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Upregulation of the Apoptosis-Associated Protein Grb3-3 in HIV-1-Infected Human CD4$^+$ Lymphocytes

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The mechanism(s) by which HIV-1 infection contributes to depletion of CD4$^+$ T cell is not well understood. In this report, we investigated whether a recently identified isoform of growth factor receptor bound protein (Grb2), named Grb3-3, a signaling molecule that is associated with the MAP kinase pathway and with apoptosis could be involved. We find that Grb3-3 is markedly up-regulated following HIV-1 infection of CD4$^+$ peripheral blood mononuclear cells undergoing apoptosis. Although IL-2 deprived CD4$^+$ cells also undergo apoptosis to a similar extent, Grb3-3 upregulation is not detected under these experimental conditions. Transient overexpression of Grb3-3 in Jurkat T-cells also causes apoptosis. Upon staurosporine stimulation, Grb3-3 predisposes Sup-T1 cell to apoptosis. Finally, analysis of the HIV-1 genes responsible for Grb3-3 expression demonstrates that Tat and Nef can independently induces its expression, suggesting these two earliest viral gene products of HIV-1 may share some common pathway(s) in up-regulating Grb3-3 expression.

Key Words: **apoptosis; CD4$^+$ T cell; Grb3-3; HIV-1.**

The fundamental issue of how HIV-1 infection results in depletion of CD4$^+$ cells is yet to be fully understood. Several mechanisms have been proposed to explain the loss and functional defects of CD4$^+$ T lymphocytes from HIV-1-infected individuals. Direct HIV-1-mediated cytopathic effects include syncytia formation and single-cell killing, which appear to be viral envelope glycoprotein-dependent (1–3). Other mechanisms may involve immunologic elimination of HIV-1 infected CD4$^+$ T lymphocytes by HIV-1-specific cytotoxic lymphocytes (CTL) or by nonspecific cytotoxic mechanisms (4–5). There are also several lines of evidence suggesting that apoptosis may play an important role in depletion of CD4$^+$ T cells (6–7).

While a variety of HIV-1 proteins have been found to cause apoptosis in T cells, the mechanism(s) involved in apoptosis of HIV-1-infected CD4$^+$ T lymphocytes remains largely unclear, especially the role of host signaling molecules that may be altered in level of expression and/or activity following HIV-1 infection. Since signaling molecules in the MAP kinase pathway are known to be activated following HIV-1-infection (8–11), we investigated whether a recently identified isoform of growth factor receptor bound protein (Grb2), named Grb3-3, a putative signaling molecule that is associated with the MAP kinase pathway and with apoptosis could be involved (12–13).

Grb3-3 was initially identified as an isoform of Grb2 (12, 14), an adapter protein that is known to participate in a variety of signal transduction pathways including MAPK pathway and T-cell activation (15–17). Structural analysis indicates that Grb2 consists of a SH2 domain flanked by two SH3 domains, and Grb3-3 is structurally similar to Grb2 except that it has a truncated SH2 domain (deleted in exon 3, residues of 60–100 in Grb2) but retains two intact SH3 domains (12, 14). As compared to Grb2, functional activities of Grb3-3 remains largely unknown except that overexpression can cause apoptosis in NIH-3T3 cells (12). In this study, we report that Grb3-3 is markedly upregulated following HIV-1 infection of CD4$^+$ peripheral blood mononuclear cells undergoing apoptosis. Although IL-2 deprived CD4$^+$ cells also undergo apoptosis to a similar extent, Grb3-3 upregulation is not detected under these experimental conditions. Analysis of the HIV-1 genes responsible for Grb3-3 expression demonstrated that Tat and Nef could independently induce Grb3-3 expression. Transient overexpression of Grb3-3 in Jurkat T-cells also causes apoptosis but constitutive expression at later times appears to be toler-

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ated. In addition, Grb3-3 also sensitizes Sup-T1 cells to undergo apoptosis upon stimulation by staurosporine.

MATERIALS AND METHODS

Plasmids and proviral DNA. pSVTat, pSVNef, pSVEnv, and pSVRev were obtained from Drs. J. Sodroski and D. Gabuzda (Dana-Farber Cancer Institute). PCDNA-Vpr was a gift from Dr. Nat Landau (Salk Institute). pNL4-3 (an infectious HIV-1 proviral clone) was obtained from NIH AIDS Reagents and Resource Repository. pSVGrb2 and pSVGrb3-3 has been described previously (12).

