cells were harvested after an additional 24 h of incubation for luciferase and β-galactosidase assays.

Cell Transfections and Reporter Assays—HCT116 cells, purchased from the American Type Culture Collection (Manassas, VA), were maintained in MacCoys’s 5A medium supplemented with 10% fetal bovine serum, 50 units of penicillin, and 50 µg/ml streptomycin. The cells were transfected using Lipofectin (Invitrogen) as described previously (31). For the experiments using pMMTV-Luc or (1B±)3-Luc as a reporter plasmid, 1.0–2.0 µg/well of FLASH-expressing plasmids, 1.0 µg/well of pCDM-IκBS2.36A and/or 1.0–2.0 µg/well of pSG5-GRIP1 fl were used with 0.8 µg/well of pMMTV-Luc or 1.5 µg/well of (1B±)3-Luc. 0.1 µg/well of pShSGR was cotransfected in all experiments since HCT116 cells do not contain functional GR. 0.5 µg/well of pSV40-β-Gal was also included to normalize luciferase activity in all transfections. Empty vectors were used to reach the same amount of transfected DNA. 0.1 µg/well of pMMTV-Luc, 0.5 µg/well of pSV40-β-Gal, and 0.1 µg/well of pShSGR, and increasing concentrations of dexamethasone were added to the medium 24 h later. The cells were harvested after an additional 24 h of incubation for luciferase and β-galactosidase assays.

Antisense Experiment—The morpholino antisense oligonucleotide for FLASH expression, encoding 5′-CACATTGTCTACATGCTGCTCAT-3′, which targets the first 25 bases of the FLASH coding region, was generated by Gene Tool LLC (Philomath, OR). The control the morpholino oligonucleotide, which contains the target sense sequence of FLASH antisense oligonucleotide, was also purchased from the same company. To introduce the antisense oligonucleotide, we employed the ethoxyethyl polyethyleneimine-based special delivery protocol prepared by the same company. 24 h after transfection, cells were treated with 0.8 µg/well of pMMTV-Luc, 0.5 µg/well of pSV40-β-Gal, and 0.1 µg/well of pShSGR, and increasing concentrations of dexamethasone were added to the medium 24 h later. The cells were harvested after an additional 24 h of incubation for luciferase and β-galactosidase assays.
Mammalian Two-hybrid Assay—HCT116 cells were transfected with 0.5–2.0 μg/well of pME18S-FLAG-FLASH, 0.2 μg/well of pM-GRIP1(596–774), 1.0 μg/well of pVP16-GR-LBD, 1.5 μg/well of pGAL4-E1B-Luc, and 0.5 μg/well of pSV40-β-Gal, using Lipofectin. 10^{-6} \text{M} of dexamethasone was administered 24 h after transfection. The cells were harvested after an additional 24 h of incubation for luciferase and β-galactosidase assays.

GST Pull-down Assay—GST-fused FLASH(1575–1982) was produced in BL21 bacteria from pGEX-4T3-FLASH(1575–1982). FLASH(1575–1982) was liberated by thrombin treatment. The protein was dialyzed and concentrated in a buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, and 0.1 mg/ml bovine serum albumin as described previously. After vigorous washing with the buffer, proteins were eluted and separated on 8% SDS-PAGE gels. 5% of total input of labeled GR was loaded as a marker. Gels were fixed and exposed to film.

Western Blot Analysis of FLASH—Cells were lysed in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol and 5% glycerol. [(35S)S]-labeled GRs were generated by in vitro translation from pGR107 using reticulocyte lysate (Promega). After separation of 10 μg of proteins in SDS-PAGE gels and blotting on nitrocellulose membrane, FLASH was detected by anti-FLASH (M-300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection and Localization of FLASH or EGF-fused FLASH and Statistical Analyses—For detecting endogenous FLASH by indirect immunofluorescent staining, cells were cultured in a multi-well slide glass, and FLASH was stained with anti-FLASH followed by fluorescein isothiocyanate-labeled secondary antibody. For detecting EGF-fused FLASH, cells were plated on 20-mm dishes in phenol red-free MacCoY's 5A medium containing 10% fetal bovine serum and antibiotics and then transfected with pME18S-FLASH-EGFP. The cells were analyzed using regular inverted or confocal microscopes. Statistical analysis was carried out by analysis of variance followed by Student's t-test with Bonferroni correction for multiple comparisons.

