Granulin and Granulin Repeats Interact with the Tat-P-TEFb Complex and Inhibit Tat Transactivation*

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The cellular positive transcription elongation factor b (P-TEFb), containing cyclin T1 and cyclin-dependent kinase 9 (CDK9), interacts with the human immunodeficiency virus, type 1 (HIV-1) regulatory protein Tat to enable viral transcription and replication. Cyclin T1 is an unusually long cyclin and is engaged by cellular regulatory proteins. Previous studies showed that the granulin/epithelin precursor (GEP) binds the histidine-rich region of cyclin T1 and inhibits P-TEFb function. GEP is composed of repeats that vary in sequence and properties. GEP also binds directly to Tat. Here we show that GEP and some of its constituent granulin repeats can inhibit HIV-1 transcription via Tat without directly binding to cyclin T1. The interactions of granulins with Tat and cyclin T1 differ with respect to their binding sites and divalent cation requirements, and we identified granulin repeats that bind differentially to Tat and cyclin T1. Granulins DE and E bind Tat but do not interact directly with cyclin T1. These granulins are present in complexes with Tat and P-TEFb in which Tat forms a bridge between the cellular proteins. Granulins DE and E repress transcription from the HIV-1 LTR and gene expression from the viral genome, raising the possibility of developing granulin-based inhibitors of viral infection.

The granulins are emerging as multifunctional regulators of cell growth, development, wound repair, and transcription. Full-length granulin (granulin/epithelin precursor (GEP)) was first purified as a growth factor from conditioned tissue culture media (1, 2). GEP has been identified several times and is also known as PC-cell-derived growth factor, progranulin, proepithelin, and acrogranin. The murine teratoma PC cell line secreted granulin in the form of an 88-kDa glycoprotein, which acts as an autocrine growth factor. A 68-kDa form of granulin produced by rat liver cells is sufficient to support the growth in serum-free medium of 3T3 cells null for the type 1 insulin-like growth factor receptor. No other growth factor can by itself stimulate the growth of these cells (3). Granulin mRNA is prominent in several human cells and tissues (4–6). High levels of GEP expression are found in several human cancers, and its involvement in breast cancer has been examined in some detail (reviewed in Refs. 7–9). Its expression is correlated with tumorigenicity, and the proliferation of malignant human breast carcinoma cells is inhibited by GEP antisense RNA or anti-GEP antibodies. GEP can substitute for estradiol in stimulating DNA synthesis in breast cancer cells, rendering them estrogen-independent and tamoxifen-resistant (10). GEP also stimulates the growth of several other cell lines, including epithelial cells and rat adrenal gland pheochromocytoma PC12 cells (4). In normal tissues full-length granulin and individual granulins mediate the wound response and coordinate host defense and wound repair (11, 12).

GEP contains seven and a half repeats of a cysteine-rich motif \( (CX_{n\alpha}CC_{X_{\alpha}}CC_{X_{\alpha}}CCDX_{\alpha}HCPCX_{\alpha}C) \) in the order P-G-F-B-A-C-D-E, where A-G are full repeats and P is the half motif (Fig. 1A). The C-terminal region of the consensus sequence contains the conserved sequence CCDX_{\alpha}HCCP and is suggested to have a metal binding site and to be involved in regulatory function (13). Several of the repeat units have been isolated from tissue culture medium, blood, and urine as ~6-kDa peptides known as granulins (or epithelins) and are individually biologically active. In some systems they stimulate cell proliferation, whereas in others they inhibit mitosis (7). The GEP repeats are joined by short intergranulin linkers of diverse sequence, containing the cleavage site for the serine protease elastase, which is secreted in large quantities by neutrophils during infection and inflammation (12).

The mode of action of GEP is not well understood. Granulin binding sites have been demonstrated, although cell surface receptors have not yet been characterized (14, 15). Recently GEP was reported to interact with perlecan, a heparan sulfate proteoglycan found in basement membranes and cell surfaces. The perlecan-GEP interaction was suggested to modulate tumor growth (16). GEP leads to activation of the mitogen-activated protein kinase pathway and to stimulation of cyclin D1 protein expression. This can account for the cellular proliferation activity of granulin and its ability to replace estrogen in inducing the growth of breast cancer cells (17). More recently, we found that GEP can modulate transcription elongation by interacting with human cyclin T1, a component of positive transcription elongation factor b (P-TEFb) (18).

