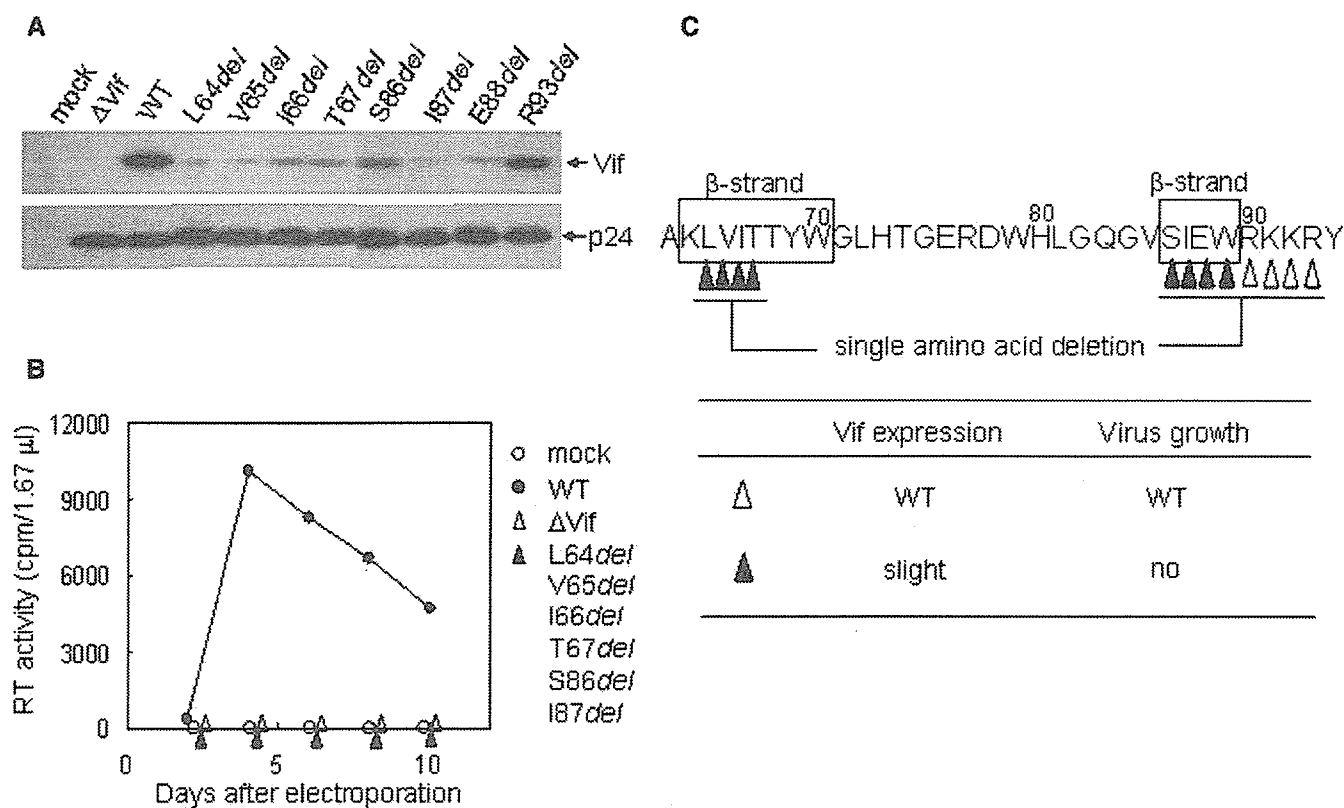

Fig. 4. Deletion analysis of HIV-1 Vif. (A) Steady-state expression of Vif in 293T cells transfected with various proviral mutant clones. 293T cells were transfected with 20 µg of various pNL clones indicated, and cell lysates were prepared at 48 h post-transfection for western blot analysis using appropriate anti-Vif and anti-Gag (p24) antibodies, as previously described [22,23]. ΔVif, pNL–Nd [34]; WT, pNL432 [21,34]. (B) Growth kinetics in H9 cells of various proviral mutant clones. H9 cells were electroporated with 10 µg of various pNL clones indicated, and RT production in the culture supernatants was determined at intervals. WT, pNL432 [21,34]; ΔVif, pNL–Nd [34]. (C) Phenotype of various mutant clones. The results in (A) and (B) are summarized. Characteristics of E88*del*, W89*del*, R90*del*, K91*del*, K92*del*, and R93*del* mutants have been previously described [22] and are included in this summary.

