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lck-Independent Inhibition of T Cell Antigen Response by the HIV gp120

Sophie Gratton, Michael Julius, and Rafick-Pierre Sékaly

Binding of the HIV envelope glycoprotein gp120 to CD4 inhibits T cell activation. We have used a murine T cell clone transfected with either wild-type human CD4 or mutated forms of CD4 to characterize the pathways involved in this inhibitory effect of gp120. Ag-induced proliferation of T cell clones transfected with human CD4 was completely inhibited in the presence of gp120, even though stimulation of this clone is independent of a CD4/MHC class II interaction. In addition, our results demonstrate that the inhibition by gp120 is not due to the sequestration of lck from TCR and does not require activation of lck by gp120. This suggests that CD4 can regulate the initiation of T cell activation independently of its interaction with lck. Moreover, we demonstrate that the nonresponsiveness induced by gp120 can be reversed by soluble CD4 when added early after onset of stimulation and that gp120 exerts its inhibitory effect when cells are in the G0 phase of the cell cycle.

gp120 was previously demonstrated to increase the tyrosine kinase activity of CD4-associated lck (20–23, 28). To evaluate the potential contribution of lck activity in inhibition by gp120, we have transfected the 2.10 clone with a chimeric molecule consisting of the extracellular domain of the epidermal growth factor (EGF) receptor and the transmembrane and cytoplasmic domains of human CD4. We have previously demonstrated that this chimera is associated with a similar amount of lck as wild-type CD4. Moreover, binding of EGF to the chimera activates lck tyrosine kinase activity. Two independently derived clones expressing the chimer were tested several times in functional assays for their capacity to respond to Ag. Results of a representative experiment are shown in Figure 2. These clones responded to Ag as efficiently as cells expressing wild-type CD4 molecules, further confirming the lack of requirement of a CD4-MHC class II interaction. Interestingly, addition of saturating levels of EGF (100 nM), which induces the tyrosine kinase activity of lck, does not have any effect on the response to OVA. Based on these results, it was tempting to speculate that the inhibition by gp120 of T cell activation did not require activation of lck associated with the cytoplasmic tail of CD4.

The inhibition of antigenic stimulation by gp120 does not require the association of lck with CD4

To verify if the association of CD4 with lck is required for the inhibition of gp120, several 2.10 clones expressing a mutant CD4 molecule that is not associated with lck, C4202A, were stimulated in the presence or in the absence of gp120. Each clone was tested at least twice, and a representative experiment is shown in Figure 3B. Interestingly, gp120 inhibited proliferation of C4202A clones induced by stimulation with OVA. This result clearly demonstrates that the inhibition of antigenic stimulation induced by gp120 does not require the association of lck with CD4.

Binding of gp120 to CD4 has been reported to induce the internalization of CD4, leading to the suggestion that this internalization plays a role in the inhibitory effect of gp120 (24). Three

4 Abbreviations used in this paper: EGF, epidermal growth factor; FSC, forward light scatter; SSC, side light scatter.

5 S. Gratton, L. Haughn, R.-P. Sékaly, and M. Julius. The extracellular domain of CD4 regulates the initiation of T cell activation. Submitted for publication.
serine residues located in the cytoplasmic domain of CD4 are responsible for the internalization and degradation of CD4 molecules (33). These serine residues were mutated to alanine residues to abrogate the internalization of CD4 (32). Several clones expressing this mutant were derived. Interestingly, these clones still responded to Ag stimulation (Fig. 3B) whereas this mutation was previously found not to restore a CD4-dependent response (4), further confirming the CD4-independent nature of this clone. Furthermore, gp120 inhibited Ag-induced proliferation of these transfectants (Fig. 3B). This result suggests that inhibition of antigenic stimulation by gp120 does not require the triple serine endocytosis motif of the cytoplasmic tail of CD4, providing further evidence for the lack of role of the cytoplasmic domain of CD4 in this function.

**gp120 prevents Ag-mediated blastogenesis**

To further determine the mechanism leading to gp120-mediated inhibition of T cell activation, we verified whether gp120 was capable of preventing early activation signals, such as blast transformation. Clones expressing wild-type CD4 were stimulated with Ag in the presence or absence of gp120 for 24 h, and their size was monitored using the FACScan. As a control, an aliquot of cells was incubated without Ag or IL-2 for the same period. As depicted in Figure 4, A and B, cells exposed to Ag in the absence of gp120 had an increased FSC as compared with cells stimulated with Ag in the presence of gp120, indicating that the presence of gp120 prevented blast transformation in response to antigenic stimulation. Further analysis was conducted using FSC/SSC contour plots of total cells (Fig. 4, A–C) or with dead cells gated out using propidium iodide (Fig. 4, D–F). The lower left quadrant of each plot contains the splenocytes used to present Ag while blasts accumulate in the upper right quadrant. Fig. 4, A and D, indicates that cells stimulated with Ag undergo blast transformation and exclude propidium iodide. On the other hand, cells stimulated by Ag in the presence of gp120 did not increase in size (Fig. 4B). Figure 4E indicates that cells die in the presence of gp120, since they are totally excluded by propidium iodide staining. This is further confirmed by the fact that the profiles are similar to those observed with cells that were incubated without any Ag or IL-2 (Fig. 4, C and F). This T cell clone is dependent on exogenous IL-2 for growth and, upon deprivation of IL-2, cells undergo apoptosis (data not shown). Hence, cell death observed in the presence of gp120 may be due either to a direct effect of gp120 on T cells or to apoptosis resulting from the lack of stimulation and the absence of autocrine IL-2.