P-TEFb is a general transcription factor required for the production of mRNA by RNA polymerase II (19). In addition to cyclin T1, P-TEFb contains the cyclin-dependent kinase CDK9, which phosphorylates the C-terminal domain of the large subunit of polymerase II, thereby facilitating the transition of polymerase II into a productive elongation mode (reviewed in Ref. 20). P-TEFb, and cyclin T1 in particular, is a target for cellular and viral transcription regulators. For example, cyclin T1 binds the HIV-1
transcription activator Tat (21) and allows the generation of full-length rather than prematurely terminated RNA chains (19, 22, 23). Cyclin T1 is an exceptionally large cyclin with unique sequences in its C-terminal region (Fig. 1B) that interact with regulatory proteins (24–26). GEP binds specifically to the histidine-rich domain of cyclin T1 (18), which is also necessary for RNA polymerase II binding (27). GEP forms complexes with intact P-TEFb inside cells and represses transcription from the HIV-1 LTR, a promoter that is highly dependent on P-TEFb (18). The C-terminal region of granulin, GrnCDE, serves as a substrate for P-TEFb in kinase assays. Phosphorylation of a C-terminal domain peptide in vitro is inhibited when P-TEFb is bound to GST-GrnCDE, suggesting that granulin may act by repressing the phosphorylation of RNA polymerase II C-terminal domain by P-TEFb (18).

Remarkably, GEP also binds to the Tat proteins of HIV-1, HIV-2, and caprine arthritis encephalitis virus (18, 28, 29). HIV-1 Tat is synthesized as a protein of 72–101 amino acids (aa), depending on the viral strain and alternate splicing of its mRNA. The one-exon 72-aa form, which is sufficient to activate transcription from the HIV-1 promoter (30, 31), is composed of five functional domains (Fig. 1C). GEP interacts with the cysteine-rich domain of Tat, which forms part of the activation domain required for binding to P-TEFb and for transactivation. These data raised the possibility that granulins could inhibit HIV-1 transcription by binding the Tat activation domain.

Previous work indicated that granulins can inhibit the HIV-1 promoter via tethered cyclin T1 in the absence of Tat (18). In the present study, we aimed first to determine whether granulins can serve as specific inhibitors of Tat transactivation via direct interactions with Tat. We found that the interaction of granulin with Tat can be sufficient for transcriptional repression and that the interactions of GEP with cyclin T1 and Tat are separable. Granulins inhibited Tat transactivation mediated by truncated cyclin T1 that lacks the histidine-rich domain essential for binding granulin. The interactions of granulins with Tat and cyclin T1 are distinguishable with respect to their requirement for zinc ions, and distinct Tat sequences are involved in interactions with granulin and cyclin T1. Next, we assessed the contribution of specific granulins to Tat and cyclin T1 binding and to the inhibition of expression from the HIV-1 promoter. We found that granulins DE and E interact differentially with cyclin T1 and Tat: these granulins bind to Tat in cell extracts and in living cells but do not interact directly with cyclin T1. Granulins DE and E repressed transcription from the HIV-1 LTR and gene expression from the viral genome and are found in complexes with Tat and P-TEFb. Thus granulins DE and E are specific inhibitors of the Tat-P-TEFb complex and of viral gene expression.

**Experimental Procedures**

**Cell Lines and Culture**—Adherent 293T, 3T3, and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Jurkat cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 2 mM l-glutamine and 10% fetal bovine serum (Sigma-Aldrich).