Cell lines and transfections. T cell lines including Sup-T1 and Jurkat cells were purchased from ATCC. A Jurkat cell line constitutively expressing the tetracycline-controlled transactivator (tTA)-expression plasmid pUHD15-1 was provided by Drs. J. Cao and J. Sodroski (Dana-Farber Cancer Institute, Boston, MA) and was described previously (2). The responsive plasmid pUHD10-3 was reconstructed by inserting a hygromycin resistant gene and modification of cloning site (pUHD-JR, J. Richardson and W. Marasco, unpublished data). The cDNA clones encoding Grb3-3 and Grb2, as described (12), were digested with BamHI and HindIII and ligated into pUHD-JR to yield pUHD-Grb3-3 and pUHD-JR-Grb2, respectively. The pUHD-JRGrb3-3 and pUHD-JR-Grb2 plasmids were introduced into Jurkat cells by electroporation and cultured in the presence of hygromycin (250 μg/ml) for selection of stable cell lines. For detection of inducible Grb3-3 and Grb2 expression, the cells were extensively washed four times with PBS to remove tetracycline, followed by cultivation of these cells in fresh media without tetracycline. For transient transfection, Lipofectamine (Life Technologies) was used as transfection mediator. Total amounts of plasmid (10 g) were normalized with empty vectors, and pcDNA- -gal expression vector (Invitrogen) was used to standardize transfection efficiency.

Generation MAbs against Grb2/Grb3-3 and Western blot assay. GSTGrb3-3 or a 13mer peptide, EMKPHPFGNDVQ, spanning the junction between the first SH3 domain and the truncated SH2 domain of the Grb3-3 gene (12) and covalently coupled to keyhole limpet hemocyanin (KLH), were used separately to immunize female Balb/c mice. Hybridoma cell lines generated from the mice immunized with GSTGrb3-3 were screened for positive clones by ELISA against GSTGrb2, GSTGrb3-3, and GST coated microtiter plates. Only those clones secreting MAbs with binding activity to BSA-13mer and GSTGrb3-3 were recovered for further subcloning. Binding specificity of those antibodies to GSTGrb2 and/or GSTGrb3-3 was further verified in a Western blot assay on purified GSTGrb2, GSTGrb3-3 and GST proteins as well as by Western blot and immunoprecipitation on stably transfected NIH3T3 cells expressing similar levels of Grb2 and Grb3-3. Hybridoma 3B5 recovered from the GSTGrb3-3 immunized mice bound Grb2 and Grb3-3 by ELISA, Western blot and immunoprecipitation, whereas hybridoma 4F2 recovered from the KLH-13mer immunized mice bound specifically to Grb3-3 alone in all three assays. Western blot assays were performed as described previously (18).

Detection of apoptosis. Assessment of apoptotic DNA fragments was essentially performed by a method described previously with slight modification (19). In general, 2×10^6 cells were recovered at various times after infection and used for isolation of DNA fragments. The isolated DNA fragments were observed and photographed under UV light. In some cases, TdT-mediated dUTP nick end labeling (TUNEL) was also used for assessment of cell death (20). The fluorescence *in situ* cell death detection kit (Boehringer-Mannheim) was used according to the manufacturer's instructions to examine cells at various times following HIV-1 infection in the presence or absence of IL-2. Positive controls were prepared by treating the cells with camptothecin at a final concentration of 2 μg/ml (Sigma) for 5 h at 37°C.

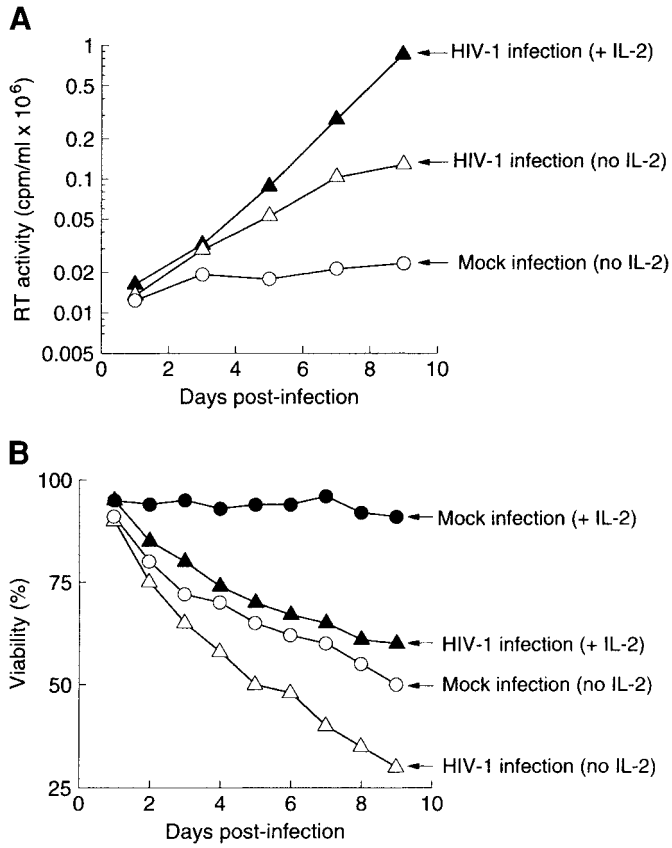

FIG. 1. (A) Determination of reverse transcriptase activity (RT) in $CD4^+$ peripheral blood mononuclear cell cultures following HIV-1 infection. Cells (10^7) were infected with 5 ng p24 of HIV-1 for 3 h, followed by washing three times with PBS. The cells were then resuspended in fresh RPMI-1640/10% FCS. To test the effect of interleukin-2 on HIV-1 infection and apoptosis, some cultures were treated with 40 U/ml of IL-2 (Collaborative Research, Bedford, MA). Samples of cell-free supernatants were collected at regular intervals and assayed for virus content by RT assay as described elsewhere (18). (B) Determination of cell viability. The viability of $CD4^+$ mononuclear cells harvested at various times following IL-2 treatment of HIV-1 infection was determined using trypan blue exclusion assay.

