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Enhancement of SIV Infection with Soluble Receptor Molecules

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The CD4 receptor on human T cells has been shown to play an integral part in the human immunodeficiency virus type 1 (HIV-1) infection process. Recombinant soluble human CD4 (rCD4) was tested for its ability to inhibit SIVagm, an HIV-like virus that naturally infects African green monkeys, in order to define T cell surface receptors critical for SIVagm infection. The rCD4 was found to enhance SIVagm infection of a human T cell line by as much as 18-fold, whereas HIV-1 infection was blocked by rCD4. Induction of syncytium formation and de novo protein synthesis were observed within the first 24 hours after SIVagm infection, whereas this process took 4 to 6 days in the absence of rCD4. This enhancing effect could be inhibited by monoclonal antibodies directed to rCD4. The enhancing effect could be abrogated with antibodies from naturally infected African green monkeys with inhibitory titers of from 1:2,000 to 1:10,000; these antibodies did not neutralize SIVagm infection in the absence of rCD4. Viral enhancement of SIVagm infection by rCD4 may result from the modulation of the viral membrane through gp120-CD4 binding, thus facilitating secondary events involved in viral fusion and penetration.

RECEPTOR-MEDIATED INFECTION of T cells by HIV-1 has been studied extensively, and reports have shown that the interaction of HIV-1 gp120 with CD4 is the primary event required for infection of susceptible human T cells (*1, 2*). Subsequent events responsible for penetration are theorized to include direct fusion processes by way of a region within the transmembrane protein (TMP) (*3*). The exact sequence of events leading to penetration, uncoating, and replication of HIV is unknown. Members of the immunodeficiency virus family that were originally isolated from nonhuman primates also replicate efficiently in human CD4-positive T cells (*4–10*), although some restriction of infection has been reported for SIVagm and SIVmac (*8–11*). We began these studies to define the nature of receptor-mediated infection by SIVagm viruses and to evaluate the biological relation of SIVagm with HIV-1 in their requirements for gaining cell entry and replication.

Monoclonal antibodies (MAbs) to CD4 (including anti-Leu3a and OKT4A) define major epitopes on CD4 that overlap those regions responsible for gp120 binding and therefore efficiently block HIV-1 infection of CD4-bearing T cell lines (*2, 12*). To determine if SIVagm(tyo-1), an isolate previously characterized and shown to share approximately 50% nucleic acid sequence homology with HIV-1 (*8*), also uses similar epitopes on CD4 for gp120 binding, a panel of mouse MAbs to CD4 (anti-CD4) were tested for their ability to block infection of a CD4-positive T cell line (MOLT-4 cl.8) by SIVagm(tyo-1) and HIV-1. This cell line is highly permissive for SIVagm infection as assessed by syncytium formation, unlike most other human CD4-bearing T cell lines (*6*). Those antibodies capable of blocking HIV-1 were also able to block SIVagm(tyo-1) infection, as measured by a total reduction in syncytium formation (Table 1). Monoclonals that bind to regions other than the NH_2-terminal V1 domain of CD4 did not block either HIV-1 or SIVagm(tyo-1) infection. These data suggest that SIVagm(tyo-1) uses essentially the same CD4 epitopes as HIV-1 for binding and that binding through the CD4 molecule is a prerequisite for infection.

Recombinant soluble human CD4 (rCD4) blocks HIV-1 infection of human T cell lines (*13, 14*). Incubation of rCD4 (25 μg/ml) with HIV-1 resulted in complete inhibition of syncytium formation in MOLT-4 cl.8 cells. In contrast, incubation of rCD4 with SIVagm(tyo-1) resulted in syncytium formation within the first 24 hours of infection rather than 4 to 6 days after infection as normally observed in the absence of rCD4 (Fig. 1). The enhanced rate of syncytium formation in the presence of rCD4 makes it unlikely that the lack of inhibition is due to a lower affinity of human-derived CD4 for SIVagm. The effects of rCD4 on SIVagm were concentration dependent; concentrations as low as 250 ng/ml enhanced syncytium formation by SIVagm at 1 to 2 days after infection. At concentrations below 250 ng/ml, there was no enhancement, and syncytium formation was observed at 6 days. The effect of rCD4 on HIV-1 was also concentration depen-