**gp120 inhibition is reversible by soluble CD4**

To determine whether cell death observed in the presence of gp120 was due directly to the binding of gp120 to CD4, we verified whether soluble CD4 could reverse the inhibition induced by gp120. As shown in Figure 5A, soluble CD4 completely prevented inhibition of antigenic stimulation by gp120 when added at the onset of stimulation. Interestingly, soluble CD4 completely restored Ag-induced proliferation when added 6 h after start of stimulation, while partially restoring the response when added 18 or 24 h after addition of Ag and APC. This partial reversal of inhibition cannot be due to lack of Ag, since addition of fresh splenocytes and Ag with the soluble CD4 at 24 h still does not completely restore the response (data not shown).

These results argue against a direct cytopathic effect of gp120 on T cells but rather support the hypothesis that gp120 prevents the initiation of T cell activation. The observed cell death would thus be due to a consequent lack of IL-2, and ensuing apoptosis. Moreover, the state of nonresponsiveness induced by gp120 is not permanent, since it can be reversed by the addition of soluble CD4.
The presence of gp120 is required at the G0/G1 phase of the cell cycle for efficient inhibition of T cell activation.

In light of the above results demonstrating only partial rescue of gp120-mediated inhibition by soluble CD4 when added 24 h after the onset of stimulation, we were interested in determining the time frame in which gp120 had to be present during stimulation to inhibit T cell activation. It was also interesting to verify if cells expressing the different CD4 mutants exhibited the same requirements. As demonstrated in Figure 5B, gp120 inhibited the response of clones expressing wild-type or mutant forms of CD4 (C4202A and CD4 3S) when added at the onset of stimulation. Remarkably, when gp120 was added at 24 h poststimulation, we could still observe a significant inhibition of antigenic stimulation. As shown in Figure 6A, at the beginning of stimulation, cells are distributed in all phases of the cell cycle. We thus hypothesized that cells that are in the S, G2, and M phases at the onset of stimulation must thus inhibit T cell activation.
complete their cycle back to G0/G1 before encountering Ag and becoming susceptible to inhibition by gp120.

To verify this hypothesis, we enriched for G0/G1 CD4-positive cells and compared their susceptibility to inhibition by gp120 with that of unsynchronized population. To synchronize cells, we first starved them of IL-2 for 2 h and then let them grow for 12 to 16 h in limiting amounts of IL-2 (5% of concentration used for expansion) before stimulation. As shown in Figure 6, A and B, this treatment led to an accumulation of cells in the G0/G1 phase of the cell cycle (75–80%), as opposed to unsynchronized cells that were distributed more evenly in all phases of the cycle (40–45% in G0/G1). Both populations were stimulated with Ag, and gp120 was added at different time points after the onset of stimulation (Fig. 6C). While gp120 profoundly inhibited stimulation of both populations when added at initiation of stimulation, addition of gp120 9 h later inhibited only 56% of the response of the “synchronized” population, whereas it inhibited 95% of the response of the unsynchronized population. Addition of gp120 at 24 h poststimulation further differentiated the two populations; while we could observe a 75% inhibition in the unsynchronized population, the “synchronized” population was only weakly inhibited (13%). These results suggest that all “synchronized” cells have seen Ag and become committed for activation before the 24-h time point and thus cannot be inhibited by addition of gp120 at that time point. On the contrary, in the unsynchronized population, some cells are not stimulated yet at the 24-h time point. These cells are the ones that were in the S/G2/M at time 0 and are now in G0/G1 and susceptible to inhibition by gp120 24 h after start of stimulation. Analysis of the populations 24 h after onset of stimulation could not allow a distinction between cells committed to G1 and cycle progression and those not stimulated and residing in G0 (data not shown). These results support the hypothesis that gp120 must be present in the G0/G1 phase of the cycle to inhibit antigenic stimulation.

