Prostratin Antagonizes HIV Latency by Activating NF-κB*

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Samuel A. Williams‡§, Lin-Feng Chen‡, Hakju Kwon‡, David Fenard‡, Dwayne Bisgrove‡,
Eric Verdin§, and Warner C. Greene‡¶**

From the 3Gladstone Institute of Virology and Immunology and Departments of *Physiology, Medicine,
and Microbiology and Immunology, University of California, San Francisco, San Francisco, California 94141

A subset of quiescent memory CD4 T cells harboring integrated but transcriptionally silent proviruses poses a currently insurmountable barrier to the eradication of the human immunodeficiency virus (HIV) in infected patients. Induction of HIV gene expression in these latently infected cells by immune activating agents has been proposed as one approach to confer sensitivity to antiretroviral therapy. Interest has recently focused on the non-tumor-promoting phorbol ester, prostratin, as a potential agent to activate latent HIV proviruses. Using multiple Jurkat T cell lines containing integrated but transcriptionally latent HIV proviruses (J-Lat cells), we now demonstrate that prostratin effectively activates HIV gene expression in these latently infected cells. We further show that prostratin acts by stimulating IKK-dependent phosphorylation and degradation of IkBα, leading to the rapid nuclear translocation of NF-κB and activation of the HIV-1 long terminal repeat in a κB enhancer-dependent manner. In contrast, NFAT and AP-1 are not induced by prostratin. Using chromatin immunoprecipitation assays to identify host transcription factors recruited to the latent HIV-1 promoter in living cells, we find that prostratin induces RelA binding. Analysis of potential upstream signal transducers demonstrates that prostratin stimulates membrane translocation of classical, novel, and atypical protein kinase C (PKC) isoforms. Studies with isoform-specific PKC inhibitors suggest that the novel PKCs play a particularly prominent role in the prostratin response. These findings provide new insights into the molecular pathway through which prostratin antagonizes HIV latency highlighting a central role for the action of NF-κB.

A small but clinically important fraction of CD4+ memory T cells in HIV-1-infected patients contain integrated but transcriptionally inactive proviruses (1). These latently infected cells retain the ability to produce infectious virus following cellular activation by specific antigen or various cytokines (2). The half-life of these CD4+ memory T cells is at least 44 months; consequently, elimination of this viral reservoir is projected to require administration of antiretroviral therapy.

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** To whom correspondence should be addressed: Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94114-9100. Tel: 415-696-3806; Fax: 415-826-1817; E-mail: wgreene@gladstone.ucsf.edu.

The abbreviations used are: HIV-1, human immunodeficiency virus; type 1; ART, antiretroviral therapy; LTR, long terminal repeat; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; TNF, tumor necrosis factor; PMA, 4-α-phorbol 12-myristate 13-acetate; PBMC, peripheral blood mononuclear cell; PKC, protein kinase C. (ART) for at least 60 years (3, 4). Accordingly, the rebound viremia routinely observed in patients terminating effective ART probably involves viral reseeding from this long-lived latent reservoir. Indeed, studies examining the genetic characteristics of rebound virus have found substantial similarities between virus present in the latent pool and that emerging after cessation of therapy (5). New approaches to the elimination of these latently infected cells are urgently needed (6).

One proposed strategy to eliminate this pool of latently infected cells is to purge the virus by activating the CD4+ memory lymphocytes in concert with ART administration (7). However, clinical efforts aimed at eliminating the pool of latently infected cells by induction with IL-2 or anti-CD3 OKT3 antibodies have yielded discouraging results (8, 9).

The failure of initial attempts to deplete the latently infected pool of CD4 T cells reflects in part our limited understanding of the molecular mechanisms governing HIV latency. HIV latency is characterized by transcriptional inactivity, but the underlying cause of this inactivity remains unknown and may be multifactorial. The HIV long terminal repeat (LTR) is a well-characterized transcriptional regulatory element that contains DNA-binding domains for a broad range of transcription factors, including AP-1, NFAT, Sp1, and NF-κB (see Ref. 10 for a review). NF-κB and Sp1 have been demonstrated to be key factors in the stimulation of HIV replication, and viruses lacking binding sites for either factor display attenuated replicative capacity (11). The absence of active NF-κB in the nuclei of quiescent memory CD4 lymphocytes could play a key role in promoting proviral latency in this lymphocyte subset.

Study of the latently infected reservoir in vivo is greatly hampered by the fact that infected patients may contain only 105 to 106 latently infected CD4 memory T cells and that in their latent state these cells are virtually impossible to distinguish from uninfected CD4 memory T cells (12). We have employed a recently developed Jurkat T-cell model of postintegration HIV latency termed J-Lat (13). J-Lat T-cell clones are infected with full-length HIV proviruses and contain the Aequorea victoria green fluorescent protein (GFP) gene in lieu of Nef, thus permitting epifluorescence monitoring of viral transcriptional activity. Under basal conditions, little or no GFP expression is detected; however, transcriptional activation of the latent provirus leads to GFP expression, which can be detected at the single-cell level by flow cytometry. In contrast to other previously studied models of HIV latency where mutations have been detected in the HIV Tat gene or TAR element, the J-Lat cells contain wild-type Tat and TAR and appear to be highly representative of the latently infected cells present in vivo. Because multiple latently infected clones have been independently established, clone- and integration locus-specific phenotypes can be excluded.