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Table 1. HIV-1 and SIVagm blocking by monoclonal antibodies. For evaluating the blocking effects of infection with MAb to CD4, 5×10^4 MOLT-4 cl.8 cells were incubated with antibody (5 μg/ml) for 1 hour at 25°C in a volume of 100 μl and then combined with 100 μl of virus diluted 1:2 in RPMI 1640 supplemented with 15% FBS in 96-well microtiter wells. Cells were observed on a daily basis for cytopathic effects, and 100 μl of culture medium was replaced with fresh medium at day 4. Virus concentrations for HIV-1 and SIVagm(tyo-1) used in this study reproducibly induced syncytium formation at 6 days. Scoring was as follows: + indicates greater than 99% inhibition of syncytium formation at 6 days after infection; −, complete syncytium formation at 6 days; and +/−, inhibitory effects seen before day 6, but complete syncytium formation observed at 6 days.

Monoclonal antibodies	Inhibition of syncytium formation	
	HIV-1	SIVagm
anti-Leu3a	+	+
OKT4A	+	+
L110	+	+
L34	+	+
L200	+	+
L93	+	+
L71	+/−	+/−
L83	+/−	+/−
L201	+/−	+/−
OKT4	−	−
L67	−	−
L80	−	−
L117	−	−

dent, with 250 ng/ml being the lowest concentration that blocked syncytium formation at 6 days (*15*).

Serial tenfold dilutions of SIVagm(tyo-1) virus stocks were compared in the presence or absence of rCD4 for infection of MOLT-4 cl.8 cells to evaluate the effects of rCD4 on the efficiency of virus infection (*16*). Infection by SIVagm in the presence of rCD4 could be demonstrated at a 10^{-3} dilution of virus stock in contrast to the 10^{-1} dilution needed without rCD4. This finding suggests that rCD4 acts not only by enhancing the rate of infection, but also by augmenting the infectious nature of the virus stock. However, enhancement of syncytium formation at 2 days after infection in the presence of rCD4 was only observed at the 10^{-1} dilution, indicating that there may be a critical concentration of infectious virus required for enhancement that is 100-fold greater than that required for infection.

It was still possible that the accelerated rate of syncytium formation might simply be caused by cell-to-cell fusion through bridging with SIVagm that had been stabilized with rCD4 rather than by an enhanced rate of infection. However, radioimmunoprecipitation (RIP) revealed a significant amount of viral protein synthesis in the first 24 hours after infection when rCD4 was incubated with SIVagm, whereas only weak de novo synthesis of viral proteins occurred, beginning at day 4 in the absence of rCD4. By day 4, high levels of both gp160/gp120 and p55 were observed from cells infected with rCD4-treated SIVagm (Fig. 2A). No viral proteins were observed when rCD4 was incubated with HIV-1 (Fig. 2B). The time of appearance of viral protein in T cells infected with untreated HIV-1 was comparable to that for untreated SIVagm.

Although not strictly quantitative, analysis of autoradiographs from rCD4-treated and rCD4-untreated SIVagm-infected cells by densitometer scanning of gp160/gp120 showed an approximate 18-fold increase in viral protein synthesis in rCD4-treated SIVagm-infected cells at day 4. These results suggest that the enhanced cytopathic effects related to syncytium formation were probably a result of enhanced replication and expression of viral envelope proteins at the surface of infected cells.