**Plasmids and Plasmid Construction**—The plasmids pGBT9 and pGAD GH and the yeast strain CG1945 for yeast two-hybrid system experiments were from Clontech. GrnCDE (aa 342–593) and GEP (aa 1–593) were subcloned into pGBT9. Construction of cyclin T1 full-length and truncated versions and CDK9 and HIV-1 Tat expression vectors was described previously (18). GrnCDE, GrnPGFBA (aa 18–341), GrnCD (aa 342–498), GrnDE (aa 442–593), GrnD (aa 442–498), and GrnE (aa 499–593) were cloned into the bacterial expression vector pGEX-4T-3 (Amersham Biosciences), into the mammalian expression vector pFLAG-CMV-2 (Sigma), and into the yeast vector pGAD GH. The plasmid pcDNA3-PCDGF (encoding full-length human granulin) for expression in mammalian cells, the yeast vector pCEN.PK2-HIS3, and the bacterial expression vector pGEX-4T-3 (Amersham Biosciences) were constructed by subcloning the corresponding PCR-amplified sequences from the GST-Tat72 vector (AIDS Research and Reference Reagents Program (National Institutes of Health)) into the GST-containing vector pGEX-4T-3. GST-Tat (1–101) was from K. T. Jeang (30). The HIV-1 molecular clone pNL4–3–LucE in which part of the nef gene is replaced by firefly luciferase and the envelope gene is replaced by GFP, obtained from Baltimore (37). All constructs used in this study were confirmed by sequencing.

**Yeast Two-hybrid Interactions**—Protein interactions were studied in yeast using the MATCHMAKER GAL4 system (Clontech) as described in Hoque et al. (18). The β-galactosidase liquid quantification assay was performed according to the manufacturer’s instructions.

**Expression of GST Proteins—**Escherichia coli strain BL21(DE3) was transformed with recombinant GST fusion protein-expressing plasmids. Overnight cultures (grown at 37 °C) were diluted 100-fold in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin and grown for 3–4 h until the A_{600} reached 0.6. Cells were induced with 0.1 mM isopropyl 1-thio-D-galactopyranoside and grown for an additional 4 h at 30 °C. Bacterial pellets were washed twice with phosphate-buffered saline, and the cells were lysed in 5 volumes of EBCD buffer (50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol) containing 2 mg/ml of lysozyme, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin A, and antipain. After sonication and 20-min centrifugation (13,000 rpm, 4 °C), the clear supernatants were collected and stored at –80 °C.

**In Vitro Binding Assay**—GrnCDE and GrnPGFBA were synthesized using the in vitro Tnt quick-coupled transcription and translation system (Promega). GrnDE, GrnD, and GrnE were synthesized using the coupled wheat germ extract system (TnT, Promega). In vitro synthesized [35S]Met-GrnCDE or [35S]Met-PGFBA (10 μl of reactions) were mixed with ~2 μg of GST or GST fusion proteins, bound to glutathione-Sepharose beads, and incubated for 2 h at 4 °C with gentle rocking. To test the effect of different metal ions, various concentrations of the specified salts were added to the binding buffer. The beads were washed extensively in EBCD plus 0.03% SDS. Bound proteins were released by heating to 100 °C in Laemmli sample buffer and resolved in 12% polyacrylamide-SDS gels. [35S]Met-labeled proteins were detected by autoradiography.

**Preparation of Whole Cell Extracts**—Cells were grown to 100% confluency in 100-mm tissue culture dishes, or in suspension to a density of 1 × 10^6 cells/ml, and washed twice with 10 ml of phosphate-buffered...
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RESULTS

Granulin Binding to Tat Is Sufficient for Inhibition of Tat Transactivation—Full-length granulin, as well as its N-terminal (GrnPGFBA) and C-terminal (GrnCDE) regions, inhibit Tat transactivation in mouse 3T3 cells supplemented with human cyclin T1 (18). Because granulin interacts with both cyclin T1 and Tat, the inhibition could be mediated by the binding of granulin to either or both of these transcription factors. Experiments with tethered cyclin T1 showed that granulin can inhibit expression from the HIV-1 promoter in the absence of Tat, i.e. via a granulin-cyclin T1 interaction (18). To determine whether the direct interaction between granulin and cyclin T1 is necessary for inhibition, we employed a truncated form of cyclin T1 (aa 1–333) that does not bind granulin in yeast two-hybrid or GST pull-down assays (18). The N-terminal region of human cyclin T1, containing its cyclin domain and TRM (Tat: TAR recognition motif), is sufficient to permit Tat transactivation in 3T3 cells (33, 39).