Recent interest has focused on prostratin, a nontumorigenic phorbol ester, as a potential antagonist of HIV latency (14, 15).
Prostratin is derived from the Samoan plant *Homalanthus nutans* and has been used in traditional medicine to treat yellow fever and other conditions (16, 17). Early studies of prostratin demonstrated that this agent inhibits HIV replication in vitro at micromolar concentrations via down-regulation of the cellular HIV receptors CD4 and CXCR4 (14, 18). More recent studies have shown that prostratin also promotes transcriptional activation of latent HIV viruses in latently infected thymocytes present in the SCID-hu Thy/Liv model (15). Prostratin may also not elicit the broader and less desirable changes in cellular activation associated with IL-2 and OXT3 therapies (15) and thus may be clinically useful. Currently, the precise molecular basis of prostratin antagonism of HIV latency is unknown. We now describe a series of studies dissecting the mechanism of prostratin induction of latent HIV virus in J-Lat cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions—**J-Lat clones 6.3, 8.4, 9.2, and 10.6 and sorted peripheral blood lymphocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, and $\gamma$-glutamine. Cells were stimulated with 0.1–10 $\mu$g prostratin (LC Laboratories, 100 ng/ml TNF-$\alpha$ (R&D Systems), or 10 $\mu$g a-phorbol 12-myristate 13-acetate (PMA) in the presence or absence of $\mu$g ionomycin (Sigma) for 8 or 14 h as indicated. For PKC inhibition studies, cells were incubated with 10–1000 nM Go6983, 5 $\mu$M Go6976 (Calbiochem), 5 $\mu$M Go6850 (Biomol), or 100 nM TGR14687 (Sigma) for 1 h prior to cellular activation.

**Transfection and Luciferase Assays—**J-Lat 6.3 or 9.2 cells were expanded to 0.5 to 1 x 10^6 cells/ml and resuspended in serum-free RPMI at 4 x 10^3 cells/ml. 15 $\mu$g of expression vector DNA was added (adjusted with pCMV4 carrier DNA as necessary) to 0.4-mI aliquots of the cell suspension followed by electroporation (250 V, 950 microfarads in 0.4-cm cuvettes; Invitrogen) and resuspension in 6 ml of complete RPMI. Reporter plasmids containing the luciferase gene positioned immediately downstream of the full-length, ΔB, ΔAP-1, or ΔSp1 LTR of HIV-1 or transcription cassettes containing tandem copies of the NFAT, NF-κB, AP-1, or Sp1 enhancers. These reporter plasmids were cotransfected with 0.1 $\mu$g of Renuila luciferase expression vector (Promega) to monitor differences in transfection efficiency. 15–20 h after electroporation, cells were stimulated as indicated for 5 at 37°C. The dual luciferase assay system (Promega) was employed to measure resultant luciferase activity using a Wallac microbeta 1450 luminometer.

**Flow Cytometry Analysis and FACS—**J-Lat 6.3 or 9.2 cells were transfected with 2 $\mu$g of pMACS-Kk (H2Kk) and 13 $\mu$g of empty pCMV4-Kk or pCMV4-LTR-Sp1LTR expression vector DNA 48 h prior to stimulation. Transfected and nontransfected cells were stimulated at indicated times for 14 h at 37°C. Following incubation, cells were incubated with biotin-α-H2Kk antibody, washed, and stained with streptavidin–APC (Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). For intracellular anti-Gag immunostaining, J-Lat 9.2 cells were fixed in 4% paraformaldehyde and stained with anti-p24 RD1 (Coulter) antibody in buffer containing 0.1% Triton for permeabilization. Samples were washed once and resuspended in PBS prior to analysis. Compensation was performed with monocholored cultures. Data were analyzed with FloJo software (Treestar). For FACS sorting experiments, J-Lat 9.2 cells were incubated with 2 $\mu$g prostratin or 10 ng/ml TNF-$\alpha$ for 24 h, washed twice in complete medium, and incubated for an additional 24 h prior to FACS sorting on the FACSVantage. GFP-negative cells were collected and incubated in complete medium for 3 days, followed by incubation with either prostratin or TNF-$\alpha$ for 24 h. GFP expression was assessed by flow cytometry.

**Immunoblotting Analysis—**J-Lat 6.3 or 9.2 cells were adjusted to 1 x 10^6 cells/ml and stimulated with 10 ng/ml TNF-$\alpha$ or prostratin (10 $\mu$g/ml) on ice in egg lysis buffer (50 mM HEPS, pH 7.2, 250 mM NaCl, 1% Nonidet P-40, 5 mM EDTA) for 20 min and clarified by microcentrifugation. Lysates were next added to an equal volume of 2 x Laemmli buffer (25 mM Tris, 200 mM glycerine, 0.1% SDS) and heated to 95°C for 5 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with various antibodies.