Although rCD4 appears to enhance viral infection of SIVagm, we wanted to determine if this effect could be blocked with MAbs to CD4, thus demonstrating specificity of rCD4 for gp120 receptor binding domains. Incubation of anti-Leu3a (1 μg/ml) with rCD4 (0.5 μg/ml) for 2 hours before addition to SIVagm blocked the enhancing effects of rCD4. In contrast, when this same concentration of anti-Leu3a was incubated with MOLT-4 cl.8, as in Fig. 1, and the cells were then infected with rCD4-treated SIVagm(tyo-1), no inhibition of enhancement was observed. High concentrations of anti-CD4 could block both enhancement and infection, but this was not unexpected in view of the saturating concentrations of antibody that could influence binding of SIVagm to both rCD4 and cellular CD4. Other MAbs directed to sites on CD4 that are not involved in gp120 binding did not inhibit enhancement even at relatively high concentrations (25 μg/ml), with the exception of one anti-CD4 preparation designated L117 that had been shown to have no effect on SIVagm infection in the absence of rCD4 (Table 1). This antibody was found to inhibit enhancement regardless of whether the monoclonal was incubated with rCD4 before SIVagm or with uninfected cells before the addition of rCD4-treated SIVagm (*17*). Concentrations as low as 1 μg/ml were found to inhibit this enhancing effect (*15*).

This MAb was evaluated, by RIP analysis, for its ability to co-precipitate rCD4-gp120 to determine if the L117 inhibitory effect was related to events subsequent to gp120-CD4 binding. L117 could precipitate radiolabeled gp120 in the presence of rCD4 but not in its absence (Fig. 3). Because L117 could still bind to CD4 after the interaction of CD4 with gp120, the inhibition of enhancement by L117 may be a result of steric hindrance of the virus in its association with the cell surface, thus preventing penetration events after binding. This possibility is strengthened by the fact that other nonblocking MAbs to CD4 that bind to a

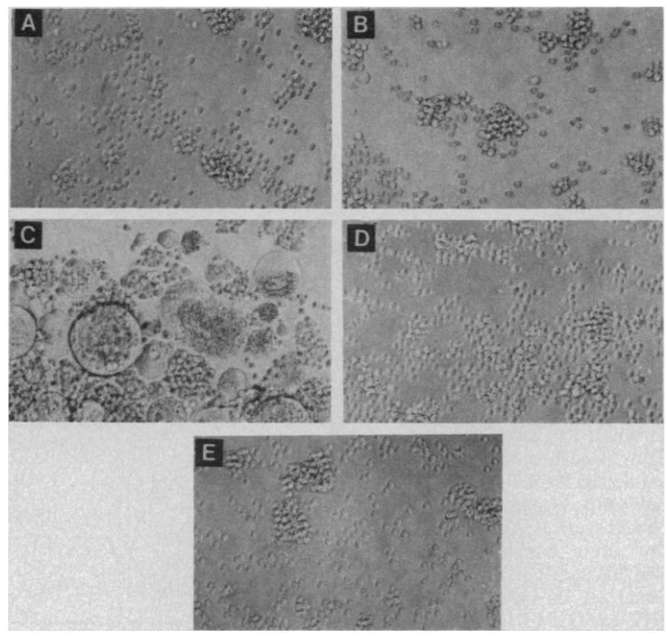

Fig. 1. Enhancement of SIVagm-induced syncytium formation by rCD4. Viruses were incubated in the presence or absence of rCD4 for 1 hour at 25°C, then mixed with an equal volume of 0.5×10^6 MOLT-4 cl.8 cells, and the cultures were observed for cytopathic effects. Photomicrographs were taken at 2 days after infection. MOLT-4 cl.8 cells were infected with the following: (**A**) culture medium without virus, (**B**) SIVagm incubated with medium alone, (**C**) SIVagm incubated with rCD4 (25 μg/ml) in culture medium, (**D**) HIV-1 (HTLV-III_B) incubated with culture medium alone, and (**E**) HIV-1 (HTLV-III_B) incubated with rCD4 (25 μg/ml). The virus concentration for SIVagm and HIV-1 for these studies was used at a final 1:4 dilution of culture supernatant fluid from infected cell lines. This concentration is ten times the infectious titer required to induce syncytia formation at 6 days after infection. Optical magnification was ×200.

similar region of CD4 as L117 failed to block the enhancing effect. In addition, these data show that rCD4 binds directly to gp120 of SIVagm, suggesting that rCD4 exerts its effects by interaction with gp120. Again, the orientation of rCD4 binding to gp120 probably mimics gp120 binding to naturally occurring CD4 expressed at the cell surface since another monoclonal antibody, anti-Leu3a, which normally blocks gp120 binding to CD4, was not able to coprecipitate gp120 in these experiments (Fig. 3, lane 2).