RESULTS

Induction of Apoptosis in $CD4^+$ Peripheral Blood Lymphocytes Infected with HIV-1 and/or Deprived of IL-2

Experiments were initially designed to compare cell viability of the $CD4^+$ cells after HIV-1 infection and IL-2 deprivation. As expected, productive infection by HIV-1 was observed in $CD4^+$ cells cultured in the presence of IL-2 (Fig. 1A), while a much lower level of viral replication was detected in $CD4^+$ cells cultured in the absence of IL-2 since these cells were dying as a result of both HIV-1 infection and lack of growth factor stimulation (Fig. 1B). It is also clear that the viability of uninfected $CD4^+$ cells deprived of IL-2 decreased with time in culture, although not as much as was seen with infected cells deprived of IL-2 (Fig. 1B).

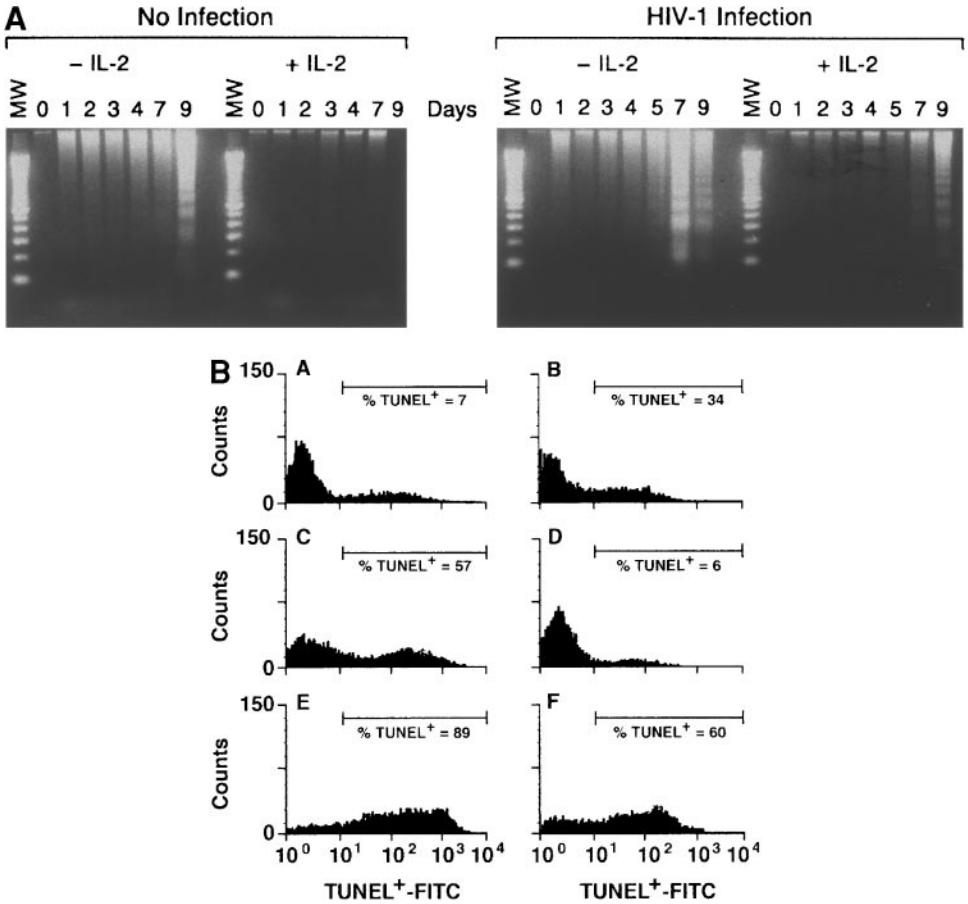

FIG. 2. (A) Detection of apoptotic DNA fragments following IL-2 deprivation and/or HIV-1-infection. $CD4^+$ mononuclear cells (from Fig. 1) were harvested at various times following IL-2 treatment or HIV-1 infection and were lysed for isolation of apoptotic DNA fragments (19). Isolated DNA was fractionated on 2% agarose containing ethidium bromide and photographed under UV light. (B) TUNEL assay. Primary $CD4^+$ mononuclear cells (2×10^6) were harvested and subjected to TUNEL assay. Panel A, HL60 cells (no treatment); panel B, HL60 cells treated with camptothecin for 3 h; panel C, $CD4^+$ cells deprived of IL-2; panel D, $CD4^+$ cells cultured in the presence of IL-2; panel E, $CD4^+$ cells deprived of IL-2 and infected with HIV-1; panel F, $CD4^+$ cells cultured in the presence of IL-2 and infected by HIV-1.