**In Vitro Kinase Assay—**J-Lat 6.3 or 9.2 cells were adjusted to 2 x 10^6 per ml in serum-free medium and stimulated with 10 $\mu$g/ml prostratin, 10 ng/ml TNF-$\alpha$, or 10 $\mu$M PMA for various times. Cells were lysed in egg lysis buffer and immunoprecipitated with anti-NEMO antibodies to obtain IKK-containing signalosomes as previously described (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (19). In vitro kinase assays were performed as described utilizing glutathione S-transferase-LexB (1–62) as an added exogenous substrate. Reactions were terminated by the addition of an equal volume of 2 x Laemmli buffer. Reaction products were separated by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane, and exposing to Hyperfilm at 80°C. Following autoradiography, membranes were probed with anti-IKK3 antibodies (Santa Cruz Biotechnology) to determine the amounts of kinase immunoprecipitated in each sample.
flow cytometry. The percentage of cells activated is presented in the GFP-positive gate. Note that prostratin and TNF-α are capable of activating latent HIV in a dosedependent manner. J-Lat clone 9.2 cells were stimulated with 0.1–10 μM prostratin. The error bars indicate S.D. C, prostratin induction of GFP expression is correlated with intracellular expression of HIV Gag. J-Lat clone 9.2 cells were stimulated with medium containing carrier MeSO₂, TNF-α, or 10 μM prostratin and stained for intracellular Gag expression with anti-p24 monoclonal antibodies conjugated to phycoerythrin (bottom row). GFP expression is plotted on the x axis, and intracellular anti-p24-phycoerythrin immunofluorescence on the y axis. The staining pattern obtained indicates that the majority of GFP-positive cells also express the HIV late gene product Gag. A similar response was observed in other J-Lat clones. D, prostratin and TNF-α induce a variegated response in J-Lat T cells. Stimulated J-Lat 9.2 cells, which failed to express GFP in response to either prostratin or TNF-α, were isolated by FACS and subjected to a second round of stimulation with either prostratin or TNF-α. Levels of GFP expression induced in initially nonreactive J-Lat clones were measured by flow cytometry. Note that levels of GFP expression induced during the second round of stimulation in initially nonresponding cells are similar to that induced in previously unstimulated, nonsorted cells.

**RESULTS**

**Prostratin Induces HIV Gene Transcription in Latently Infected J-Lat T Cells**—To examine the effect of prostratin in a lymphocyte-based model of HIV postintegration latency, J-Lat clones 6.3, 8.4, 9.2, and 10.6 were incubated for 14 h with 10 μM prostratin, 10 ng/ml TNF-α, or 0.1% MeSO₂ as a control. In the absence of stimulation, J-Lat clones 6.3, 8.4, and 9.2 cells expressed virtually no GFP, whereas J-Lat 10.6 cells were ~4% positive, indicating low-level HIV gene expression (Fig. 1A, left column). Following treatment with prostratin, GFP expression was induced to various and characteristic degrees in each of the four J-Lat T cell lines (Fig. 1A, right column). Of note, a higher proportion of J-Lat 10.6 cells displayed GFP epifluorescence following prostratin stimulation. TNF-α activated GFP expression in each of the latently infected cells lines to levels comparable with that induced by prostratin. Short term (<24 h) prostratin treatment induced little or no toxicity as assessed by the forward and side light scattering properties of the treated cells (data not shown). Prolonged exposure to prostratin (>2 days), however, induced substantial growth arrest and cell death at concentrations greater than 500 nM (data not shown). To more precisely define the dose-response relationship for prostratin, J-Lat 9.2 cells were stimulated with 0.1–10 μM concentrations of the agonist. Prostratin induced latent HIV LTR-driven expression of GFP at concentrations as low as 0.1 μM, with minimal toxicity (Fig. 1B). When concentrations of prostratin higher than 10 μM were tested, increased short-term toxicity was observed (data not shown).

Because the GFP gene is substituted for the early expressed Nef gene of HIV-1 in the J-Lat model, we investigated whether prostratin also activated HIV late gene expression. Following 14 h of stimulation with prostratin or TNF-α, immunostaining for intracellular Gag expression revealed expression of this Rev-dependent late gene product in a majority of the GFP positive subset of cells (Fig. 1C). Together, these studies demonstrate that prostratin induces dose-dependent transcriptional activation of the latent HIV provirus present in four independently derived J-Lat cell lines. Further, prostratin stimulation leads to expression of both early and late HIV gene products. However, at high doses or after prolonged exposure, prostratin induces cytotoxic effects.