3.3. Rapid and extensive degradation is unique to Vif among accessory proteins

We finally asked whether Vif is unique among the four HIV-1 accessory proteins with respect to its high sensitivity to proteasome degradation. To address this question, we performed pulse/chase analyses using wt full-length proviral clone pNL432. Transfected 293T cells were pulse-labeled and chased as described above, and the degradation kinetics of Vif, Vpr, Vpu and Nef were monitored. Gag-p24 was used as a control. As shown in Fig. 6A, Vif was unstable and decayed much more rapidly and extensively than Vpr, Vpu and Nef. The blocking effect of the proteasome inhibitor MG-132 on the degradation of accessory proteins was then examined (Fig. 6B). Transfected 293T cells were pulse-labeled for 5 min and chased for 30 min in the presence or absence of MG-132, and the ratio of expression level of accessory proteins (soluble fraction) was determined. As clearly seen in Fig. 6B, the effect was the greatest for Vif.

4. Discussion

We have recently shown that rapid turn-over of Vif in cells may be virologically important [20]. Excess amounts of Vif exert harmful effects on virus infectivity, and therefore HIV-1 has apparently evolved to control the intracellular expression level of Vif. In this study, we report for the first time on the mechanism, the cell-type independency, and the region in Vif critical for the rapid intracellular degradation. We also show that this unstable nature of Vif is unique among HIV-1 accessory proteins.

Our results here strongly suggest that the mechanism of Vif degradation involves the cellular proteasome pathway. The proteasome inhibitors MG-132 and *clasto*-lactacystin β-lactone specifically blocked the degradation (Figs. 1, 5, and 6). We have detected large amounts of polyubiquitinated derivatives of Vif that are greatly increased by the proteasome inhibitor MG-132 (Fig. 3). These results are quite consistent with those on the presence of APOBEC3G recently reported by Mehle et al. [14]. In the absence of APOBEC3G, however, they found that only a minor fraction of Vif is monoubiquitinated, and that Vif is a relatively stable protein with a half-life of approximately 90 min [14]. These observations were very different from our results presented here. In contrast to the soluble cytosolic Vif, which is rapidly degraded, the insoluble cytoskeleton-associated Vif was quite stable and accumulated in cells (Figs. 1 and 2). This phenomenon was observed in permissive 293T and HeLa cells, and in non-permissive H9 cells (Figs. 1 and 2). Whether

