Inhibition of Lymphoproliferation and Protein Kinase C by Synthetic Peptides with Sequence Identity to the Transmembrane and Q Proteins of Visna Virus

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Received 27 October 1989/Accepted 19 January 1990

A peptide sequence in the transmembrane protein of visna virus has been identified that bears a high degree of similarity to a sequence within the transmembrane protein gp41 of human immunodeficiency virus that we have previously shown to be immunosuppressive. Also within the *Q* (*vif/sor*) open reading frame of the visna virus genome is a sequence that is highly similar to the immunosuppressive sequence from the retroviral transmembrane protein p15E. We synthesized peptides containing these visna virus sequences and tested them for immunosuppressive activity, comparing them with their human immunodeficiency virus and leukemia retrovirus counterparts. Both the *Q*- and transmembrane-derived visna virus peptides inhibited lymphoproliferation stimulated by either interleukin-2 or the T-cell antigen receptor in a dose-dependent and sequence-specific manner. The two visna virus peptides also inhibited the enzymatic activity of protein kinase C, thus providing a possible molecular mechanism by which they inhibit immune function.

Visna virus is a member of the nononcogenic lentivirus subfamily of retroviruses, of which other notable members include the immunodeficiency viruses of humans (HIV), primates, and cats, as well as the arthritis-encephalitis virus of goats (24). Among the distinctive biological features of the lentiviruses are their slow courses of disease progression and etiologies that are associated with dysregulation of immune function (24). The immunosuppression caused by immunodeficiency viruses such as HIV predisposes the patient to various opportunistic infections resulting in death (for a review, see reference 7). In the case of lentiviruses that infect ungulates (including visna virus, caprine arthritis-encephalitis virus, and equine infectious anemia virus), the damage done to the infected host is the consequence of an apparent overstimulation of the immune system. This dysregulation of immune function results in inflammatory lesions in the nervous and respiratory systems (visna virus), arthritic inflammation of the joints (caprine arthritis-encephalitis virus), and immune complex-induced renal and hepatic dysfunction (equine infectious anemia virus) (24).

We (29) and others (5) have previously studied a highly conserved immunosuppressive sequence located in the transmembrane protein p15E of the oncornavirus subfamily of retroviruses, which includes murine leukemia virus, feline leukemia virus, and human T-cell lymphotropic virus types I and II. This sequence has been shown to inhibit a wide variety of immune functions in vitro when presented as a synthetic peptide (5, 13, 14, 16, 22, 26, 29). Subsequent work in our laboratory (28) and that of Cianciolo et al. (4) has demonstrated that a similar immunosuppressive sequence is present in a highly conserved region of the transmembrane protein gp41 of HIV and that this sequence also directly inhibits the enzymatic activity of protein kinase C (pkC) and Ca^{2+} influx mediated by the T-cell antigen receptor (CD3/Ti) (C. L. Ruegg and M. Strand, J. Immunol., in press). Gottlieb et al. have reported that the immunosuppressive sequence contained within p15E inhibits pkC (12).

The apparent contrasts between the biology of infection by the visna virus and the immunodeficiency groups of lentiviruses led us to determine whether the genome of visna virus encodes any immunosuppressive sequences similar to that found in HIV, with the expectation that the lack of such a sequence might explain why immunosuppression is not as pronounced in visna virus infection. However, we did identify a region of the transmembrane protein of visna virus with high sequence similarity to the immunosuppressive sequence from amino acids 581 to 597 of HIV gp41. We also found that the visna virus genome contains a highly similar sequence in the *Q* or *sor* open reading frame with significant homology to the immunosuppressive sequence of the murine retroviral p15E. We synthesized peptides encoding the sequences identified in the visna virus *Q* and transmembrane proteins and observed that lymphoproliferation and pkC enzymatic activity were inhibited by these peptides.

MATERIALS AND METHODS

Synthetic peptides. Synthetic peptides were prepared, purified, and conjugated to carrier protein exactly as described previously (29).

Cell culture. The T-cell lines CTLL-2 and EL-4.IL-2 were obtained from the American Type Culture Collection (Rockville, Md.) and maintained as described previously (29). The Jurkat clone E6-1 (21) was obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Rockville, Md.) and grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, and 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). The murine hybridoma OKT3 was obtained from the American Type Culture Collection, maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum and 2 mM glutamine, and passaged in mice to produce ascites fluid.