Both prostratin and TNF-α induce GFP expression in only a subset of the J-Lat cells. Given that the J-Lat cell lines are clonal, the nonuniform response of these lines to activating stimuli suggested a variegated response. However, it was unclear whether the unresponsive cells were permanently inactivated or able to undergo activation in a second round of stimulation. To distinguish between these possibilities, J-Lat 9.2 cells were incubated...
Prostratin Activates the HIV LTR through Induction of NF-κB. A, prostratin activates cB- and AP-1 luciferase reporter plasmids. J-Lat 9.2 cells were transfected with cB-, AP-1-, Sp1-, or NFAT-luciferase reporter plasmid DNA and stimulated with 2 μg prostratin, TNF-α, PMA, or a combination of PMA and ionomycin. Luciferase activity was measured 5 h later. The error bars indicate S.D. Note that prostratin activates the cB and AP-1 reporter plasmids but not the Sp1 or NFAT reporter plasmids. Similar results were obtained in J-Lat 6.3 cells. B, an HIV LTR lacking the cB enhancers is not induced by prostratin. J-Lat 9.2 cells were transfected with HIV1-LTR-, HIV1-LTRcB-, HIV1-LTRΔAP-1-, and HIV1-LTRΔSp1-luciferase reporter plasmid DNA and 14 h later were stimulated with 2 μg prostratin, TNF-α, PMA, or PMA/ionomycin. Luciferase activity was measured after 5 h of stimulation. The error bars depict S.D. Note the lack of prostratin activation of the HIV1-LTRΔcB reporter plasmid. Similar results were obtained in J-Lat 6.3 cells. C, prostratin induction of the HIV LTR is not inhibited by cyclosporin A. J-Lat 6.3 or 9.2 cells were preincubated with cyclosporin A for 1 h followed by stimulation with 2 μg prostratin, TNF-α, PMA, or PMA/ionomycin. Note that the prostratin response is not inhibited by cyclosporin A, whereas the PMA/ionomycin response is partially impaired, suggesting a component of NFAT-mediated stimulation under these conditions of activation.

Prostratin Activation of the HIV LTR Is Mediated through NF-κB—We next explored the signaling pathway activated by prostratin in J-Lat cells that mediates activation of the latent HIV proviruses. The HIV-1 LTR contains binding sites for several inducible transcription factors, including NF-κB, NFAT, AP-1, and Sp1 (10). To assess the effects of prostratin on the induction of these transcription factors, J-Lat 9.2 cells were transfected with NF-κB-, AP-1-, Sp1-, or NFAT-luciferase reporter plasmids and stimulated with either 2 μg prostratin, 10 ng/ml TNF-α, 10 μM PMA, or combinations of PMA and 1 μM ionomycin. Prostratin activated the NF-κB- and AP-1-responsive reporter constructs but failed to activate the Sp1 or NFAT luciferase reporters (Fig. 2A). The magnitude of the NF-κB response to prostratin was less than that obtained with PMA either added alone or in combination with ionomycin but was comparable with TNF-α.

To assess more directly the role of NF-κB/Rel factors in prostratin activation of the HIV LTR, J-Lat 9.2 cells were transfected with luciferase reporter plasmids containing either the wild type HIV-1 LTR, the LTR lacking the two cB enhancers, the LTR lacking the AP-1 enhancers, or the LTR lacking the Sp1 enhancers. Prostratin induced 4-fold stimulation of the HIV-LTRΔcB reporter relative to unstimulated controls (Fig. 2B) but failed to activate the HIV-LTRΔcB-Luc reporter. However, co-incubation with PMA and ionomycin stimulated luciferase activity with this ΔcB reporter, indicating the induction of a non-NF-κB/Rel transcription factor with LTR-activating properties. Additionally, prostratin induced ~2.5-fold stimulation of the HIV-LTRΔAP-1 and HIV-LTRΔSp1 reporters, indicating that neither AP-1 nor Sp1 is required for HIV LTR responsiveness to prostratin. Together, these findings support a central role for NF-κB/Rel induction in prostratin-mediated activation of the latent HIV LTR and exclude a necessary role of AP-1 and Sp1.

Because NFAT has been implicated in the activation of the LTR involving a site overlapping the cB sites (21), we further tested the potential involvement of NFAT as a mediator of prostratin antagonism of HIV latency by treating cells with cyclosporin A. This agent blocks the activation of NFAT by antagonizing calcineurin-mediated dephosphorylation of NFAT (22). J-Lat 6.3 or 9.2 cells were preincubated with cyclosporin A or with medium containing comparable quantities of Me2SO used to dissolve cyclosporin A and then stimulated with either prostratin or TNF-α, and the initially unresponsive cells were purified and stimulated in a second round with prostratin or TNF-α. In parallel, previously unstimulated, unsorted J-Lat 9.2 cells were stimulated with prostratin or TNF-α. The second round of either prostratin or TNF-α stimulation induced GFP expression in ~20% of originally nonresponsive cells, a fraction that was equivalent to that observed in the previously unstimulated cells (Fig. 1D). These findings indicate that an increasingly greater proportion of the entire J-Lat cell population is activated by serial stimulation, with the fractional response during each cycle remaining constant.
with prostratin, TNF-α, PMA, or combinations of PMA and ionomycin for 14 h, followed by assessment of GFP expression. Prostratin, TNF-α, and PMA each induced strong GFP responses, and these responses were not impaired by the addition of cyclosporin A (Fig. 2C). Conversely, combined stimulation of these cells with PMA and ionomycin induced higher levels of GFP expression than observed with PMA alone, and this response was partially inhibited by cyclosporin A. These findings suggest that NFAT induction plays a role in the prostratin response, J-Lat 6.3 or 9.2 cells were transfected with expression vectors encoding either an IκBαSR protein expression vector or vector alone, in combination with an H2Kk marker of transfection, followed by stimulation with 2 μM prostratin, TNF-α, PMA, or PMA/ionomycin. H2Kk-expressing cells were analyzed by flow cytometry for GFP expression. Note that the IκBαSR markedly inhibits prostratin-mediated activation of the latent provirus present in both J-Lat clones but only partially inhibits the response elicited by PMA and ionomycin.