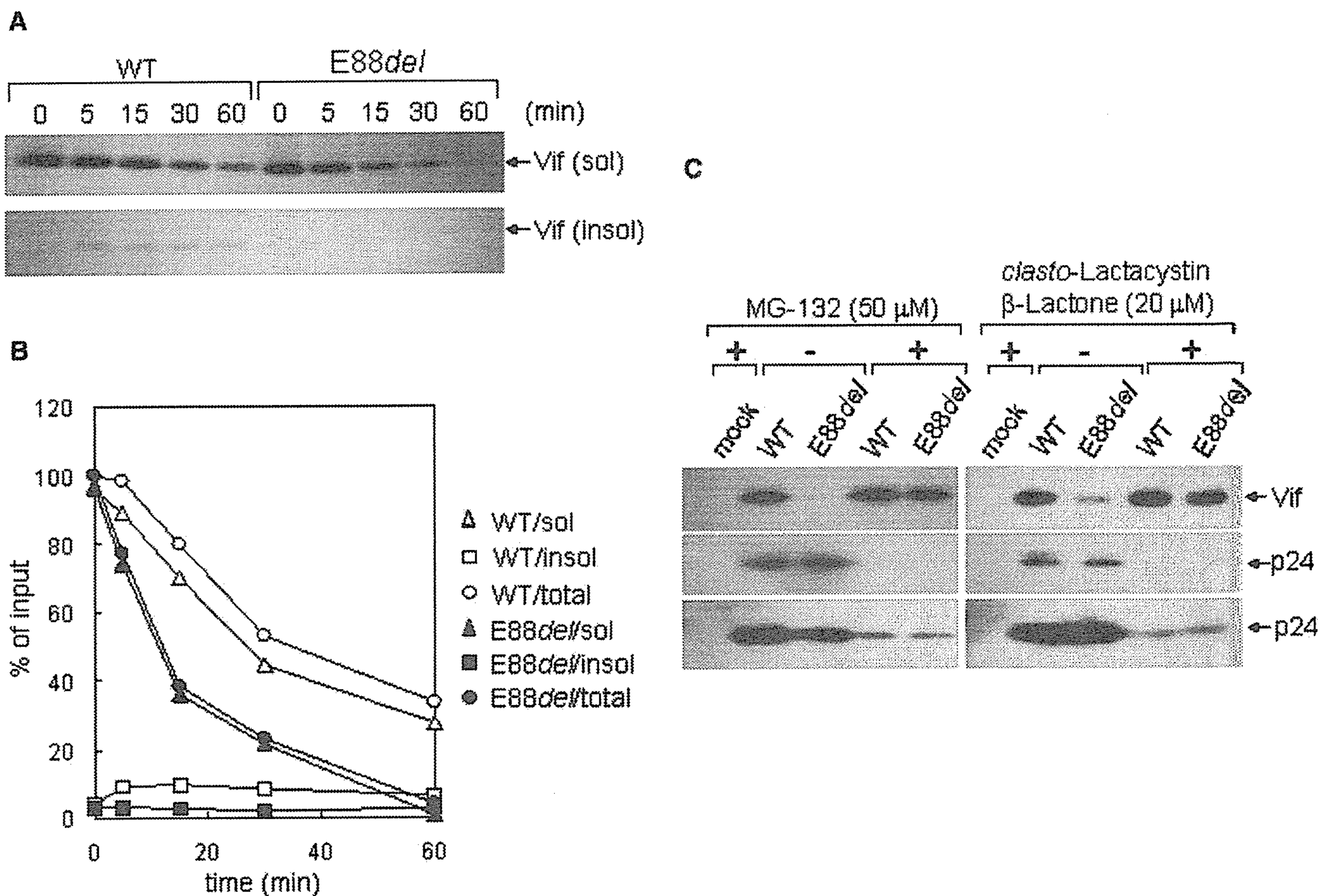

Fig. 5. Comparative analysis of wt and E88*del* mutant Vif proteins. (A) Stability of wt and mutant Vif proteins. 293T cells were transfected with 5 μg of pNL-A1 (WT) or pNLA1-E88*del*. Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for up to 60 min, as indicated above the lanes. Cell lysates were prepared as described in Section 2 and precipitated with a Vif-specific polyclonal antibody Vif93 [29]. Vif proteins were identified by SDS-PAGE followed by fluorography; sol, soluble fraction; insol, insoluble fraction. (B) Degradation kinetics of wt and mutant Vif proteins. Vif-specific bands in (A) were quantified by scanning, and relative values were plotted as a function of time. Total, sol plus insol. (C) Steady-state expression of wt and mutant Vif proteins in the presence or absence of proteasome inhibitors. The 293T cells were transfected with 3 μg of wt pNL432 or pNL-fE88*del* and cultured in the presence (+) or absence (–) of proteasome inhibitors MG-132 and *clasto*-lactacystin β-lactone. Cells were harvested at 36 h post-transfection for western blot analysis using appropriate anti-Vif and anti-Gag (p24) antibodies, as previously described [22,23]. Appropriate adjustments in sample volumes were made to give bands of similar intensity (wt Vif). Also, results of two different blots for Gag-p24 are presented to show that each lane for comparison contained approximately similar amounts. Expression level of viral proteins in cells in the presence of the inhibitors was low relative to that in the absence of the inhibitors.

the cytoskeletal Vif represents a population refractory to ubiquitination and has a pivotal role for Vif function remains to be determined. It would be vitally important to generate degradation-resistant Vif mutants and characterize them biochemically and biologically.

Deletion analysis of Vif in this study has demonstrated that there are some amino acids that confer stability on Vif. We have recently reported that mutations affecting residues 88 and 89 of NL432 Vif, located within a β-strand structure, as predicted by the PredictProtein, resulted in a reduced level of Vif expression [22]. Based on this observation, we performed mutational analysis of the area. Deletion of amino acids in the predicted β-strand structures (residues 63–70 and 86–89 in NL432 Vif), but not those located outside of the regions, diminished Vif expression to a negligible level and abolished viral infectivity (Fig. 4). Apparently, formation of the β-strand structures is important for stable expression of Vif. In addition, we showed that the E88*del* mutant Vif is degraded more rapidly than wt Vif, and that this degradation is proteasome-dependent (Fig. 5). Together with the results in our recent work [20], we can conclude that there is an optimal level of Vif expression in cells for viral replication.