RESULTS

Identification of FLASH as a Binding Partner of GRIP1 in a Yeast Two-hybrid Screening—To identify molecules regulating glucocorticoid receptor activity at the level of the p160 coactivator GRIP1, we performed a yeast two-hybrid screening using the NRB domain of the p160 coactivator GRIP1. We found four clones out of 80 colonies, which contained different C-terminal cDNA fragments of the FLASH molecule (data not shown). The shortest interactor represented the sequence included between amino acids 1636 and 1982 of FLASH. To confirm and localize the portion of FLASH that interacts with GRIP1 NRB, we constructed a series of prey vectors that contained different fragments of FLASH cDNA, and tested the interaction of their products with GRIP1 NRB in our yeast two-hybrid assay (Fig. 1A). As expected, FLASH(1709–1982), but not the other fragments, specifically interacted with the GRIP1 NRB.

Since we used the wild type GRIP1 NRB, which has three intact LXXLL motifs, it was of particular interest to determine whether FLASH interacted with GRIP1 through any of these LXXLL motifs as nuclear receptors do. To accomplish this, we examined the interaction of FLASH(1709–1982) with mutated GRIP1 NRB defective in the LXXLL motifs by replacing two leucines with alanines in each of the three LXXLL motifs in the same yeast two-hybrid assay (Fig. 1B). Both LXXLL mutant and wild type GRIP1 NRBs interacted similarly with FLASH, indicating that the FLASH and GRIP1 NRB interaction is LXXLL motif-independent. To further localize the portion of GRIP1 NRB necessary for binding to FLASH, we tested a set of bait plasmids expressing different portions of GRIP1 NRB for their binding to FLASH(1709–1982) (Fig. 1C). GRIP1(597–774), (691–743), (646–743), and (691–774), all of which are devoid of LXXLL motifs, strongly interacted with FLASH, in vitro.
indicating that GRIP1 (691 – 743), whose amino acid sequence is highly conserved in subtypes and species, is responsible for binding to FLASH (12). Since this portion of GRIP1 is located between two LXXLL motifs that are essential for the binding of GRIP1 to the GR (32, 33), we hypothesized that FLASH might modulate the binding activity of GRIP1 to GR. To examine this, we used GST pull-down and mammalian two-hybrid assays. In the former assay, we used a bacterially produced and purified C-terminal portion of FLASH peptide, while in the latter system, we employed full-length FLASH, expressed by a plasmid (Fig. 2). GR bound to GST-GRIP NRB in a ligand-dependent fashion, as previously reported. Addition of FLASH (1574 – 1982) peptide was added to the reaction containing bacterially produced GST-GRIP1 NRB WT and in vitro translated and labeled GR. After vigorous washing with buffer, samples were loaded on an 8% SDS-PAGE gel. B, FLASH suppressed the interaction of GR-LBD and GRIP1 NRB in a mammalian two-hybrid assay. The cells were cotransfected with increasing amounts of FLASH-expressing plasmid together with pMMTV-Luc and pSV40-β-Gal. Fold induction was calculated by the ratio of normalized luciferase values of cells transfected with VP16-GR-LBD versus VP16. Bars show the 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 with the baseline.