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**FIG. 3.** Prostratin activation of the latent HIV LTR involves IKK activation and degradation of IκBα. A, prostratin stimulates activation of endogenous IKKs. J-Lat 6.3 cells were stimulated with TNF-α or 2 μM prostratin for 5, 10, or 30 min, followed by immunoprecipitation of IKK complexes with anti-IKK antibodies. These complexes were analyzed for enzymatic activity in *in vitro* kinase assays utilizing glutathione S-transferase-IκBα(1–62) as an exogenous substrate. Levels of IKK1 immunoprecipitated under each condition are shown in the lower panel. Note that both TNF-α and prostratin activate the IKK complexes, although the prostratin response occurs with slightly slower kinetics. Similar results were obtained with other J-Lat clones. B, prostratin induces IκBα degradation. J-Lat 6.3 cells were stimulated with TNF-α or 2 μM prostratin for 10, 15, or 30 min, and cellular lysates were immunoblotted with antibodies specific for IκBα or β-tubulin. Similar results were obtained with other J-Lat clones. C, IκBα super repressor (IκBαSR) inhibits prostratin activation of latent HIV provirus. J-Lat 6.3 or 9.2 cells were transfected with expression vectors encoding either an IκBαSR protein expression vector or vector alone, in combination with an H2Kk marker of transfection, followed by stimulation with 2 μM prostratin, TNF-α, PMA, or PMA/ionomycin. H2Kk-expressing cells were analyzed by flow cytometry for GFP expression. Note that the IκBαSR markedly inhibits prostratin-mediated activation of the latent provirus present in both J-Lat clones but only partially inhibits the response elicited by PMA and ionomycin.

Activated IKKs target the IκBα inhibitor of NF-κB for phosphorylation on serines 32 and 36, leading in turn to the rapid ubiquitylation and degradation of the inhibitor by the 26 S proteasome (24). To assess the functional consequence of prostratin induction of IKK kinase activity, we assessed the ability of prostratin to induce IκBα degradation in J-Lat 6.3 cells. Prostratin, like TNF-α and PMA, induced degradation of IκBα (Fig. 3B). However, consistent with the observed delay in IKK activation, the degradation of IκBα induced by prostratin at 30 min was less complete than that induced by TNF-α.

To further evaluate whether IκBα degradation plays a key role in the prostratin response, J-Lat 6.3 or 9.2 cells were transfected with expression vectors encoding a nondegradable mutant of IκBα (IκBα SS32/36, termed the IκBα super repressor, or IκBαSR) or with vector alone, along with a mouse MHC H2Kk surface antigen transfection marker plasmid (Fig. 3C).
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Cell surface immunofluorescence staining for H2Kk permitted rapid identification of the transfected subset of cells. Following transfection and culture for 24 h, cells were stimulated with prostratin, TNF-α, PMA, or a combination of PMA and ionomycin for 14 h. Cells expressing H2Kk were then analyzed for GFP expression indicative of latent HIV provirus activation. Prostratin, TNF-α, PMA, and PMA/ionomycin effectively induced latent HIV LTR-mediated GFP expression in the H2Kk-transfected cells. In contrast, cells transfected with IκBαSR failed to increase GFP expression in response to prostratin, TNF-α, or PMA. Consistent with dual induction of NF-κB and NFAT by combination of PMA and ionomycin, GFP expression in dually treated cells was only partially inhibited by IκBαSR. These findings provide further evidence that NF-κB activation plays an important role in prostratin-mediated activation of latent HIV proviruses in the J-Lat cells.

Prostratin Induces NF-κB Nuclear Translocation and DNA Binding—Following the degradation of IκBα, the liberated NF-κB transcription factors translocate into the nucleus and engage cognate κB enhancers. To assess whether prostratin stimulation provided sufficient stimulus for RelA nuclear translocation and DNA binding, we assessed κB enhancer DNA binding activity in nuclear extracts from J-Lat 6.3 cells stimulated with prostratin or TNF-α for 30 or 45 min. Extracts were incubated with [γ-32P] radiolabeled κB enhancer oligonucleotides. Parallel assays were performed with γ-32P-labeled Oct-1 DNA oligonucleotides to assess loading and integrity of the nuclear extracts. Unstimulated nuclear extracts contained κB enhancer binding activity consistent in migration with p50-p50 homodimers (Fig. 4A). Following prostratin and TNF-α stimulation, a more slowly migrating complex was observed. Based on antibody supershifting, this complex corresponds to the prototypical NF-κB complex of p50-RelA heterodimers bound to the κB enhancer (data not shown). Consistent with the observed slower kinetics of both IKK activation and IκBα degradation, prostratin induction of NF-κB DNA binding occurred somewhat slower than the response elicited by TNF-α.