As shown in Fig. 2A, Tat transactivation in 3T3 cells was greatly enhanced by either full-length human cyclin T1 (1–726) or human cyclin T1 (1–333). In both cases, GrnPGFBA and GrnCDE inhibited Tat transactivation by 3- to 6-fold. Similar results were obtained with another cyclin T1 truncation (1–397; data not shown). Thus, inhibition of Tat transactivation by granulin can occur in the absence of the granulin binding site on human cyclin T1. We note that the inhibition was weaker in the absence of the granulin binding site in the cyclin T1 C terminus. Similarly, inhibition was weaker in the absence of Tat, when cyclin T1 was tethered to the promoter (Ref. 18, and see Fig. 7C below), suggesting that granulin may preferentially target the Tat-P-TEFb complex.

To verify that the truncated cyclin T1 binds to Cdk9 but not to Gcn5, we conducted co-immunoprecipitation experiments using transfected 293T cell extracts (Fig. 2B). Both the endogenous cyclin T1 and HA-tagged truncated cyclin T1 (1–333) were co-precipitated with anti-Cdk9 antibody (lanes 1 and 6), demonstrating that the truncated protein retains the ability to form P-TEFb complexes. As expected, endogenous cyclin T1 was co-precipitated with FLAG-tagged Gcn5 both in the absence (lane 4) (18) and the presence of transfected cyclin T1 (1–333) (lane 5). In contrast, the HA-tagged truncated cyclin T1 (1–333) failed to co-precipitate with FLAG-Gcn5 (lane 10). These results indicate that the interaction...
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Some Granulin Repeats Interact Differentially with Cyclin T1 and Tat—Because the interactions of Tat with granulin and with cyclin T1 are not identical, although they involve overlapping Tat sequences, we reasoned that individual regions of granulin may bind differentially to the two transcription factors. All full-length granulin repeats contain a conserved symmetrical 12 cysteine-rich motif except for the G repeat, which contains only 10 cysteines (see the introduction). Otherwise, there is considerable sequence variation among the repeats as detailed under “Discussion.” To determine the binding specificity of granulin repeats to cyclin T1 and Tat, we constructed two di-repeats (CD and DE), and three single repeats (C, D, and E), and examined their interactions with cyclin T1 and Tat.

First we tested the interactions in the yeast two-hybrid system by monitoring growth on selective medium and β-galactosidase activity (Fig. 4). As shown previously, GrnPGFBA and GrnCDE interacted with both cyclin T1 and Tat (18). GrnCD also interacted with both cyclin T1 and Tat. GrnC and GrnD did not interact detectably with either Tat or cyclin T1. Strikingly, GrnDE and GrnE interacted selectively with Tat but not with cyclin T1 compared with the control of vectors alone.

We confirmed the selective interaction of GrnDE and GrnE with Tat but not cyclin T1 in co-immunoprecipitation assays. Human 293T cells were transfected with various FLAG-tagged granulin constructs and anti-FLAG immunoprecipitates were probed for the presence of endogenous cyclin T1. GrnDE and GrnE, as well as GrnD (not shown), failed to co-immunoprecipitate with endogenous cyclin T1 (Fig. 5A). GrnDE and GrnE, but not GrnD, co-immunoprecipitated HA-tagged Tat (Fig. 5B).
The binding of GrnCD to cyclin T1 and Tat, observed in the yeast two-hybrid assay, could not be confirmed by co-immunoprecipitation because of background problems, but transactivation data support the existence of these interactions in mammalian cells (see Fig. 7 below). To determine whether the interactions are direct, we tested the binding between Tat and granulins in a GST pull-down assay. The granulins were labeled with 35S by in vitro synthesis in a wheat germ system. GrnDE and GrnE, but not GrnD, interacted with GST-Tat72 (Fig. 5C).

Finally, to document the specific interactions between GrnDE and Tat in living cells, we examined their intracellular co-localization. Previously we observed that GrnCDE co-localized in the nucleus of transfected COS7 cells with cyclin T1, but GrnDE did not (18). Like GrnCDE, GrnDE was predominantly detected in the cytoplasm in the absence of Tat, but co-localized with Tat in the nucleoli (Fig. 6). Thus, although the major N- and C-terminal segments of the granulin molecule (GrnPGFBA and GrnCDE) both bind cyclin T1 and Tat, differential specificity was observed within GrnCDE. Tat binding is a property shared by GrnCD, GrnDE, and GrnE, but only the GrnCD di-repeat is able to bind cyclin T1.