Proliferation assays. The synthetic peptides were tested for immunosuppressive activity by determining their effects on lymphoproliferation in vitro. Proliferation of CTLL-2 cells as measured by [³H]thymidine uptake was carried out as

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FIG. 1. Schematic representation of the lentiviral sequences studied and their approximate locations within the lentiviral genome. The Q- and transmembrane-derived visna virus sequences designated VQ4 and VTM.1, respectively, are aligned with their p15E- and HIV-derived counterparts, and the peptide designations appear in parentheses. :, Sequence identity; ·, conservative substitution. The external envelope protein (ext) and transmembrane protein (tm) components of the *env* polypeptide are indicated.

described previously (29), except that the serum-free cell culture supplement Nutridoma SP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at a final concentration of 1% was used in place of fetal calf serum. Proliferation of anti-CD3-stimulated human peripheral blood mononuclear cells (PBMC) was measured as described previously (28), except that Nutridoma was substituted for serum as noted above. Jurkat cells were cultured in 96-well plates at 2×10^4 cells per well in a total volume of 200 μ l of the same proliferation medium used for PBMC but lacking OKT3 ascites; the cells were incubated in the presence of synthetic peptides at the concentrations specified in Fig. 2 to 5 for 24 h at 37°C in humidified 5% CO₂, and proliferation was measured as for CTLL-2 cells by [³H]thymidine pulse during the final 4 h of culture.

pkC assays. The effects of the peptides on pkC enzymatic activity were determined by using enzyme partially purified from rat brains by DEAE-cellulose chromatography as previously described (18). Enzymatic assays were performed as described previously (19), with the following modifications: all reaction components except ATP were first preincubated for 5 min, after which the reactions were initiated by the addition of [³²P]ATP (Amersham Corp., Arlington Heights, Ill.) and allowed to proceed for 10 min (in preliminary experiments, the reaction rate was found to be linear over this time). The reactions were terminated by the addition of 100 μ l of 25% trichloroacetic acid, and the amount of ³²P incorporated into trichloroacetic acid-precipitable material was quantitated by liquid scintillation spectrofluorometry. The preparation and assay of Jurkat-derived pkC were conducted exactly as described previously (19).

RESULTS

Selection of sequences from visna virus proteins. We have previously identified a sequence of 10 residues, designated MOLV.23 (Fig. 1), from the transmembrane protein p15E of oncornaviruses that is the minimum sequence required for the inhibition of lymphoproliferation (29). We subsequently identified a sequence of 17 amino acids, from amino acid 581 to 597 (Fig. 1), from the transmembrane protein gp41 of HIV type 1 (HIV-1) that is similar but not identical to MOLV.23 and that exhibits a similar degree of inhibitory activity in lymphoproliferation assays (28). Both these sequences were used as probes in a computer-assisted sequence analysis (6)

to identify any similar sequences present in the genome of visna virus. Figure 1 shows a schematic representation of the genetic organization of the visna virus and HIV genomes. Using the HIV transmembrane sequence from amino acid 581 to 597 as the search probe, we identified a similar sequence, designated VTM.1, which consists of *env* residues 719 to 733 of visna virus (Fig. 1). Similarity analysis of the visna virus genetic sequence with MOLV.23 as the probe resulted in the identification of a region consisting of residues 74 to 87 of the Q protein of visna virus, designated VQ4, that encodes a sequence highly similar to the immunosuppressive p15E sequence MOLV.23 (Fig. 1). Peptides containing the regions of similarity identified by this analysis were synthesized (Fig. 1) and tested for inhibitory activity in assays of lymphoproliferation and pkC enzymatic activity. The peptide designated aa655-671 was derived from the HIV-1 transmembrane protein and used as a control since we had previously shown that it is devoid of activity in all assays used, although its degree of homology to the p15E sequence is similar to that of amino acids 581 to 597 (28).

Inhibition of CTLL-2 proliferation by synthetic peptides. We first tested the visna virus peptides for their effects on the murine T-cell line CTLL-2 and found that both the VQ4 and VTM.1 visna virus-derived synthetic peptides specifically inhibited interleukin-2 (IL-2)-dependent proliferation (Fig. 2). The peptides VQ4, VTM.1, and aa581-597 inhibited proliferation in a dose-dependent manner, with half-maximal inhibitory doses (ID₅₀) ranging from ~3 to 8 μ M. The difference in potency for VQ4 and MOLV.23 observed in this particular assay is exaggerated compared with the consensus observed over several separate assays (data not shown). The inhibition was sequence specific, since neither a control peptide, aa655-671, from HIV gp41 nor bovine serum albumin conjugated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in the absence of any peptide [BSA(edc)] had any effect on CTLL-2 proliferation (Fig. 2).