Prostratin Induces Direct RelA Binding to the Integrated HIV LTR—NF-κB could either directly activate the LTR of the latent proviruses present in J-Lat cells by binding to the κB enhancer or indirectly by inducing the expression of a second gene product, which in turn drives LTR transcription. To investigate whether RelA is directly recruited to the HIV LTR in vivo following prostratin stimulation, chromatin immunoprecipitation assays were performed. J-Lat clones 6.3 and 9.2 were treated with TNF-α or prostratin for 30 or 45 min, respectively. The longer time course for prostratin was selected in view of its slower kinetics of induction. Following stimulation, cells were cross-linked, and DNA was fragmented by micrococcal nuclease digestion and sonication. Lysates were immunoprecipitated with anti-RelA or anti-Sp1 antibodies and agarose A beads or agarose A beads alone, and DNA-protein cross-links were reversed. Coimmunoprecipitated DNA was isolated and input, RelA-immunoprecipitated, and agarose A control samples were probed by PCR for HIV LTR κB binding site DNA and for downstream HIV and β-actin sequences as nonspecific controls. In the absence of stimulation, samples immunoprecipitated with anti-RelA amplified low to undetectable levels of HIV LTR κB binding site DNA (Fig. 4B). Following stimulation with TNF-α or prostratin, anti-RelA immunoprecipitated samples from both J-Lat clones 6.3 and 9.2 amplified significant quantities of HIV LTR DNA. Importantly, these samples did not amplify β-actin negative controls beyond background levels, demonstrating specificity of the DNA immunoprecipitation. In contrast, Sp1 was constitutively bound to the latent HIV LTR, with no observable change in occupancy occurring in response to TNF-α or prostratin stimulation. These findings support a model of prostratin action involving the induction and direct recruitment of NF-κB to the latent HIV LTR.

Prostratin Activation of the Latent LTR Requires Activity of Novel PKC Isoforms—Previous studies of prostratin induced down-regulation of CD4 and CXCR4 identified a PKC-dependent signaling step (18). However, it remained unclear whether single or multiple PKC isoforms were involved in this response. To characterize the profile of PKC isoforms induced by prostratin, we prepared cytoplasmic and membrane fractions from J-Lat 6.3 cells treated with prostratin, TNF-α, or PMA and analyzed which PKC isoforms were induced to translocate from the cytoplasm to the membrane. Such translocation serves as an early marker of PKC activation (25). Prostratin induced rapid translocation of PKC-δ and -β from the cytoplasm to the membrane fraction, with similar efficiency to PMA (Fig. 5A). Membrane translocation of PKC-α, -γ, -ζ, and -η was also induced by prostratin; however, this translocation response was incomplete and greatly reduced in comparison with the response induced by PMA. In contrast, TNF-α did not induce membrane translocation of any of these PKC isoforms. Immuno-
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Fig. 5. PKC activation and prostratin-mediated stimulation of latent HIV provirus. A, prostratin induces membrane translocation of multiple PKC isoforms. J-Lat 6.3 cells were treated with Me2SO (DMSO), TNF-α, 2 μM prostratin, or PMA for the indicated times. Cytoplasmic and membrane extracts were prepared, and proteins were separated by SDS-PAGE followed by immunoblotting with antibodies specific for various PKC isoforms. β-Tubulin was employed as a loading control for these extracts. Similar responses were observed in other J-Lat clones. B, PKCs participate in prostratin antagonism of HIV latency. J-Lat clones were incubated with a pan-PKC inhibitor, G6986, prior to stimulation with 2 μM prostratin, PMA, or TNF-α. Results for J-Lat 6.3 and 9.2 are shown. The percentage inhibition of total GFP expression was measured by flow cytometry. Note the inhibition of the prostratin and PMA responses with G6986 but no effect of this inhibitor on the TNF-α induced response. C, novel PKCs probably play a role in prostratin-mediated activation of latency. J-Lat 6.3 or 9.2 cells were incubated with medium, G6976 (an inhibitor of conventional PKC isoforms only), G6850 (an inhibitor of novel and conventional PKC isoforms), or TER14687 (an inhibitor the novel PKC isoform PKC-θ). Cells were stimulated with 2 μM prostratin, TNF-α, or PMA. The percentage inhibition of total GFP expression was measured by flow cytometry. Note that an inhibitor of conventional PKC isoforms failed to impair the prostratin or PMA response, whereas the inhibitor of conventional and novel PKC isoforms effectively inhibited these responses, and the inhibitor of PKC-θ failed to inhibit prostratin induction of GFP expression.