**Granulins DE and E Inhibit Tat Transactivation and Viral Gene Expression**—The transfection experiments of Fig. 2 showed that granulins can inhibit expression from the HIV-1 LTR without binding cyclin T1, implying that the granulin-Tat interaction is also inhibitory. Having found that GrnDE and GrnE cannot directly bind cyclin T1 but can bind Tat, we were able to test this inference by asking whether these granulins are able to repress the HIV-1 promoter.

Vectors expressing GrnDE and GrnE and other granulin constructs were co-transfected into 3T3 cells with human cyclin T1 and an HIV-1 LTR-luciferase reporter. Firefly luciferase expression was normalized to expression of Renilla luciferase from the RSV promoter. GrnDE and GrnE reduced gene expression by 4- to 6-fold, comparable to the effects of GrnCD and GrnCDE, which interact with both Tat and cyclin T1 (Fig. 7A).

GrnD, which does not interact with either Tat or cyclin T1, did not inhibit to a significant degree. Thus interaction of GrnDE and GrnE with Tat, or perhaps with the Tat-P-TEFb complex, is sufficient for inhibition of transcription from the HIV-1 promoter. We note that GrnDE and GrnE, despite their relatively low expression (Figs. 5B and 7A), repress Tat transactivation to an extent comparable to that of the other inhibitory granulins. The granulins PGFBA, CDE, DE, and E, but not GrnD, were also inhibitory toward luciferase expression from a transfected noninfectious HIV-1 molecular clone (Fig. 7B).

We showed previously that full-length granulin and GrnCDE inhibit reporter gene expression by about 2-fold when the Gal4-cyclin T1 fusion protein is tethered to a modified HIV-1 promoter via Gal4 binding sites (18). GrnPGFBA, which also interacts with cyclin T1, gave a similar inhibition, but GrnDE did not cause a significant inhibition in this tethered system (Fig. 7C). These
results are consistent with a model in which GrnDE and GrnE inhibit expression from the HIV-1 LTR via interaction with Tat.

**GrnDE and GrnE Interact with the Tat\(\text{His}18528\)P-TEFb Complex—**

Results presented above (see Fig. 2) suggested that granulin targets the Tat\(\text{His}18528\)P-TEFb complex, which is the modulator of HIV-1 transcription. Therefore, we sought to determine whether granulin interacts with this complex in cells. The inability of GrnDE and GrnE to bind directly to cyclin T1 (Figs. 4 and 5A) allowed us to determine whether Tat can form a bridge between these proteins by forming ternary complexes containing Tat, P-TEFb, and granulin. To this end, 293T cells were co-transfected with GrnDE (or GrnE) and HA-Tat. GrnDE (or GrnE) immunoprecipitates should contain cyclin T1 if, and only if, P-TEFb is complexed with Tat bound to granulin. Fig. 8A shows that FLAG-tagged GrnDE and GrnE precipitated with endogenous cyclin T1 in the presence of HA-Tat. GrnD, which does not bind to cyclin T1 or Tat, served as a negative control and GrnPGFBA and GrnCDE, which bind both cyclin T1 or Tat, were positive controls. CDK9 was also detected in the immunoprecipitated complex containing GrnDE and HA-Tat (Fig. 8B), confirming that these complexes contain both subunits of P-TEFb. Thus, Tat can bridge between P-TEFb and GrnDE (or GrnE).

This finding was confirmed using a Tat mutant, TatK41A, that fails to interact with cyclin T1 (21, 41) but still binds to granulin (see Fig. 8D). Cells were co-transfected with wild-type or mutant HA-Tat together with FLAG-tagged GrnCDE or GrnDE. Immunoprecipitates with anti-FLAG antibody were probed for the presence of endogenous cyclin T1 and HA-Tat. Fig. 8C shows that the mutant Tat bound both granulins (top panel). Cyclin T1, which co-precipitated with GrnDE in the presence of wild-type Tat (Fig. 8, A and C), was absent from complexes containing GrnDE and Tat K41A (second panel). Thus, when the Tat-cyclin T1 interaction is abrogated by the mutation, ternary complexes containing granulin DE, P-TEFb, and Tat are not observed. Cyclin T1 was also present in com-