Inhibition of anti-CD3-stimulated PBMC proliferation by synthetic peptides. We then examined the effects of the visna virus peptides on the proliferation of human PBMC stimulated via the CD3/Ti receptor expressed on T cells. In agreement with the results observed with the CTLL-2 assay, both the VQ4 and VTM.1 peptides inhibited this assay of immune function in a manner similar to that observed for

FIG. 2. Inhibition of proliferation of CTLL-2 cells by synthetic peptides. Percent inhibition was calculated by using the following formula: percent inhibition = $[1 - (\text{exp cpm} - \text{bkgd cpm}) / (\text{total cpm} - \text{bkgd cpm})] \times 100$, where bkgd cpm is $[^3\text{H}]$ thymidine uptake in the absence of IL-2 and/or other mitogens and synthetic peptides ($2,919 \pm 346$), total cpm is uptake in the presence of IL-2 without peptides ($106,108 \pm 2,353$), and exp cpm is the uptake in the presence of IL-2 and peptides in the concentrations specified. Each point is expressed as the mean $\pm$ standard error of the mean of quadruplicate samples and is representative of three separate experiments. Values for the concentration of BSA(edc) are those that correspond to an equivalent bovine serum albumin concentration in the peptide conjugate samples.

aa581-597, with ID_{50} s of 5 to 20 μM (Fig. 3). No inhibition was observed with the negative controls aa655-671 and BSA(edc), indicating that this inhibition of human lymphocytes by the visna virus peptides was also sequence specific.

Inhibition of proliferation of Jurkat T cells by synthetic peptides. The peptides were then tested for their effects on the proliferation of the well-characterized Jurkat human T-cell line. In agreement with the results obtained in the previous lymphoproliferation assays, visna virus peptides VTM.1 and VQ4, as well as aa581-597, displayed similar

FIG. 3. Inhibition of proliferation of OKT3-stimulated human PBMC by synthetic peptides. Data were obtained and calculated exactly as they were for Fig. 1. Values for total cpm and bkgd cpm were $98,197 \pm 5,646$ and $1,254 \pm 177$, respectively.

FIG. 4. Inhibition of proliferation of Jurkat T cells by synthetic peptides. Data were obtained and calculated exactly as they were for Fig. 1, except that percent inhibition was calculated by the following formula: percent inhibition = $[1 - (\text{exp cpm} / \text{total cpm})] \times 100$. The value (mean $\pm$ standard error of the mean) obtained in the absence of peptide (total cpm) was $71,104 \pm 4,256$. These data are representative of two separate experiments.

potencies in the inhibition of Jurkat cell proliferation, with ID_{50} s of $\sim 10 \mu\text{M}$ (Fig. 4).

As was previously observed for the MOLV.23 and aa581-597 peptides (28, 29), the VQ4 and VTM.1 peptides were not cytotoxic (as determined by trypan blue exclusion), and their inhibitory activity was dependent on conjugation to carrier protein, since neither VQ4 nor VTM.1 inhibited any of the lymphoproliferation assays when added as a free peptide. Furthermore, the inhibitory effects of VQ4 and VTM.1 on cellular proliferation appeared to be specific for cells of immune lineage, because neither VQ4 nor VTM.1 inhibited the proliferation of NIH 3T3 cells (data not shown). The inhibition of lymphoproliferation observed for the VQ4 and VTM.1 peptides in this study was not restricted by the species origin of the target cell; a similar lack of restriction has previously been shown for the MOLV.23 and aa581-597 peptides (28, 29).

Inhibition of pkC activity by synthetic peptides. To examine further the molecular mechanism by which these visna virus-derived peptides inhibit lymphoproliferation, we tested them for their effects on the enzymatic activity of pkC. We have previously shown by using an in vitro phosphotransferase assay of pkC that the peptide aa581-597 from HIV directly inhibits partially purified pkC in a sequence-specific and dose-dependent manner (Ruegg and Strand, submitted). We found that both VQ4 and VTM.1 directly inhibited pkC in a manner similar to that observed for aa581-597, exhibiting ID_{50} s of ~ 2 to $20 \mu\text{M}$; aa655-671 and BSA(edc) had no effect (Fig. 5).