Prostratin Activates NF-κB and Induces NF-κB-dependent Gene Expression in Primary Memory CD4 Lymphocytes—The
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In this study, we have explored the mechanism by which the non-tumor-promoting phorbol ester, prostratin, antagonizes HIV latency. Considerable interest has recently focused on prostratin as an in vivo agent to purge latent HIV proviruses (6, 14, 15). As a cellular model of HIV latency, we employed the J-Lat T cells, which contain a full-length latent HIV provirus. This provirus contains GFP in place of Nef; thus, transcriptional activation of the latent provirus can be readily detected in individual cells by flow cytometry. We find that prostratin effectively activates the latent provirus in multiple independently derived J-Lat T-cell clones. These results are in agreement with recently published results demonstrating prostratin-mediated activation of latent virus in the SCID-HU Thy/Liv system and in the blood of patients with HAART-suppressed HIV (14, 15). We observed that prostratin induces similar levels of proviral gene transcription as TNF-α, although the kinetics of the prostratin-mediated response are slower. Intriguingly, neither prostratin nor TNF-α induced proviral reactivation in the entire clonal population of latently infected cells. Indeed, it appears that activation of the latent HIV LTR occurs in a variegated manner, a hallmark of epigenetic regulation. When initially nonresponsive cells were restimulated with prostratin or TNF-α, a similar fraction of previously unresponsive cells were induced to express GFP. The underlying mechanism of this fractional responsiveness may lie in heterochromatin epigenesis or alternatively could reflect selective responsiveness of cells at a particular stage in the cell cycle. These findings suggest that the reversal of HIV latency will probably require repeated administration of agonists. Thus, to be clinically useful, such inducers must exhibit relatively low toxicity, permitting patients to withstand long-term, multiround treatments (33).

Prostratin was observed to lack toxicity when applied for short time courses; however, it induced substantial growth arrest and cell death if administered in a concentration of >500 nM for greater than 2 days. If prostratin is to be considered as a human therapeutic, it is unlikely that high-dose or protracted treatment will be tolerated. Consequently, short-term, low-dose treatments will probably be the only alternative, since sustained administration will probably result in dramatically negative side effects.

To delineate the molecular basis for prostratin-mediated activation of the latent HIV LTR, we first examined the ability of prostratin to activate various signaling pathways using reporter plasmids containing κB, AP-1, Sp1, or NFAT enhancer cassettes. We observed that prostratin effectively activated the κB- and AP-1-luciferase reporters but displayed no stimulatory effects on the Sp1 or NFAT reporter constructs. Consistent with a central role for NF-κB stimulation, prostratin failed to induce a mutated version of the HIV LTR lacking the tandem κB enhancers, whereas it promoted expression of AP1- and Sp1-deficient LTR constructs. These findings are consistent with the prior observation that prostratin induces up-regulation of a number of NF-κB-responsive genes, including IL-1β, IL-8, and osteoprotegerin (14). The observed induction of AP1 by prostratin is probably reflective of the phorbol ester nature of this compound. It is possible that in addition to the NF-κB and AP-1 pathways, prostratin also stimulates other signaling cascades that either modulate the NF-κB response or function independently. The fact that the profile of gene expression induced by prostratin fails to completely overlap with that found with classical agonists of NF-κB like TNF-α strengthens this possibility.
Further investigation of the pathway of NF-κB activation stimulated by prostratin revealed that prostratin activates the IKKs residing in the macromolecular signaling complex. Notably, this response occurs with somewhat delayed kinetics compared with the response elicited by TNF-α. We further found that prostratin induces phosphorylation and degradation of IκBα and that expression of a degradation-resistant mutant of IκBα (IκBα super repressor) inhibits prostratin activation of the latent HIV LTR. These findings indicate that the classical NF-κB signaling pathway is stimulated by prostratin.

NFAT has also been proposed to function as a key transcriptional activator of the HIV-1 LTR (34, 35); however, the NFAT inhibitor cyclosporin A failed to impair prostratin-mediated activation of the latent HIV LTR. Of note, latent provirus was activated in a greater fraction of cells when agonists that stimulated both the NFAT and NF-κB pathways were employed. This observation is consistent with early studies demonstrating cooperative induction of the HIV LTR by NF-κB and NFAT (36). These results suggest that strategies to activate latent HIV proviruses involving the induction of both NF-κB and NFAT merit further investigation. Such approaches might also help mitigate the problem posed by the variegated nature of latent HIV provirus activation.

Since the NF-κB pathway is activated by prostratin, and HIV-1 LTR constructs lacking the κB enhancers are not stimulated by prostratin, it was important to confirm that Rel proteins were directly recruited to the HIV-1 LTR in vivo following prostratin stimulation. Using chromatin immunoprecipitation assays to study this question, we observed that prostratin stimulation promoted rapid recruitment of RelA to the HIV LTR. In contrast, we observed that Sp1 is constitutively associated with the latent HIV promoter. These findings argue for a direct role of RelA in the prostratin-induced transcriptional response leading to activation of the latent HIV LTR in cells stimulated with either prostratin or TNF-α. Additionally, these observations demonstrate that Sp1 binding is insufficient to promote transcriptional activation of the latent HIV LTR. In the setting of the HIV LTR, RelA synergizes with Sp1 to induce gene expression, probably involving chromatin remodeling steps (37). In the case of the A20 gene, Sp1 independently recruits TFIIID to the site of transcriptional initiation (38).

Nonetheless, RelA is required to promote efficient transcriptional elongation by the RNA polymerase II complex, probably through its ability to recruit the pTEFb complex that phosphorylates the C-terminal heptapeptide repeat of RNA polymerase II. Indeed, in the absence of Sp1, RelA is probably less effective in driving LTR transcription (39). Determining the full range of transcription factors bound to the transcriptionally repressed and activated LTR in vivo will be instrumental in enhancing our understanding of the molecular events that govern HIV latency. Additionally, analyses of the impact of transcription factor binding on localized chromatin structure may reveal insights into the molecular underpinnings of HIV latency. Such insights may also suggest additional strategies for activation of the latent LTR in a greater fraction of the cells.