To analyze the individual contributions of HIV-1 infection and IL-2 deprivation to apoptosis, cell samples were harvested from the above cultures for isolation of apoptotic DNA fragments. As is shown in Fig. 2A (left panel), in the absence of IL-2 typical apoptotic DNA fragments were detected in uninfected cells one day after culture initiation and the amounts of these DNA fragments increased with time. In contrast, little apoptotic DNA fragmentation was detected if IL-2 was included in the culture, confirming the dependence of lymphocytes on IL-2 for survival (21). As expected, HIV-1 infection causes extensive apoptosis of $CD4^+$ cells irrespective of whether IL-2 was present or absent, although more apoptotic DNA fragments were detected from cells deprived of IL-2.

To further verify relative numbers of $CD4^+$ cells undergoing apoptosis, we employed the TUNEL assay (20). Figure 2B is a representative experiment showing results from TUNEL assay of cell samples collected one week after initiation of the culture. Figure 2B (Panel A) shows a negative control (untreated HL60 cells) in which 7% of the cells were stained positive, while Fig. 2B (Panel B) is a positive control (HL60 cells treated with camptothecin), in which 34% of the cells were stained positive. In the absence of IL-2, 57% of $CD4^+$ cells were undergoing apoptosis (Fig. 2B, Panel C), and inclusion of IL-2 in culture prevented apoptosis (Fig. 2B, Panel D). Clearly, following HIV-1 infection the highest percentage (89%) of the cells were stained positive in the absence of IL-2 (Fig. 2B, Panel E), while IL-2 slightly protected the cells from undergoing apoptosis (Fig. 2B, Panel F), 60% of the cells were positive). These results are consistent with those obtained from the DNA fragmentation assays (Fig. 2A).

Upregulation of Grb3-3 Expression in $CD4^+$ Peripheral Blood Lymphocytes Infected with HIV-1

We next analyzed Grb3-3 protein expression from these same cultures by Western blot. As shown in Fig.