Neutralizing antibodies to HIV-1 are theorized to be important in controlling the spread of virus within the infected host, and most vaccine strategies are directed toward the induction of neutralizing antibodies (*18*). If rCD4 modulates the viral membrane by exposing secondary sites important for viral fusion and penetration, perhaps these sites could be blocked with antibodies to SIVagm. A panel of SIVagm seropositive and seronegative serum samples from African green monkeys was evaluated for antibodies that could block enhancement of SIVagm by rCD4 (Table 2). Incubation of SIVagm(tyo-1) with rCD4 followed by various concentrations of serum samples resulted in the inhibition of enhancement in all ten seropositive serum samples, but in none of the ten seronegative samples, suggesting that antibodies directed toward viral components may be involved in this inhibitory effect. In some cases, dilutions of serum samples to 1:10,000 were able to block in vitro rCD4 enhancement (Table 2). These serum samples did not neutralize the virus in the absence of rCD4 (*19*). Serum samples from HIV-1–infected humans did not block enhancement, but one SIVmac-seropositive serum sample from an experimentally infected rhesus monkey was able to block enhancement at high concentrations (1:5).

Evidence of enhancement of SIVagm-(tyo-1) infection by rCD4 was observed with one other human T cell line (Jurkat, Fig. 4C). In this case, reverse transcriptase levels were observed earlier after infection than occurred upon infection with untreated SIVagm, although the time course was considerably slower than for MOLT-4 cl.8–infected cells. Also, the degree of cytopathic effects varied from cell line to cell line but was generally correlated with the ability to recover virus from culture supernatants. A low level of virus expression was detected in culture fluids from untreated SIVagm–infected MOLT-4 cl.8 cells as compared to rCD4-treated virus-infected cells (Fig. 4A). MOLT-4 cl.8 cells are extremely sensitive to the cytopathic effects of viral infection, with few cells surviving the initial cell-to-cell fusogenic event. This results in a low titer of cell-free virus in non-rCD4–treated SIVagm. On the other hand, cell-free virus appears coincident with cytopathology in MOLT-4 cl.8 cells infected with rCD4-treated virus, consistent with the idea that virus replication may be accelerated because of the enhancing effects of rCD4. For C8166 cells, equivalent virus expression was observed both in the presence and absence of rCD4 (Fig. 4D), in contrast to the SIVagm blocking effects of rCD4 reported by Clapham *et al.* (*20*) for this cell line. The discrepancy in these findings may result from variations in both the assay conditions