**FLASH Suppresses GR Transactivation**—Based on the above binding results, we next examined the effect of FLASH on GR-induced transactivation in a transient transfection reporter system (Fig. 3). Full-length FLASH suppressed dexamethasone-stimulated GR transactivation on the MMTV promoter in a dose-dependent fashion (Fig. 3A). On the other hand, FLASH (1697 – 1982), which was devoid of its C-terminal portion necessary for the binding to the GRIP1 NRB domain, did not suppress GR activity. FLASH also suppressed GRIP1 enhancement of GR transactivation, indicating that FLASH antagonized GRIP1 coactivation of the GR (Fig. 3B). We next examined the role of FLASH on GR transactivation employing a FLASH antisense morpholino oligonucleotide to suppress endogenous levels of FLASH (Fig. 3, C and D). This oligonucleotide targeted the translation start site of the FLASH mRNA and efficiently suppressed levels of FLASH protein measured by Western blot analysis after 48 h of incubation (Fig. 3C). FLASH antisense shifted the dexamethasone dose-dependent curve to the left and upward, suggesting that endogenous FLASH functions as an inhibitory factor on glucocorticoid-induced GR transactivation (Fig. 3D).

**Subcellular Localization of FLASH**—How does FLASH, a cytoplasmic protein associated with the TNF-R and Fas receptor, interact with GRIP1, which resides mainly in the nucleus (7, 27)? To address this, we examined the intracellular distribution and localization of FLASH. Using indirect immunofluorescent staining with anti-FLASH and regular inverted microscopy, this protein was predominantly located in the cytoplasm, although a small fraction of immunofluorescence was also detected in the nucleus (Fig. 4A, a and b). In subsequent confocal microscope analysis, we confirmed that a small proportion of FLASH was located in the nucleus (Fig. 4A, c). On the other hand, when we employed transfected FLASH-EGFP, we detected both cytoplasmic and nuclear localizations of the
protein (Fig. 4B, a–c). Inside the nucleus, FLASH-EGFP was distributed in small speckles (Fig. 4B, a and b). The discrepancy of FLASH cytoplasmic versus nuclear localization in the two methods employed may indicate that FLASH shuttles between the cytoplasm and the nucleus.

Since FLASH is downstream of TNFα/H9251 and Fas, we examined the effect of TNFα on FLASH localization by indirect immunofluorescent staining. Incubation of cells with TNFα induced strong nuclear localization of FLASH in some cells, suggesting that TNFα may suppress GR transactivation by promoting the nuclear localization of FLASH (Fig. 5).

Action of FLASH on GR Is Independent of the NF-κB Pathway—TNFα antagonizes the effects of glucocorticoids through activation of NR-κB (23, 34). Since TNFα induced FLASH nuclear localization and FLASH suppressed GR transactivation, we examined the relative contribution of FLASH on TNFα-induced suppression of GR-induced, GRE-mediated transactivation by dissecting its activity from that of NF-κB (Fig. 6, A and B). For this purpose, we used a super-repressor IxBα, which contains serine to alanine mutations at residues 32 and 36, which inhibit signal-induced phosphorylation and subsequent proteasome-mediated degradation (29). This IxBα mutant constitutively binds to NF-κB and suppresses its nuclear translocation and subsequent DNA binding. A concentration of 30 ng/ml of TNFα suppressed dexamethasone-stimulated GR-induced, GRE-mediated transactivation on the MMTV promoter by 60%. Coexpression of super-repressor IxBα partially eliminated the TNFα effect, while it almost completely abolished TNFα-induced NF-κB transactivation. When both the FLASH antisense and super-repressor IxBα were introduced into the cells, TNFα completely lost its suppressive effect while FLASH suppressed GR-induced, GRE-mediated transactivation regardless of presence or absence of super-repressor IxBα. These results strongly suggest that FLASH suppresses GR transactivation independently of NF-κB. To demonstrate additivity between the FLASH and NF-κB effects on GR transactivation, we examined coexpression of FLASH and the p65 component of NF-κB on the MMTV promoter. As expected, the suppressive effect of FLASH and p65 on the MMTV promoter was additive (Fig. 6C), further supporting the conclusion that FLASH and NF-κB function separately on blocking GR-induced, GRE-mediated transactivation.