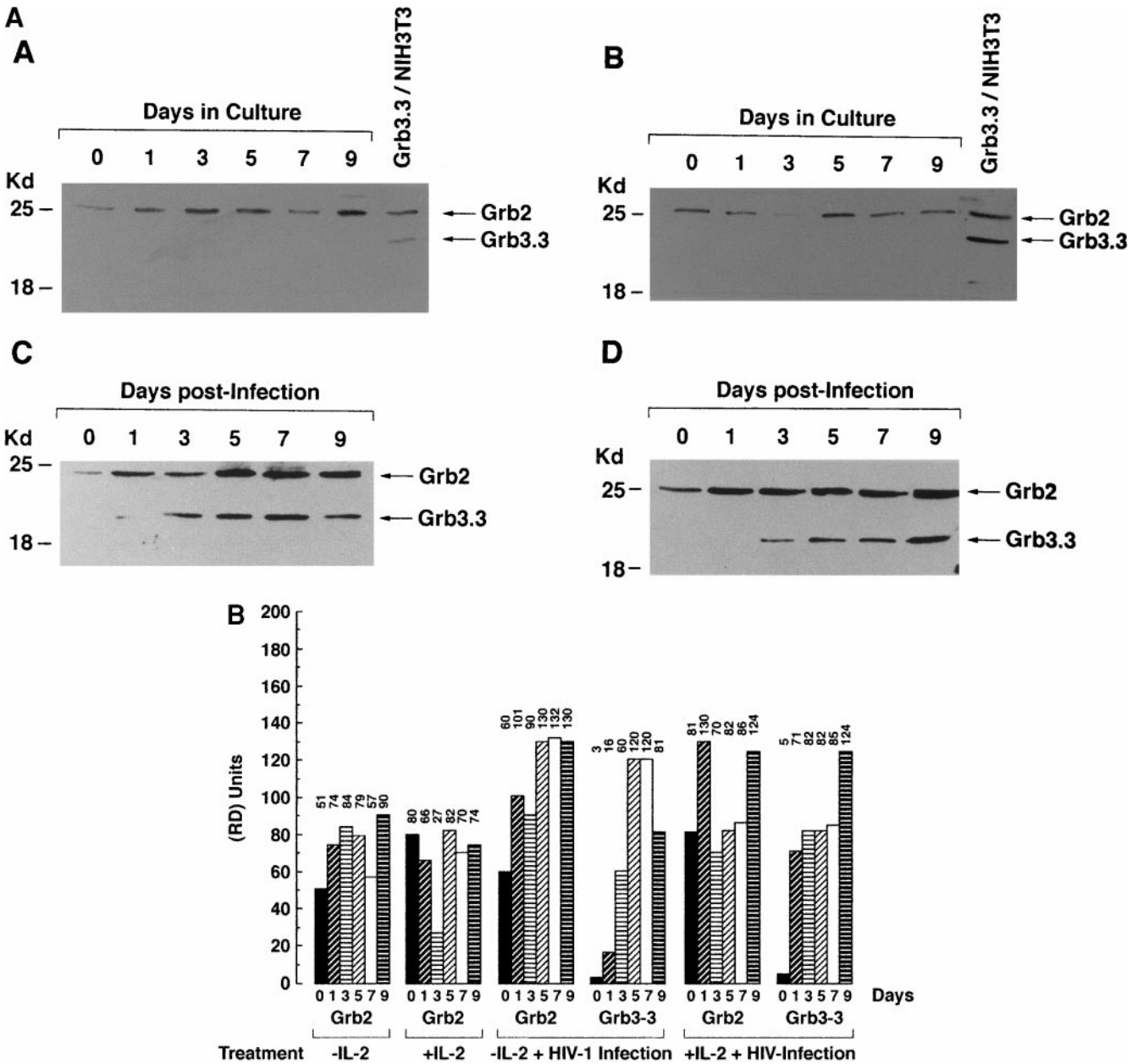

FIG. 3. (A) Determination of Grb2 and Grb3-3 protein levels in primary $CD4^+$ mononuclear cells during HIV-1-infection and/or IL-2 deprivation. Cells were harvested for isolation of total cellular protein. Equal amounts of protein were fractionated in 12.5% PAGE in the presence of SDS, followed by transferring to nitrocellulose filter. Detection of the protein was achieved by Western blot using MAb 3B5 which binds to both Grb2 and Grb3-3. Panel A, $CD4^+$ cells deprived of IL-2; panel B, $CD4^+$ cells cultured in the presence of IL-2; panel C, $CD4^+$ cells deprived of IL-2 and infected by HIV-1; panel D, $CD4^+$ cells cultured in the presence of IL-2 and infected by HIV-1. (B) Quantitative densitometry of Grb2 and Grb3-3 protein levels in $CD4^+$ mononuclear cells during HIV-1-infection and/or IL-2 deprivation. The Western blot shown in Fig. 3A was subjected to quantitative densitometry measurements. The values shown represent relative density measurements over the nine-day experiment and are expressed as relative density (RD) units.

3A, Grb3-3 was not detected in uninfected cells regardless of the presence or absence of IL-2 during the entire 9-day culture period (Fig. 3A, Panels A and B). Densitometry measurements (Fig. 3B) showed less than a two-fold increase in Grb2 levels in the absence of IL-2 (Panel A) and essentially no change in Grb2 levels in the presence of IL-2 (Panel B). Infection of $CD4^+$ cells with HIV-1 in the absence (Fig. 2A, Panel C) or presence (Fig. 2A, Panel D) of IL-2 also caused a circa two-fold increase in Grb2 levels, however in striking contrast, a 120-fold increased level of Grb3-3 was seen irrespective of IL-2 inclusion (Fig. 3B). These results demonstrate that HIV-1 infection selectively upregulates Grb3-3 expression since an increased level of

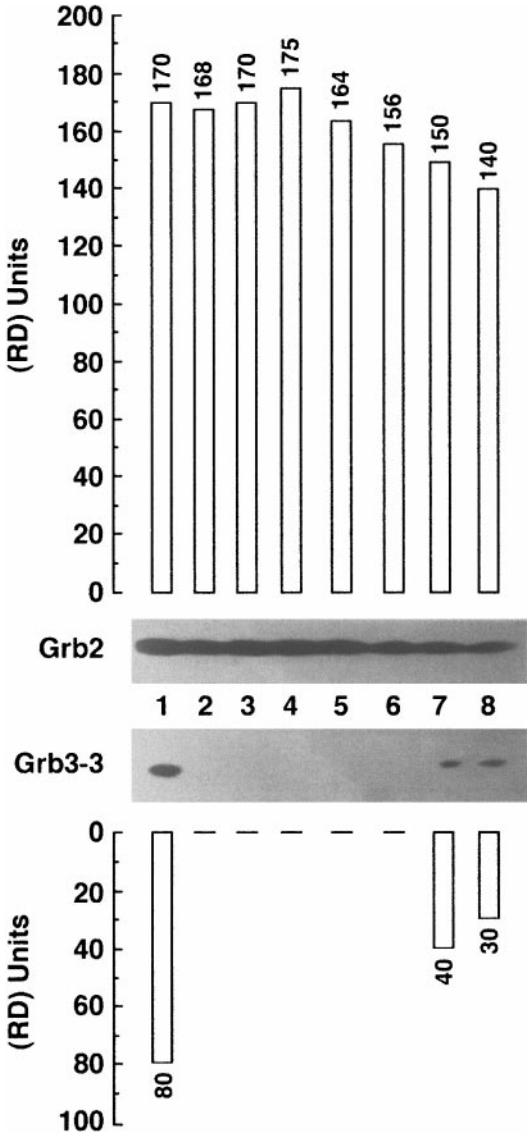

FIG. 4. Detection of Grb2 and Grb3-3 protein in Jurkat cells transfected with various constructs. 72 h posttransfection, cells were isolated for isolation of cellular proteins, which were subsequently analyzed in Western blot using MAb 3B5. Lane 1, cells transfected with pSV-Grb3-3 (positive control); lane 2, transfection with empty vector; lanes 3–8, cells were transfected with either empty vector, pSV-Env, pSV-Rev, pcDNA-Vif, pcVpr, pSV-Tat, or pSV-Nef (lanes 3 through 8), respectively.