To ensure that inhibition of pkC isolated from rat brain was relevant to our investigation of the effects of the peptides on immune function, we tested the VQ4 and VTM.1 peptides for their effects on the enzymatic activity of pkC partially purified from the cytoplasmic compartment of Jurkat T cells and found that the peptides inhibited Jurkat-derived pkC in a manner indistinguishable from that of rat brain pkC (data not shown). Furthermore, conjugation of peptides to bovine serum albumin was required for the inhibition of pkC, since free peptide had no effect on pkC activity at concentrations as great as 1 mM (data not shown).

FIG. 5. Inhibition of pK by synthetic peptides. Percent inhibition was calculated by the following formula: percent inhibition = $[1 - (\text{exp cpm} - \text{bkgd cpm}) / (\text{total cpm} - \text{bkgd cpm})] \times 100$, where exp cpm is the counts per minute of trichloroacetic acid-precipitable ^{32}P obtained in the presence of peptides at the concentrations specified; bkgd cpm represents the counts per minute precipitated from reactions in which Ca^{2+} , phospholipids, and peptides were excluded and EGTA [ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid] was added ($2,858 \pm 267$); and total cpm is the counts per minute precipitated from reactions performed in the presence of Ca^{2+} and phospholipid and in the absence of peptides ($60,289 \pm 1,465$). Each point represents the mean $\pm$ standard error of the mean of triplicate samples and is representative of three separate experiments. Values for the concentrations of BSA(edc) were derived as they were for Fig. 1.

A similar requirement had previously been found for aa581-597 (Ruegg and Strand, submitted) and was also observed for the VQ4- and VTM.1-mediated inhibition of lymphoproliferation described above.

DISCUSSION

In this study, we have identified two sequences within the visna virus genome that, when presented as synthetic peptides, inhibit lymphoproliferation and the enzymatic activity of pK in a dose-dependent and sequence-specific manner. The inhibition mediated by these visna virus peptides is in agreement with that previously observed for similar sequences derived from HIV and the *Oncovirinae* by ourselves (28, 29; Ruegg and Strand, in press) and others (4, 5, 12). In studies using the HIV peptide (Ruegg and Strand, in press), we observed that the inhibition of pK was mediated at the level of the catalytic domain of the enzyme and thus was independent of any regulatory effects of Ca^{2+} and diacylglycerol. Furthermore, we found that in lymphocytes exposed to the HIV peptide, pK-mediated phosphorylation of the CD3 γ -chain was inhibited. These observations suggest that biologic functions that are dependent on pK in vivo may be affected by these inhibitory retroviral sequences. The potency with which these retrovirally derived synthetic peptides inhibit both lymphoproliferation and pK enzymatic activity is consistently observed at micromolar concentrations, yet it is not expected that the corresponding native retroviral proteins would be found at such concentrations in vivo. However, because of a failure to assume the proper conformation, the affinity of these synthetic peptides for their cellular targets may be substantially reduced, compared with that observed for the native protein. In fact, we

have previously observed that the potency of the inhibitory sequence from the murine retroviral transmembrane protein increases 10-fold when presented in the form of a recombinant protein monomer and as much as 1,000-fold when presented as a multimeric complex of the native protein (29).

The inhibitory activities exhibited by the visna virus peptides in this study suggest that the respective Q and envelope transmembrane proteins of the virus might mediate similar activities in vivo. Whereas infection with lentiviruses such as HIV and simian immunodeficiency virus results in clinical symptomatology that can be categorized as immunosuppressive, ungulates infected with visna virus and caprine arthritis-encephalitis virus lentiviruses do not manifest immunodeficiency, at least as a hallmark symptom, but rather display pathology that is the result of unchecked inflammation (24). The inflammatory pathology observed in visna virus infection is highly reminiscent of that seen in autoimmune disease (23). The inflammatory pathology exhibited in autoimmune disease is often accompanied by the loss of various immune functions such as IL-2 production and responsiveness (1, 2, 20), granulocyte function (27), and natural killer cell function (3). Similar disabling forms of cellular immune dysfunction are also observed in patients infected with HIV (7). A recent study of the clinical immune status of sheep infected with visna virus showed that naturally infected animals that exhibited the disease displayed a significantly depressed ratio of $\text{CD4}^+/\text{CD8}^+$ T lymphocytes that resulted primarily from a decrease in the absolute number of CD4^+ cells (15). The depressed $\text{CD4}^+/\text{CD8}^+$ ratio phenotype, which is a notable symptom of patients with acquired immunodeficiency syndrome, was not observed in sheep that had been experimentally infected and showed no clinical signs of disease.