Discussion

In this report, we have extended the characterization of the effect of gp120 on T cell activation by demonstrating that gp120 can also abrogate antigenic stimulation of a murine T cell clone transfected with human CD4. The concentrations of gp120 required to observe this inhibition (5 μg/ml) are in the range of levels found in HIV-infected patients (34). Whether the monomeric form or an oligomeric form of gp120 is required to observe inhibition of activation is still controversial. In our hands, the monomeric form of gp120 was sufficient to completely inhibit Ag-induced stimulation, confirming that aggregation of CD4 is not required for this effect. A decrease in T cell stimulation by mitogens or recall Ags is observed in HIV-infected patients (35, 36). The HIV env glycoprotein gp120 has been suggested to play a role in this phenomenon of the inhibition of T cell activation. Indeed, soluble or membrane-bound forms of gp120 can inhibit in vitro TCR-triggered activation of both human primary PBL and T cell lines. This inhibition could result in vivo from the interaction of gp120 at the surface of infected cells or of gp120 on virions with CD4 molecules. The presence of viral particles in lymph nodes where T cells are activated by their encounter with specific Ag supports this model.

The inhibitory effect of gp120 was completely reversible in the presence of soluble CD4 when added early after triggering of T cell activation. This result shows that, to inhibit stimulation by Ag, a prolonged interaction between CD4 and gp120 is required. However, synchronization experiments and kinetics of addition of either gp120 or soluble CD4 suggest that gp120 exerts its inhibitory effect when cells are in the G0/G1 phase of the cycle, i.e., when TCR recognition of Ag occurs (reviewed in Ref. 37). Indeed, when cells have become committed to cycle at time t = 24 h poststimulation, gp120 was no longer able to abrogate the proliferation of activated T cells. Several cell cycle-regulating genes, such as the cyclins and their kinases, have been implicated in cell cycle progression (reviewed in Ref. 38). Modulation of their activity occurs both at transcriptional and posttranscriptional levels and involves phosphorylation events. Blocking of these kinases by chemicals such as rapamycin and FK506 results in abrogation of cell cycle progression leading to T cell anergy (reviewed in Ref. 39). Interestingly, signals generated through CD4 have been implicated in the regulation of early T cell activation events, and gp120 could thus disrupt one or more of these signals to abrogate T cell stimulation by Ag (40, 41).

The response of this T cell clone to antigenic stimulation is not enhanced by the presence of CD4 (14). The inhibitory effect of gp120 cannot thus be explained by the disruption of an adhesion interaction between CD4 and MHC class II molecules. Binding of gp120 to CD4 has been previously reported to induce intracellular biochemical events such as an increase in tyrosine kinase activity of lck and down-regulation of cell surface expression of CD4. However, our results clearly show that, although inhibition by gp120 is accompanied by an increase in lck tyrosine activity (20–23, 28), neither its association with CD4 nor an increase in its tyrosine kinase activity are required for the abrogation of Ag-induced T cell proliferation. Indeed, stimulation of cells expressing either the mutant that has lost the association with lck or the endocytosis mutant was inhibited by gp120, whereas activation of lck through the EGFR/C4 chimera had no effect on T cell stimulation. We have previously shown that CD4 can sequester lck and inhibit anti-TCR-induced proliferation if not coaggregated with the TCR (14). The mechanism of inhibition by gp120 was also proposed to involve lck sequestration in T cell lines (42). The inhibition of Ag-induced stimulation by gp120 that we here observe cannot be explained by such a sequestration of lck since clones expressing a mutant of CD4 that does not associate with lck are also inhibited by gp120. Interestingly, treatment with CD4-specific Ab also inhibits antigenic stimulation of T cells expressing a mutant of CD4 that is not associated with lck (29). Taken together, the results presented here define a functional role of CD4 that is independent of its association with lck and its interaction with MHC class II molecules, although mapping to its external portion. Binding of gp120 to the external domain of CD4 could mask this regulatory domain and prevent T cell activation.

It was recently reported that gp120 binds to monomeric forms of CD4 whereas MHC class II molecules induce the dimerization of CD4 molecules through their external domain (43). A possible mechanism of inhibition of gp120 could be the disruption of CD4 dimers, thus modulating CD4 signaling capacity. Alternatively, gp120 could modulate the function of another T cell surface molecule which is associated with CD4 and implicated in early T cell activation events. Indeed, CD4 molecules have been found associated with the tyrosine phosphatase CD45 and the TCR complex (30, 31, 44–48). It is thus conceivable that binding of gp120 to CD4 could prevent an extracellular interaction between CD4 and the TCR necessary to stabilize the interaction between the TCR and MHC class II molecules (30, 31). Alternatively, gp120 binding to CD4 could sterically prevent an Ag-specific TCR/MHC class II interaction or disrupt a regulatory interaction between CD4 and CD45. This last scenario would lead to modulation of CD45 activity and inhibition of T cell activation (49, 50). Modulation of T cell activation through the CD4-CD45 interaction has been previously reported (49, 50–52).
Overall, we have demonstrated that inhibition of antigen stimulation by gp120 is not mediated through direct activation of the CD4-associated lck and occurs in the absence of lck association with CD4. We propose that gp120 modulates in a reversible manner, a regulatory function of CD4 that occurs in the G0/G1 phase of the cell cycle and that is mapped to its extracellular domain.

Acknowledgments

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