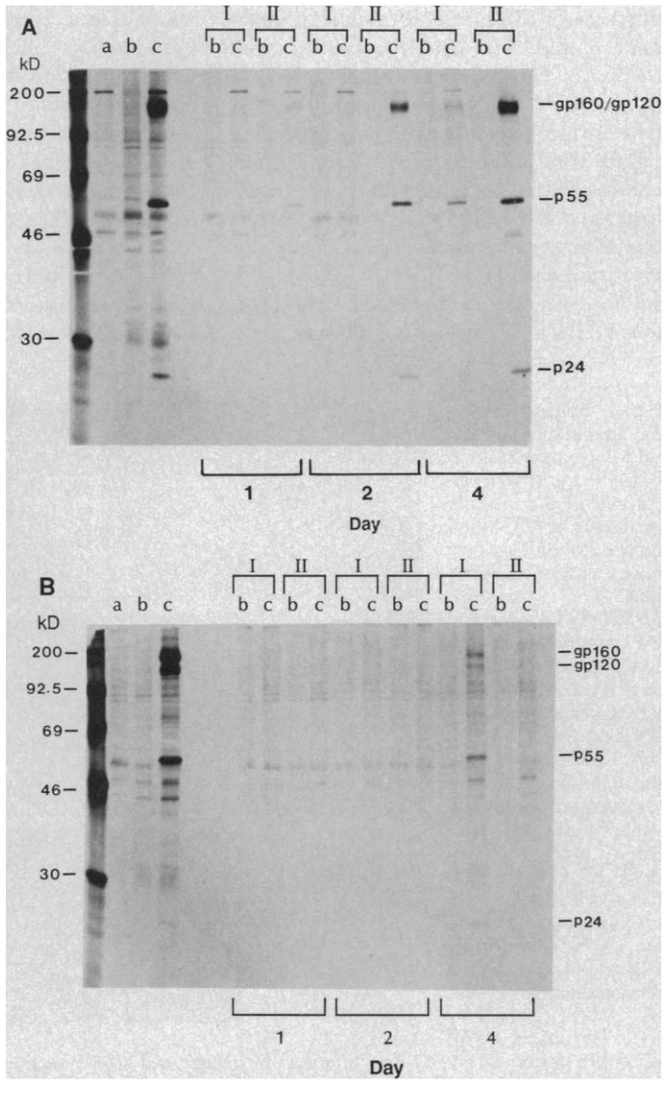

Fig. 2. Enhancement of SIVagm protein synthesis. Metabolic incorporation of [^{35}S]cysteine into viral proteins by cells infected with SIVagm (**A**) or HIV-1 (**B**) in the presence (II) or absence (I) of rCD4 (5 μg/ml). MOLT-4 cl.8 cells were infected with equivalent amounts of virus, and the cells were labeled for 6 hours at day 1, 2, and 4 after infection in the presence of [^{35}S]cysteine (100 μCi/ml) in RPMI 1640 (cysteine-free) supplemented with 15% FBS. Cell lysates were prepared, and radiolabeled proteins were immunoprecipitated with 10 μl of seronegative (b) or seropositive (c) serum samples and protein A–sepharose 4B; the immunoprecipitates were separated on 11.3% SDS-polyacrylamide gels, and the proteins visualized by autoradiography as described (*21*). Control radiolabeled cell lines are shown at the left of the figure. Cell lysates from uninfected MOLT-4 cl.8 cells were immunoprecipitated with a seropositive serum sample (a). The SIVagm(tyo-1)–infected cell line (A) or HIV-1 (HTLV-III$_B$)–infected cell line (B) was immunoprecipitated with a seronegative serum (b) or seropositive serum (c). The seropositive serum sample was from an experimentally infected SIVagm rhesus monkey in (A) and from an HIV-1–infected human in (B).

Table 2. Inhibition of rCD4 enhancement of SIVagm infection. For inhibition of enhancement, SIVagm was incubated with rCD4 (5 μg/ml) or culture medium before addition of various concentrations of heat-inactivated serum samples. Inhibition of enhancement was assessed by a total loss of syncytium formation at 2 days. Virus incubated with medium alone, or with rCD4 alone, were used as controls for observation of cytopathic effects. Virus concentrations were used at a final dilution of 1:4 from culture supernatants. Ten SIVagm seronegative serum samples and ten HIV-1 seropositive samples were also tested and did not inhibit enhancement.

Serum sample	Inhibition
SIVagm$^+$	
1	1:10,000
2	1:10,000
3	1:2,000
4	1:2,000
5	1:10,000
6	1:10,000
7	1:2,000
8	1:2,000
9	1:2,000
10	1:10,000
SIVmac$^+$	
1	1:5

and SIVagm viral strain used for infection of C8166 cells. Viral enhancement was also observed for a second isolate [SIVagm(gri-1)] in MOLT-4 cl.8 cells (Fig. 4B). Again, reverse transcriptase activity could be detected earlier in CD4-treated viral preparations. These results suggest that the enhancing effect of rCD4 is not limited to one virus or to one cell type. The rCD4 also blocked HIV-2 and SIVmac infection (*15, 20*). In addition, other non-CD4 cell types, including HeLa cells and CV-1 cells, could not be infected either in the presence or absence of rCD4 (*15*).