Extended latency and persistence are characteristic of lentiviral infection (24). In addition, visna virus exhibits a remarkable restriction of its replicative potential to mature macrophages; infection of monocytoic precursors of this host cell leads to silent infection or greatly repressed viral replication (11). Indeed, cytokines and other factors that induce the differentiation of monocytes to the mature macrophage phenotype cause infected cells to undergo a sudden burst of viral replication as the host cell restriction is released (10). Recent studies have dissected further the genetic regulation of visna virus replication in molecular terms. It is now known that the transcription of the visna virus genome is tightly regulated by agents that affect pK (9). This information regarding the involvement of pK in the regulation of visna virus replication is particularly interesting in light of the interaction of the visna virus-encoded peptides with pK described in the present study. It is possible that these pK inhibitory sequences, present in the transmembrane and Q proteins of visna virus, mediate a negative feedback signal during the replicative burst phase of the viral life cycle, when the amount of viral polypeptides present would be predicted to be at a maximum, and that these sequences may reach a concentration sufficient to inhibit pK. The concept that lentiviruses mediate their own negative feedback signals is not novel to this study; it has been shown by J. E. Clements and her colleagues that visna virus induces the production of a unique form of interferon in T lymphocytes that are incubated with visna virus-infected macrophages (25). Furthermore, this lentivirus-induced interferon inhibits the differentiation of monocytes to the more permissive macrophage cell type as well as viral transcription in infected macrophages, in addition to blocking fusion of infected macrophages with synovial membrane cells (32).

Thus, it appears that the lentiviruses have evolved a complex system of signals that regulate the life cycle of their interaction with the host cell.

The biologic properties of the polypeptides of visna virus are still in the process of being defined. While it is known that the transmembrane protein of lentiviruses serves to anchor the external envelope protein to the viral surface and has been implicated in viral fusion (17), it also possesses other activities, as demonstrated in this study. In contrast, no function has yet been attributed to the Q protein in visna virus (30). The highly basic charge on this protein has led to the speculation that it may bind DNA and thus serve to regulate gene expression (30), although such activity has not yet been demonstrated. The one function that has been attributed to Vif (virion infectivity factor), the HIV homolog of the Q protein, is that it is required for the production of infectious virus (8, 31). Deletion mutations introduced into the *vif* gene of HIV lead to the loss of infectious cell-free virus but have no obvious effect on viral transcription or translation or cell-to-cell virus transmission (8, 31). Despite the conserved position of the Q and *vif* genes in the genomes of these lentiviruses and the similar size and basic charge of the encoded proteins, there is no significant degree of sequence homology between Q of visna virus and *vif* of HIV (30). Furthermore, there is no region of *vif* that is similar to that of Q shown here to inhibit pKc and lymphoproliferation.

Because of the general degree of homology between the genomes of the lentiviruses HIV and visna virus, it is perhaps not surprising that the alignment of the HIV sequence from amino acid 581 to 597 identifies a homologous sequence in visna virus (designated in this study as VTM.1) and that these two sequences occur at roughly the same location in the respective genomes of the two viruses. Less predictable, perhaps, is the finding that the highly conserved immunosuppressive region of the transmembrane protein p15E of the retroviral subfamily *Oncovirinae* shares a high degree of similarity with a sequence not in the transmembrane protein but rather in the Q protein of visna virus, for which there exists no homolog in the genomes of the *Oncovirinae*. The apparent conservation of this short but functional peptide sequence not only across viral subfamilies but also within the respective genes of these viruses supports the hypothesis that this sequence plays a biologic role in the life cycle of these retroviruses. The information gained from this study should enable the rational design of experiments to elucidate the function(s) of these sequences in the viral genome by examining the properties of mutant viruses in which these sequences have been selectively altered by methods of recombinant DNA technology and related approaches.

ACKNOWLEDGMENTS

The Jurkat clone E6-1 was obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases (Rockville, Md.) from contributor Arthur Weiss. We are grateful to Deborah McClellan for editorial assistance and J. Thomas August for critical reading of the manuscript.

This work was supported by Public Health Service research grants AI-28206 (M.S.), NS-23039, and NS-21916 (J.E.C.) and by research training grant CA-09243 (C.L.R.) from the National Institutes of Health.

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