**Fig. 6.** **GrnCDE and GrnDE co-localize with Tat in the nucleus.** A, COS7 cells were transfected singly with a vector expressing HIV-1 Tat fused to the RFP fluorescent tag (red) or GrnCDE or GrnDE fused to the EGFP fluorescent tag (green), as indicated. Single fluorescence, red or green, is shown in each picture in the upper panel. DAPI staining of the cell nuclei is shown in the lower panel. B and C, cells were co-transfected with the plasmids indicated. Green, red, and dual fluorescence are shown from left to right. Yellow color signifies co-localization of the two proteins.
plexes formed with GrnCDE regardless of the Tat mutation (Fig. 8, A and C), as a result of the direct binding of GrnCDE to cyclin T1 (Figs. 4 and 5A). The existence of ternary complexes

FIG. 7. GrnDE and GrnE inhibit Tat transactivation and gene expression from HIV-1 molecular clone. A, NIH 3T3 cells were co-transfected with 1.5 μg of vector expressing equivalent amounts of the FLAG-granulins specified, together with 20 ng of RSV-Tat2, 100 ng of FLAG-cyclin T1 (T1), and 100 ng of the reporter plasmids pLTR-luciferase (firefly) and pRSV-luciferase (Renilla) as indicated. Cell extracts were assayed for firefly and Renilla luciferase activities. Results are expressed as -fold activation of firefly luciferase activity normalized to Renilla luciferase activity and relative to the control lacking Tat. Data are the average of three experiments in duplicate with standard deviations shown. The inset panel shows expression of granulins PG-FBA, CDE, CD, and DE, detected by immunoblotting. Granulins D and E, which are expressed at low levels in 293T cells (Fig. 5B), were undetectable by immunoblotting of 3T3 cell extracts. B, Jurkat cells were co-transfected with 1 μg of HIV-1 molecular clone, 1 μg of pRSV-luciferase (Renilla), and 10 μg or equivalent amounts of the granulin plasmids specified. Firefly luciferase expression from the molecular clone was normalized to Renilla luciferase activity. Data are the average of three experiments in duplicate with standard deviations shown. C, NIH 3T3 cells were co-transfected with 0.25 μg of Gal4-cyclin T1, 100 ng of GS-HIV-luciferase (firefly) reporter plasmid, and 1.5 μg or equivalent amounts of the specified FLAG-granulins. Results are expressed as -fold activation of firefly luciferase relative to the control lacking Gal4-cyclin T1. Data are normalized to protein levels as some granulins caused a small decrease (~30%) in the expression of Renilla luciferase from the RSV promoter. Data are the average of two experiments in duplicate with standard deviations shown.

FIG. 8. Granulins E and DE bind to the Tat-P-TEFb complex in vivo. A, extracts of 293T cells co-transfected with plasmids expressing HA-Tat and the FLAG-granulins indicated were immunoprecipitated with anti-FLAG antibody. Immunocomplexes were analyzed by immunoblotting using anti-cyclin T1 antibody. Input was 293T whole cell extract (WCE). B, same as in A, except that immunocomplexes were analyzed by immunoblotting using anti-CDK9 antibody. C, human 293T cells were co-transfected with plasmids expressing wild-type (WT) or mutant Tat (HA-Tat WT or HA-Tat K41A) and FLAG-GrnCDE or FLAG-GrnDE as indicated. Complexes containing the FLAG-granulins were immunoprecipitated with anti-FLAG antibody and examined by immunoblotting using anti-Cyclin T1 antibody (second panel) or anti-cyclin T1 antibody (second panel). The expression of HA-Tat and FLAG-GrnCDE or FLAG-GrnDE in the cells was detected by direct immunoblotting of WCE with the corresponding antibodies (bottom two panels). D, human 293T cells were co-transfected with vectors expressing FLAG-GrnCDE and HA-Tat WT or the HA-Tat mutants C30G and K41A. Complexes containing the HA-Tat proteins were immunoprecipitated with anti-HA antibody and examined by immunoblotting using anti-FLAG (GrnCDE), anti-cyclin T1, and anti-HA (Tat) antibodies as indicated.
containing granulins DE or E, Tat and P-TEFb, is consistent with the ability of these granulins to inhibit HIV-1 transactivation by targeting the Tat-P-TEFb complex.