Grb3-3 was observed only in HIV-1-infected $CD4^+$ cells undergoing apoptosis, but not in $CD4^+$ cells undergoing apoptosis as a result of IL-2 deprivation.

The Viral Proteins Tat and Nef Can Independently Induce Grb3-3 Expression

We next determined which viral component(s) was responsible for upregulation of Grb3-3 using constructs expressing various viral proteins. As is shown in Fig. 4, transfection of the *tat* and *nef* genes resulted in an increased expression of Grb3-3 (lanes 7 and 8), but no upregulation of Grb3-3 was detected in cells transfected with either empty vector, pSV-Env, pSV-Rev, pcDNA-Vif or pcVpr (lanes 3 through 6, respectively) despite comparable levels of viral protein expression (data not shown). Also shown in Fig. 4, is the positive control (lane 1) in which Jurkat cells were transfected with a Grb3-3 expression plasmid. These results indicate that two of the earliest viral proteins Tat and Nef can independently induce Grb3-3 expression in Jurkat T cells and they may share some common cellular activation pathways (see Discussion).

Overexpression of Grb3-3 Is Temporally Associated with Apoptosis in $CD4^+$ Lymphocytes

Since HIV-1 infection of $CD4^+$ cells induces apoptosis concomitantly with an increased level of Grb3-3 protein, we sought to determine whether overexpression of this protein could result in apoptosis in $CD4^+$ lymphocytes. To this end, a tetracycline-inducible system was used to express Grb3-3 following removal of tetracycline from the culture. As is shown in Fig. 5A, Grb3-3 can be detected 24 h after tetracycline removal, and the protein remained stable through 72 h. Densitometry measurements demonstrated that while Grb2 levels remained unchanged, Grb3-3 levels increased over 50-fold during the first 48 h after removal of tetracycline from the culture medium (Fig. 5B).

Coincident with Grb3-3 protein expression, apoptosis was detected in these cells (Fig. 5C). However, program cell death in these cells appears to be transient since 72 h after removal of tetracycline little apoptotic DNA fragmentation was detected. The transient nature of Grb3-3 induced apoptosis presumably results from activation of a feedback or compensatory pathway by constant exposure of the cells to high levels of Grb3-3 (see Discussion). However, since no significant DNA fragmentation was detected in Jurkat cells harboring the empty vector or a vector encoding Grb2 during the same time course (Fig. 5C), we conclude that transient Grb3-3 expression is temporally associated with apoptosis in these lymphocytic cells.

Augmentation of Staurosporine-Induced Apoptosis in SupT-1 Cells Overexpressing Grb3-3

Staurosporine has been shown to reliably induce Bcl-2-inhibitable cell death in a variety of cell lines (22–23). We assessed the effects of Grb3-3 overexpression in $CD4^+$ SupT-1 cells treated with staurosporine (serine/threonine kinase inhibitor) or camptothecin (topoisomerase inhibitor) (24). Figure 6A confirms that stable SupT-1 cells expressed either Grb3-3 or the control Grb2 protein as detected by Western blot assay. It is also noted that in the cells overexpressing Grb2,