Additional events occurring during the activation of NF-κB by prostratin may play an important role in the transcriptional activation of the latent HIV LTR. For example, recent studies have demonstrated that NF-κB activation by TNF-α results in the nuclear translocation of IKK1, which subsequently promotes phosphorylation of serine 10 on the tail of H3 histones surrounding NF-κB-regulated genes (40, 41). This post-translational modification by IKK1 may help to promote changes in chromatin structure associated with transcriptional activation. Whether IKK1 or other recognized serine 10 kinases like Rsk2 or MSK1 are similarly recruited to the latent HIV-1 LTR following prostratin stimulation is currently under investigation. Additionally, signal-dependent post-translational modification of RelA by phosphorylation and acetylation may also stimulate transcriptional activation of the repressed HIV LTR by enhancing the transcriptional potential of RelA or by impairing the inhibitory action of resynthesized IκBα (42).

Our studies have also shed light on the proximal signaling components involved in the prostratin response. Because prostratin is a nontumorigenic phorbol ester, we focused initial attention on prostratin-mediated activation of various PKC isoforms. When PKC translocation from the cytoplasm to the membrane was evaluated, we observed that conventional, novel, and atypical isoforms were effectively mobilized, indicating a broad response. However, translocation of PKC α, δ, and ζ by prostratin was attenuated relative to that induced by PMA. This differential activation of PKC isoforms may provide a mechanistic basis for the non-tumor-promoting nature of prostratin. The activation of PKCs appears necessary for prostratin induction of the latent HIV LTR, since the generalized PKC inhibitor Go6983 blocks prostratin induction of GFP expression in J-Lat cells. We additionally found that the treatment of the prostratin-stimulated cultures with an inhibitor that selectively blocks conventional PKC isoforms, Go6976, did not impair the response, indicating that conventional PKC isoforms are dispensable for this response. Conversely, the addition of Go6850, which inhibits both conventional and novel PKC isoforms, effectively inhibited the prostratin response. These findings suggest that the novel subfamily of PKC isoforms may play a key role in induction of the latent HIV LTR by prostratin. Whether prostratin signaling is dependent on a single novel PKC isoform or is rather mediated through multiple isoforms remains an open question. In this regard, the addition of inhibitor TERI4687, a selective PKC-δ inhibitor, failed to inhibit prostratin antagonism of latency. These findings raise the possibility of functional redundancy in the novel subset of PKCs.

Previous studies of prostratin antagonism of HIV latency have elucidated the ability of prostratin to stimulate p24 Gag production and release from HAART-suppressed human PBMCs or thymocytes present in a SCID-hu Thy/Liv experimental model (15). These systems contain heterogeneous populations of uninfected, actively infected, and latently infected cells, which complicate examination of the overall efficacy of candidate antilatency compounds. The clonal latency-infected J-Lat model has the advantage that changes at the single-cell level can be conveniently monitored by flow cytometry. Our studies in fact show that prostratin induces the transcriptional activation of the latent LTR only in a portion of the population. However, a second round of stimulation activates a response in a similar fraction of the previously unresponsive cells. These findings suggest that repeated rounds of stimulation may be required to purge the entire pool of latently infected cells. Differences in the chromatin environment immediately surrounding the HIV integration locus may play an important role in controlling this functional response as well as the basal and induced levels of transcription (43). HIV postintegration latency in infected patients is probably most stable within the resting memory population of CD4+ T-lymphocytes. To confirm that our observations within the continuously dividing J-Lat model are applicable to primary resting memory T cells, we analyzed NF-κB induction and its activation of a prototypical NF-κB-responsive gene within sorted primary CD4+ CD45RO+ T-lymphocytes. Our observations demonstrate that prostratin is capable of both activating NF-κB and driving NF-κB-dependent gene expression within this biologically relevant population of cells. Additionally, work...
by Kulkosky et al. (14, 44) has demonstrated that prostratin is capable of inducing outgrowth of latent viruses from the blood of HAART-suppressed patients. Taken together, the proliferating J-Lat model recapitulates many of the responses observed in primary resting memory T cells. Complete elimination of the latently infected reservoir in infected patients will undoubtedly be a difficult task. Efforts to eliminate this pool of latently infected cells with interleukin-2 and OKT3 antibodies yielded disappointing results (8, 9). For this lofty goal to be reached, more efficient and less toxic antagonists of HIV latency must be identified, and the variegated nature of the response must be successfully dealt with. Although prostratin is promising on some fronts, its cytotoxic properties may limit its utility in human use. The identification of truly effective antagonists of HIV latency could be propelled by a better understanding of the molecular basis of HIV latency. The J-Lat cells used in these studies provide a powerful cellular model for hypothesis testing and biochemical and molecular analysis. However, it is essential that any promising agents ultimately be vetted for their efficacy in primary CD4 T cells latently infected with HIV.

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