We have shown that rCD4 enhances in vitro SIVagm infection and subsequent cell fusion by altering both the potency of SIVagm and the kinetics of infection. One explanation for the receptor-induced enhancement would be the ability of rCD4 to activate determinants on the viral membrane responsible for events at the cell surface after gp120-CD4 binding. As gp120 is occupied by rCD4, it is unlikely that cellular CD4 would play a significant role in this observed enhancement. Normally, SIVagm infection would proceed in a stepwise fashion requiring gp120-CD4 binding at the cell surface, followed by as yet unknown secondary events in viral penetration. In the case of enhancement, soluble receptors may abrogate the requirement for CD4 on the cell. Activation of viral binding sites by rCD4 might potentiate the binding of the virus to secondary receptors on the cell surface. However, our results do not rule out the possibility that other sites on the CD4 molecule may be important in subsequent fusogenic events, nor can we exclude the possibility that rCD4 may itself modulate other molecules on the cell surface. In this latter case, the lack of HLA-DR expression on MOLT-4 cl.8 cells would preclude this class II molecule as a target for cellular modulation by rCD4.

The TMP represents one such candidate that could function in binding to putative secondary receptors involved in viral fusion. Support for a direct role of the TMP in second receptor binding follows from evidence that the fusogenic region appears to reside on the TMP (*3*). Furthermore, the ability of antibodies from SIVagm-infected monkeys to inhibit the enhancing effect of rCD4 suggests a more conserved and immunodominant determinant that is more likely to reside on the TMP.

Unlike SIVagm, HIV-1 infection is not enhanced by binding of rCD4. HIV-1 may not require secondary receptor binding sites, and thus fusion may occur directly with the membrane after CD4 binding at the cell surface. Alternatively, rCD4 interaction with HIV-1 may not expose viral domains that are responsible for putative secondary binding events required for in vitro enhancement. Reports that HIV-1 can infect CD4-negative cells (*20*) and that SIVagm is restricted in its ability to infect some CD4-positive cell types (*6–9*) suggest that cell tropism for immunodeficiency viruses may be determined by the expression of these putative secondary cell surface molecules.

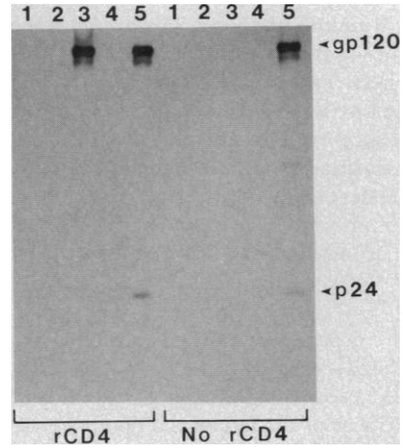

Fig. 3. Co-precipitation of SIVagm gp120 with a MAb to CD4. [^{35}S]cysteine-labeled purified SIVagm(tyo-1) was incubated in the presence or absence of rCD4 at 5 μg/ml for 2 hours at 25°C. The mixture was solubilized by the addition of Triton X-100 (1% final concentration), and the proteins were immunoprecipitated with no antibody (lane 1), anti-Leu3a (50 μg/ml) (lane 2), L117 (50 μg/ml) (lane 3), 10 μl of an SIVagm seronegative serum sample (lane 4), and 10 μl of an SIVagm seropositive serum sample from an experimentally infected rhesus monkey (lane 5) incubated with protein A–sepharose 4B as described (*21*). Immunoprecipitates were washed and denatured in sample buffer and the proteins separated by SDS–polyacrylamide gel electrophoresis (11.3% polyacrylamide gels), and autoradiography was performed. For metabolic labeling of SIVagm-infected cell lines, 20×10^6 cells were labeled for 16 hours with 2 mCi of [^{35}S]cysteine (Dupont Biotechnology Systems) in cysteine-free RPMI 1640 medium supplemented with 15% FBS. Supernatant culture fluid was filtered through a 0.45-μm filter, and the virus was sedimented through a 20% sucrose–phosphate buffered saline (PBS) interface at 20,000 rpm in an SW28 Beckman rotor. The virus pellet was resuspended in PBS before incubation with rCD4.