Recent work proposed that Vif induces proteasome-dependent degradation of APOBEC3G [13–16], but the precise molecular mechanism for this activity of Vif is still unclear. We have already shown by pulse/chase analyses that there is no significant difference in the stability of APOBEC3G in the presence or absence of Vif [17] and that there is no significant difference in the stability of Vif in the presence or absence of APOBEC3G [20]. Furthermore, we found that the half-life of Vif in non-permissive H9 cells (Fig. 2) is very different from that of APOBEC3G in the presence of Vif, which was recently reported [13,15,16, 18,19]. Based on these findings, it seems unlikely that Vif and APOBEC3G form a complex that leads to subsequent simultaneous degradation of both proteins by the proteasome path-

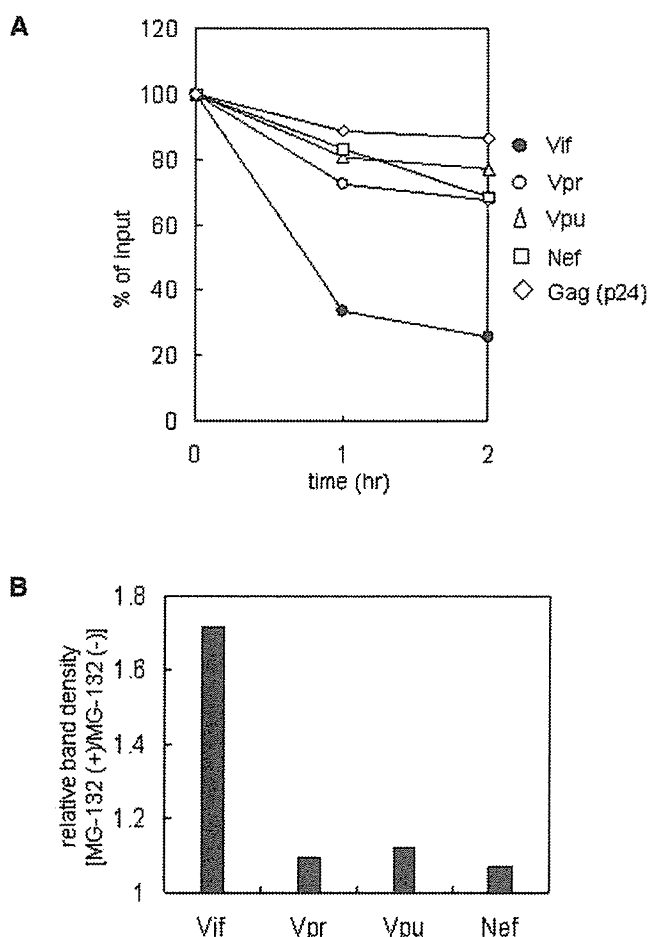

Fig. 6. Comparative analysis of HIV-1 accessory proteins. (A) Degradation kinetics of the HIV-1 accessory proteins. The 293T cells were transfected with 5 μg of pNL432 or a control Gag-p24 expression vector pSG-Gag (p24) cFLAG. Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for up to 2 h as indicated. Cell lysates (soluble fraction) were prepared and precipitated with an antibody against each viral protein (anti-Vif, Vif93; anti-Vpr, raised against full-length synthetic Vpr peptide (Strebel et al., unpublished); anti-Vpu, NIH catalog no. 969; anti-Nef, NIH catalog no. 2949; anti-Gag-p24, NIH catalog no. 6521). Viral proteins were identified by SDS-PAGE followed by fluorography. Specific bands of viral proteins were quantified by scanning, and relative values were plotted as a function of time. (B) Blocking effect of proteasome inhibitor MG-132 on the degradation of accessory proteins. 293T cells were transfected with various clones, as described in (A). Cells were collected 24 h after transfection, labeled for 5 min with [^{35}S]-methionine and [^{35}S]-cysteine, and chased for 30 min. Part of cells were incubated in the presence of MG132 (50 μM) during the labeling and chasing time, as indicated. Cell lysates were prepared and precipitated with an antibody against each accessory protein as above. Accessory proteins were identified by SDS-PAGE followed by fluorography. Specific bands of accessory proteins were quantified by scanning, and the ratio of values for each accessory protein is shown.

way. We and others have reported that Vif affects APOBEC3G translation [17–19]. However, the reduction of intracellular APOBEC3G in the presence of Vif does not fully account for the Vif-induced reduction of virus-associated APOBEC3G [17]. Further investigations are required to elucidate the molecular mechanism by which Vif counteracts the activity of APOBEC3G.

Acknowledgments

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