Lastly, we asked whether granulin can bridge between Tat and P-TEFb. Like TatK41A, TatC30G does not significantly interact with cyclin T1 (35), but neither mutation interfered with the binding of Tat to granulin. Cells were co-transfected with constructs expressing FLAG-GrnCDE and HA-tagged Tat wild-type and mutants. Tat-containing complexes were immunoprecipitated with anti-HA antibody and probed with anti-FLAG antibody. GrnCDE communoprecipitated with Tat C30G and TatK41A, indicating that the point mutations allow interaction with granulin (Fig. 8D, top panel, lanes 4 and 5). Cyclin T1 was not detected, however, suggesting that GrnCDE cannot bridge between Tat and cyclin T1 (Fig. 8D, middle panel, lanes 4 and 5). The amount of GrnCDE complexed with each of the mutant Tat proteins was considerably greater than that with wild-type Tat (Fig. 8D, top panel, lane 3), implying that GrnCDE and cyclin T1 compete for binding to wild-type Tat. This competition could also contribute to the inhibition of Tat transactivation.

**DISCUSSION**

Granulin interacts with both cyclin T1 (18) and HIV-1 Tat (18, 28) and inhibits the P-TEFb-dependent activation of the HIV promoter (18). This inhibition can occur via cyclin T1 in the absence of Tat (18). We show here that granulin binding to Tat can also be sufficient for inhibition of Tat transactivation. Furthermore, we discovered that two granulins, GrnDE and GrnE, bind to Tat but not to cyclin T1, and inhibit Tat transactivation and viral gene expression from the HIV-1 molecular clone. As discussed below, granulin E contains specific amino acids that are unique and conserved.

**Specificity of Granulin Interactions with Tat and Cyclin T1**—The activation domain of Tat (aa 1–48), which is sufficient for binding to cyclin T1 (21), also binds granulins (18). The interactions of Tat with the two proteins are different, however. First, the acidic domain of Tat (aa 1–21) is necessary for binding to cyclin T1 but not to granulin (Fig. 3B). Second, the cysteine-rich region of Tat is sufficient for binding to granulin but not to cyclin T1 (Fig. 3B). Third, specific point mutations in the cysteine-rich and core domains of Tat that abrogate its interaction with cyclin T1 do not affect its binding to granulins (Fig. 8D). Fourth, Tat interactions with cyclin T1 are zinc-dependent (38, 39), whereas zinc has no effect on Tat binding to granulins (Fig. 3C). On the other hand, as predicted (18), zinc greatly enhances the binding of granulins to full-length cyclin T1 or its His-rich domain (Fig. 3, D and E). These differences suggest that different surfaces of Tat are involved in its interactions with cyclin T1 and granulin, allowing it to bind simultaneously to both cellular proteins. Substitution of three cysteine residues in the cysteine-rich region of HIV-1 Tat had no discernible effect on granulin binding (Fig. 8D). In contrast, the substitution of each of three cysteines of caprine arthritis encephalitis virus Tat with serine residues results in loss of granulin binding activity (29). The cysteine-rich region of HIV-1 Tat contains 7 cysteines so we speculate that a minimum of three may be required for the Tat-granulin interaction.

Comparison of the interactions between various granulin repeats and cyclin T1 or Tat revealed additional specificities: several granulin repeats interact with both Tat and cyclin T1 (namely GrnCDE, GrnPFGFBA, and GrnCD), but GrnE and GrnDE interact only with Tat. GrnC and GrnD did not bind to either Tat or cyclin T1. Because GrnD alone does not bind to Tat, the Tat interaction with GrnDE is probably via GrnE (Figs. 4 and 5). Based on their shared 12-cysteine motif sequence, all granulins are predicted to form the same three-dimensional structure, a novel super-helix consisting of four stacked β-hairpins linked by an axial rod containing six intramolecular disulfide bridges (13). The findings that Tat binds to GrnE but not to GrnD or GrnC (this report), or to GrnB or GrnA (28), indicate that, in addition to the general structure of the granulin motif, one or more specific structural features that are present in GrnE are responsible for its interaction with Tat (see below). None of the single granulins tested (C, D, and E) interacted with cyclin T1, suggesting that more than one repeat is required for this interaction. Although the di-repeat GrnCD bound to cyclin T1, GrnDE did not, arguing that the general structure of the di-repeat is not sufficient per se for the interaction with cyclin T1. Presumably, specific residues and structural features to be defined are responsible for the binding differences observed.