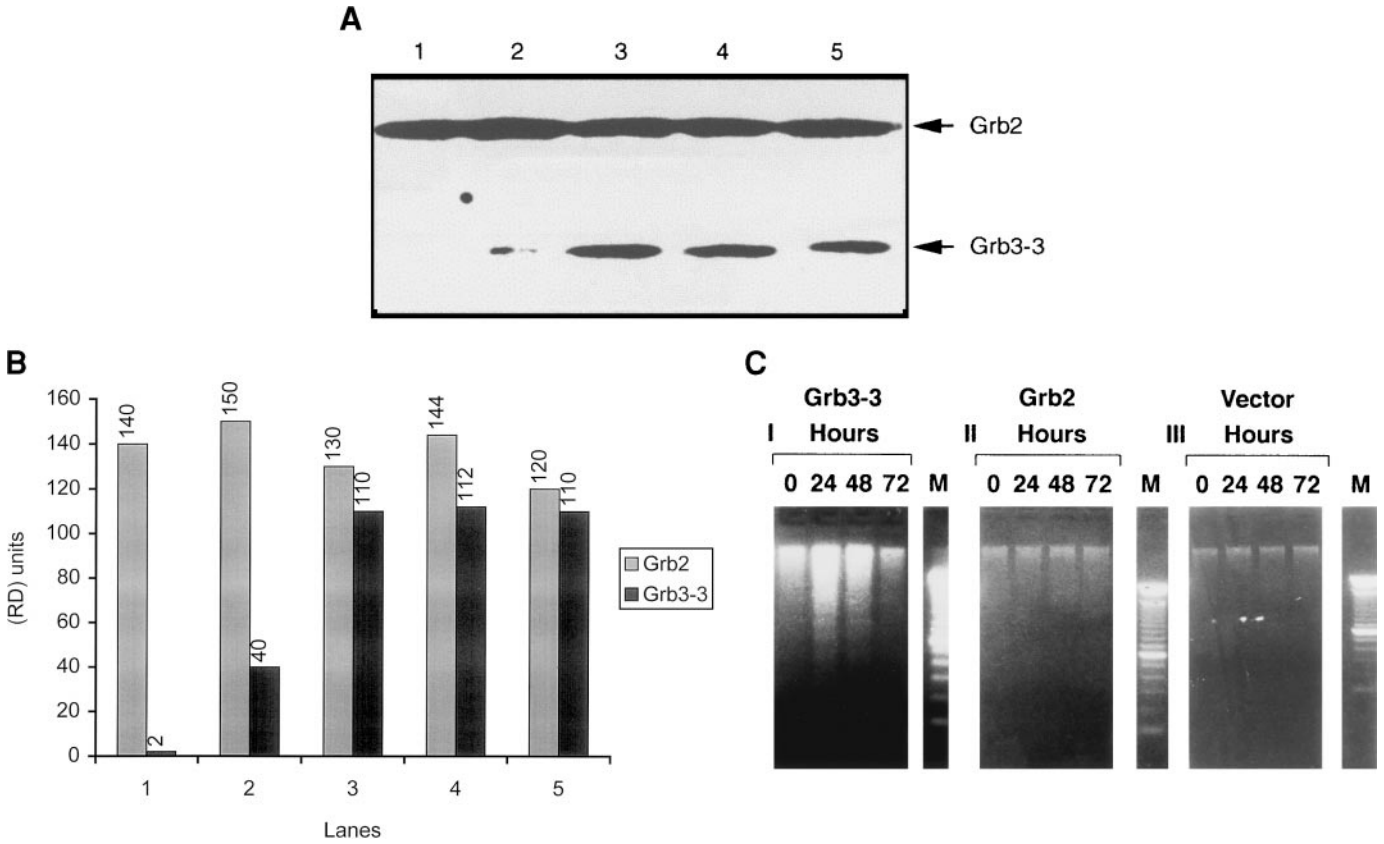

FIG. 5. (A) Detection of Grb3-3 protein expression and apoptosis in tetracycline-inducible stable Jurkat T cells by Western blot using MAb 3B5. Stable Jurkat cells expressing Grb3-3 under the control of tetracycline was analyzed for Grb3-3 expression following removal of tetracycline. Lane 1 cells cultured in the presence of tetracycline; lanes 2–4 cells from cultures 24, 48, 72 h after removal of tetracycline, respectively; lanes 5 stable NIH3T3/Grb3-3 cells. (B) Quantitative densitometry of Grb2 and Grb3-3 protein levels in these Jurkat cells inducibly expressing Grb3-3. The values shown represent RD units. (C) Detection of apoptotic DNA fragments in stably transfected Jurkat cells inducibly expressing Grb3-3 in the presence of (time 0) or 24, 48, 72 h after removal of tetracycline (m) 100 bp DNA ladder. M, molecular weight markers; V, stable SupT-vector cells; 2, stable SupT-Grb2 cells; 3-3, stable SupT-Grb3-3 cells.

Grb3-3 was not detected, suggesting that accumulation of Grb2 does not necessarily lead to generation of Grb3-3. After treatment of these cells with either staurosporine or camptothecin, SupT-1 cells overexpressing Grb3-3 had increased sensitivity to staurosporine-induced apoptosis (Fig. 6B), as compared to cells harboring vector DNA, while Grb2 expressing cells showed a lesser increased sensitivity to staurosporine treatment. From these studies, we conclude that Grb3-3 overexpression increases sensitivity to staurosporine-induced apoptosis.

DISCUSSION

Grb3-3 arises by alternative splicing of the *grb2* mRNA with complete deletion of exon 3 and as a result carries a deleted presumably non-functional SH2 domain but retains the two intact, flanking SH3 domains (12). In adult tissues, *grb3-3* mRNA is 10–50 less abundant than *grb2* mRNA, however, their relative levels appear to be developmentally regulated. For example, during the development of the rat thymus which undergoes involution after birth, *grb2* mRNA remains fairly constant whereas *grb3-3* mRNA reaches a transient peak after five weeks of age, which coincides temporally with the elimination of immature thymocytes by apoptosis (12). As we report here, in at least under one pathogenic condition, i.e., HIV-1 infection, Grb3-3 is upregulated and is also temporary associated with apoptosis. Furthermore, we have observed elevated levels of Grb3-3 in PBMCs from HIV-1 infected subjects but not the healthy donors suggesting that Grb3-3 overexpression may play a role in depletion of $CD4^+$ cells undergoing apoptosis in HIV-1-infected individuals (Li *et al.*, submitted).