DISCUSSION

The GR coactivator GRIP1 interacted specifically with the TNF-R- and Fas-associated FLASH (Fig. 7). The latter, a large protein, was originally cloned as a transducer of TNFα and FasL apoptotic signals (27). The C-terminal portion of FLASH, corresponding to amino acids 1709–1982, located just C-termi- nally to the death-effector domain-recruiting domain (DRD), bound GRIP1 at its region enclosed between amino acid 691 and 743, which is highly conserved in subtypes and species and located between the second and the third LXXLL motifs in the NRB region of the coactivator. FLASH interacted with GRIP1 in a LXXLL motif-independent fashion and suppressed both GR-induced transactivation and GRIP1 enhancement on the glucocorticoid-responsive MMTV promoter by possibly inhibi-
FLASH binds a p160 coactivators through their mutual competition for the other hand, suppresses GR action by inhibiting its binding to transcriptionally modulating transcription (23, 24) (Fig. 7). FLASH, on the venting the GRE-bound GR coactivator complex from effec-

FLASH independently and in addition to that produced by NF-

Since these two motifs are essential for the binding of the coactivator to nuclear receptors (32), FLASH must be exerting steric hindrance, preventing access of the GR to these motifs. Glucocorticoids may function both as inducers and as inhibitors of apoptosis, depending on the target tissues/cells and the resting or activated state of these cells (35–40). Therefore, FasL, a strong inducer of apoptosis upstream of FLASH, might promote or prevent apoptotic actions of glucocorticoids through FLASH, depending on the target tissues/cellular state.

Since other nuclear receptors also interact with p160 coactivators, it is quite possible that FLASH also regulates their functions by changing their binding activity to p160 proteins (5, 6). Estrogen and progesterone, for example, have strong effects on proliferation/differentiation and apoptosis in the uterus, mammary glands, ovaries, bone, and central nervous system that may also be influenced by cytokines, such as TNFα and FasL (41–44). Thus, FLASH might play an important role in the actions of these hormones or other nuclear hormones as well. FLASH might exert diverse influences on the actions of different nuclear hormones, given that the various nuclear receptors employ different sets of LXXLL motifs in the NRB domains of various p160 nuclear receptor coactivators in their interactions with them, and given that the relative concentrations of p160 coactivators are different between types of cells and between cells at varying functional states (32, 33). We have obtained preliminary data on such diversity of FLASH effects in different nuclear receptor systems.2

We showed that FLASH shifted its subcellular localization from the cytoplasm into the nucleus in response to TNFα (Fig. 7A). The underlying mechanism is not known; however, FLASH contains two nuclear localization signals around amino acids 1200 and 1760 and one nuclear export signal around

NF-κB antagonizes GR action through mutual protein-protein interactions by inhibiting its binding to GREs or by preventing the GRE-bound GR coactivator complex from effectively modulating transcription (23, 24) (Fig. 7). FLASH, on the other hand, suppresses GR action by inhibiting its binding to p160 coactivators through their mutual competition for the NRB domain of the coactivators (Fig. 7). FLASH binds a portion of GRIP1 NRB, located between the second and the third LXXLL motifs. Since these two motifs are essential for the binding of the coactivator to nuclear receptors (32), FLASH might promote or prevent apoptotic actions of glucocorticoids through FLASH, depending on the target tissues/cellular state.

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2 T. Kino and G. Chrousos, unpublished information.
Specific Binding of FLASH and GRIP1 NRB

1180, which could facilitate nucleocytoplasmic shuttling (27). In some cells expressing EGFP-fused FLASH, this protein showed a speckled pattern in the nucleus. The FLASH-binding partner GRIP1 and other nuclear receptor coactivators, such as p300 and CBF, also show similar subnuclear localization accumulating in ND10 nuclear bodies containing 26 S proteasome (7, 8, 45, 46). Thus, FLASH might also be located in the same nuclear bodies with these coactivators.

One of the p160 coactivators, SRC-3, was recently shown to be located in the cytoplasm in a serum-depleted condition and to translocate into the nucleus in response to TNFα (9, 10). Although we have not tested the association of FLASH and SRC-3, it is possible that these two proteins might form a complex in the cytoplasm and translocate into the nucleus in response to TNFα, FasL, or other extracellular factors.

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