**Ternary Complex Formation and Inhibition of HIV-1 Gene Expression**—The existence of multiple dual interactions among the three proteins, granulin, Tat and cyclin T1, raised the possibility that granulin can form a ternary complex with both Tat and cyclin T1 as well as binary complexes with either cyclin T1 or Tat alone. Taking advantage of the inability of GrnDE and GrnE to bind cyclin T1, we identified a ternary complex in which Tat acts as a bridge between the cyclin T1 and granulin components (Fig. 8).

The co-localization of Tat with GrnCDE and GrnDE (Fig. 6) demonstrates that these proteins interact in cells and that Tat can recruit these granulins to the nucleus and nucleolus. Granulins can inhibit HIV-1 transcription by binding to Tat, as evidenced here by experiments using cyclin T1 deletions that cannot bind to granulin (Fig. 2) and experiments using granulins DE and E that cannot bind full-length cyclin T1 (Figs. 4, 5, and 7). In principle, the mechanism of transcription inhibition via Tat binding could involve Tat sequestration and/or the formation of an inhibitory ternary complex containing P-TEFb, Tat, and granulin. Our data suggest that the dominant interaction is between Tat and P-TEFb. In pull-down experiments, P-TEFb binds more avidly to GST-Tat than to GST-granulin (18), and much less granulin is co-immunoprecipitated with Tat when the Tat is also able to bind P-TEFb (Fig. 8). Thus, Tat sequestration is not likely to be the predominant mechanism of suppression.

The transcriptional inhibition extends to expression from the HIV-1 molecular clone and displays specificity in terms of both the granulin effector and the target promoter. GrnD, which does not bind Tat or cyclin T1, had no significant effect on gene expression from the HIV LTR or molecular clone (Fig. 7). Furthermore, in the tethered system, granulins that bind to cyclin T1 (GrnPFGFBA and GrnCDE) inhibited cyclin T1-dependent transcription from the LTR whereas GrnDE had little effect (Fig. 7). GrnDE and GrnE are not toxic to cells and had no effect on transcription from the cellular PCNA promoter.\(^2\)

**Granulin Repeat Comparisons**—To discern unique features in GrnE, we aligned the sequences of the human granulins and looked for conserved amino acids (shown shaded in Fig. 9A). In addition to the extended cysteine-rich motif cited above, all the granulins share a conserved threonine residue immediately before the first double cysteine. The alignment reveals differences between granulin E and the other granulins, for example at positions 8 and 10 of GrnE. In granulin E positions 8 and 10 are occupied by phenylalanine and histidine, respectively, whereas the other granulins have serine and proline in these positions. Although the overall sequence identity among E granulins of human, mouse, and rat is 72%, phenylalanine and histidine are fully conserved at positions 8 and 10 (Fig. 9B).

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\(^2\) M. Hoque, M. B. Mathews, and T. Pe’ery, manuscript in preparation.
Inspection of the sequence logos in Fig. 9C reveals the existence of additional amino acids that are unique to the E granulins, as emphasized by the large dominant letters in the logo. It is also apparent that the C-terminal region of the E granulins is rich in basic amino acids compared with the other granulins. The non-E granulins share several distinct common features, such as the proline in position 10, although serine in position 5 is not highly conserved.

It is well established that the HIV-1 host range can be determined by single amino acid changes in cellular proteins that interact with viral components (42). A classic example lies in the interactions of HIV-1 Tat and TAR with cyclin T1, which is essential for the formation of the ternary complex Tat/TAR with cyclin T1, and that human GrnE sequences, displayed in the same format. C, sequence logos for GrnE of human, mouse, and rat (top) and all other granulin motifs (bottom) were created by WebLogo (available at weblogo.berkeley.edu).

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