Although several lines of evidence implicate a role of Grb3-3 in induction of apoptosis, the mechanism(s) remains unknown. Grb3-3 may trigger apoptosis by either acting as a dominant negative over Grb2 for binding to some common partners or exerting its effects through distinct signaling pathways not shared by Grb2. A variety of cellular factors have been found

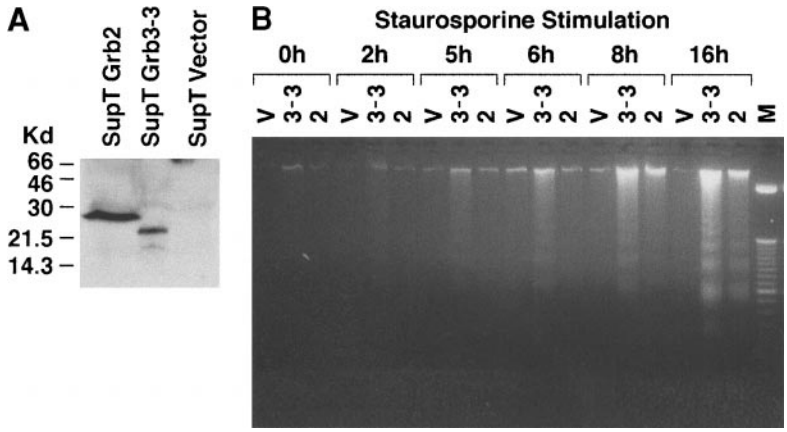

FIG. 6. (A) Augmentation of staurosporine induced-apoptosis in Sup T-1 cells overexpressing Grb3-3. Carboxy-terminal HA-tagged Grb2 and Grb3-3 were cloned into pcDNA-3 (Invitrogen) and stable Sup T-1 cells were established. Western blot analysis of stable SupT-Grb2, SupT-Grb3-3, Sup-T vector cells. Mab anti-HA (Babco, Richmond, CA) and HRP-goat anti-mouse IgG (Sigma) were used together with ECL. (B) 2×10^6 cells stably transfected with vector, Grb2, or Grb3-3 expressor DNA were treated with 300 nM staurosporine. The cells were incubated at 30°C for varying times and samples were harvested for analysis of apoptosis as described (19).

to bind both Grb2 and Grb3-3. These factors, including hSos1, Vav, dynamin, and hnRNP C (12–14, 25–26), are known to be involved in important cellular activities such as cell growth, cell differentiation or mRNA splicing. Presumably, normal functional activities of these factors could be affected as a result of interactions with Grb3-3, leading to apoptosis in cells that have elevated level of Grb3-3. Another possibility is that Grb3-3 may promote apoptosis by activating specific signaling pathways not shared by Grb2. Specific binding partners of Grb3-3 include adenosine deaminase (ADA), an enzyme involved in purine metabolism whose deficiency is associated with severe combined immunodeficiency (27). It is unclear, at present, whether Grb3-3 promotes apoptosis by antagonizing Grb2, or by interacting with other cellular factors, or by both mechanisms (see below).

In the tetracycline-inducible expression system presented in this study (Fig. 5), overexpression of Grb3-3 in Jurkat T lymphocytes causes apoptosis, especially 24–48 h postremoval of tetracycline. It is unclear why after 72 h the cells became resistant to apoptosis although the Grb3-3 level remained elevated at this time point. One possible explanation for this observation is that persistent exposure of these tumor cells to Grb3-3 may activate a compensatory signaling pathway that could counteract the apoptosis-inducing effects imposed by Grb3-3 and this would allow cells to survive continuous Grb3-3 exposure. Indeed, establishment of Grb3-3 expressing stable cell lines in Jurkat T cells and Sup-T1 cells, as shown here (Fig. 6A), is possible, while repeated attempts in establishing PBMCs overexpressing Grb3-3 have failed (data not shown). Apparently, tumor cell lines such as Jurkat T and Sup-T1 cells, as compared to PBMCs, are more resistant to apoptosis-inducing effects caused by Grb3-3. However, Grb3-3 could still predispose these refractory tumor cells to apoptosis upon stimulation by staurosporine, a serine/threonine kinase inhibitor that has been shown to reliably induce Bcl-2-inhibitable cell death in a variety of cell lines. While questions remain open as to whether Grb3-3 acts predominantly by interacting with common binding partners of Grb2 or other specific cellular proteins, it is noteworthy that there is a growing body of experimental evidence suggesting that control of RNA splicing could be an important mechanism in regulating cell growth (28).

Although the current study demonstrates that Tat and Nef can independently induce Grb3-3 expression, the study did not address the issue of how these viral proteins cause upregulation of this isoform of Grb2, however, it is noteworthy to mention that a recent report has shown that expression of the essential nuclear splicing factor SC35 is upregulated following HIV-1 infection (29). In addition, although Tat and Nef have been reported by other investigators to induce apoptosis (31–34), it is premature to conclude that these two viral proteins cause apoptosis only through Grb3-3. Apoptosis involves numerous cellular factors participating in a variety of signaling pathways and as a result, different stimuli can cause cell death via distinct pathways (35–37). For instance, other viral proteins such as Vpr and Env that have previously identified to be inducers of apoptosis (31, 38) were unable to upregulate Grb3-3 expression. All these results imply that HIV-1 induced apoptosis may involve activation of other signaling pathways that are distinct from the apoptosis pathway associated with Grb3-3. Indeed, further insight into the role of Grb3-3 in the apoptosis pathway should be obtained once the pathways involved in interaction amongst Tat, Nef and Grb3-3 are better understood and the identities of other interme-

diates that might be involved in this process are revealed.

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