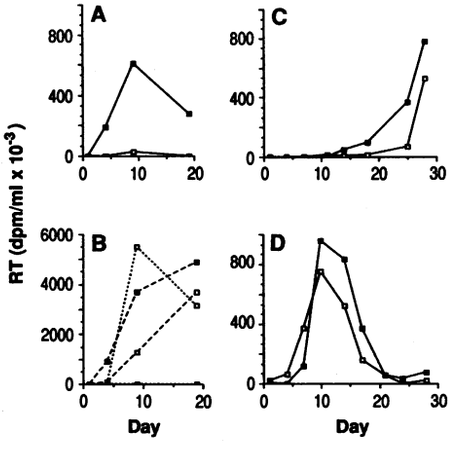

Fig. 4. Effect of rCD4 on viral strains and human T cell lines. Cell lines at 5×10^5 cells per milliliter in 24-well plates were infected with virus treated with rCD4 (5 μg/ml) for 2 hours at 25°C. Sixteen hours after infection the cells were washed extensively, and cell culture supernatants were harvested for reverse transcriptase (RT) activity as described (*22*); the cultures were observed daily for cytopathic effects. Solid squares indicate rCD4-treated preparations, and open squares represent nontreated virus. (**A**) SIVagm(tyo-1) infection of MOLT-4 cl.8 cells, (**B**) HIV-1 (dotted line) and SIVagm(gri-1) (dashed lines) infection of MOLT-4 cl.8 cells, (**C**) SIVagm(tyo-1) infection of Jurkat cells, and (**D**) SIVagm(tyo-1) infection of C8166 cells. In (A), (C), and (D) HIV-1 infection was also performed. In each case, HIV-1 infection could only be demonstrated in the absence of rCD4. All infections were performed in duplicate, and RT values reflect the mean from duplicate wells. SIVagm(gri-1) represents a strain originally isolated on SUPT1 cells by cocultivation with peripheral blood lymphocytes from a naturally infected green monkey (*9*). Infectious titers of virus were normalized by titration on MOLT-4 cl.8 cells. Individual RT values from duplicate experiments did not vary by more than 15% from the mean.

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16. Previously titrated frozen virus stocks were serially diluted tenfold from 10^0 to 10^{-5} in complete RPMI 1640 medium and incubated for 2 hours at 25°C with rCD4 (5 μg/ml) or media before being used to infect 5×10^4 MOLT-4 cl.8 cells in 96-well plates. All tests were performed in duplicate. Cells were observed daily for cytopathic effects until 2 weeks after infection, and the cultures were given fresh medium every 3 to 4 days.
17. For inhibition of viral enhancement, monoclonal antibodies at a concentration of 25 μg/ml in RPMI

1640, supplemented with 15% fetal bovine serum (FBS), were incubated for 2 to 4 hours with MOLT-4 cl.8 cells, and SIVagm(tyo-1) was incubated with rCD4 (5 μg/ml) before infection in 96-well plates, essentially as described in the legend to Fig. 1. Enhancement or inhibition was scored at 2 days after infection and was based on total inhibition of syncytium formation.
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19. For neutralization, heat-inactivated serum samples were incubated beginning at a dilution of 1:5 in complete medium with SIVagm for 2 hours at 25°C before the addition to MOLT-4 cl.8 cells. Cells were observed for cytopathic effects on a daily basis. Criterion for neutralization was a total loss in syncytium formation at 6 days.
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Calcium-Sensitive Cross-Bridge Transitions in Mammalian Fast and Slow Skeletal Muscle Fibers

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The fundamental mechanism underlying the differing rates of tension development in fast and slow mammalian skeletal muscle is still unknown. Now, in skinned (membrane-permeabilized) single fibers it has been shown that the rate of formation of the strongly bound, force-producing cross-bridge between actin and myosin is calcium-sensitive in both fast and slow fibers and that the rate is markedly greater in fast fibers. The transition rates obtained at high calcium concentrations correlated with myosin isoform content, whereas at low calcium concentrations the thin filament regulatory proteins appeared to modulate the rate of tension development, especially in fast fibers. Fiber type–dependent differences in rates of cross-bridge transitions may account for the characteristic rates of tension development in mammalian fast and slow skeletal muscles.

FAST AND SLOW MUSCLES ARE CHARacterized on the basis of differences in the duration of the isometric twitch and the maximum shortening velocity of unloaded shortening (*1*). During contraction in fast muscle, both the increase in myoplasmic Ca^{2+} concentration from $10^{-7}M$ to a peak of around $10^{-5}M$ (*2*) and the binding of Ca^{2+} to the low-affinity sites of troponin are too rapid to limit the rate of force development (*3, 4*). The Ca^{2+}-induced conformational changes in the thin filament are essentially complete before the initiation of tension development (*5*) and are thus unlikely to limit the rate of tension development in skeletal muscle.

We tested the hypothesis that the rate of formation of the strongly bound, force-producing cross-bridge state differs between fast and slow fibers from rat skeletal muscles, which could provide a basis for the difference in the rate of tension development between these fibers in vivo. We also determined whether the thin filament regulatory protein troponin is involved in conferring Ca^{2+} dependence to transition rates of attached cross-bridges.

We used a method that permits the measurement of the rate of the limiting step in the cross-bridge cycle leading to the production of force (*6*). Chemically skinned (sarcolemma permeabilized) single fibers are used so that the concentration of Ca^{2+} in the vicinity of the contractile proteins can be controlled directly. The measurement protocol involves a rapid release and subsequent reextension of fiber length during steady Ca^{2+} activation, resulting in the dissociation of cross-bridge connections to actin. Subsequently, tension redevelops to the initial value at a rate that is well fit by a single exponential curve. The observed rate constant of tension redevelopment (k_{tr}) reflects a rate-limiting step or steps in the transition of cross-bridges to the force-generating state.

Currently accepted models of the actomyosin kinetic scheme (*7*) consist of a series of reversible-state transitions, in which the rate-limiting steps are as yet unknown. In this scheme, k_{tr} is a measure of the rate of approach to steady-state isometric force. Thus, k_{tr} is determined by the arithmetic sum of the forward and reverse rate constants for those steps that are rate limiting in the process of force generation. The k_{tr} is Ca^{2+}-sensitive, increasing with Ca^{2+} concentration in the physiological range (*8, 9*). However, the influence of Ca^{2+} on k_{tr} is independent of the effect of Ca^{2+} to increase the number of cross-bridge attachments to the thin filament (*10*); thus, Ca^{2+} regulates k_{tr} via effects on specific rate constants that characterize cross-bridge transitions.

The value of k_{tr} differed in fast and slow muscle at maximal activation by Ca^{2+} (that is, at *p*Ca 4.5, where *p*Ca is the $-\log Ca^{2+}$ concentration) (Fig. 1). Mean k_{tr} values (± SEM) at *p*Ca 4.5 were 22.9 ± 0.5 s^{-1} (n = 20) in rat fast-twitch (type II_b) superficial vastus lateralis (svl) skinned fibers and 3.0 ± 0.1 s^{-1} (n = 12) in rat slow-twitch (type I) soleus skinned fibers. In both svl and soleus fibers, k_{tr} was maximal at *p*Ca 4.5, since the rate constant was unaffected by increasing Ca^{2+} concentration to *p*Ca 4.0.

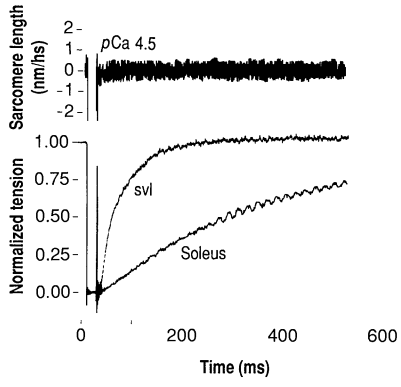

Fig. 1. Records of sarcomere length (upper traces) and tension (lower traces) obtained during the protocol to determine k_{tr}. Records were obtained during maximal Ca^{2+} activation of a fast svl skinned fiber and a slow soleus skinned fiber; sarcomere length traces from the svl and soleus are superimposed. The k_{tr} was 23.4 s^{-1} in the svl fiber and 3.0 s^{-1} in the soleus fiber. In both fibers, sarcomere length was clamped at 2.50 μm. Steady isometric tension was 55 mg in the svl fiber and 50 mg in the soleus fiber. Fiber type was determined on the basis of protein subunit composition by SDS–polyacrylamide gel electrophoresis (*14*). All soleus and svl fibers contained the slow and fast isoforms, respectively, of contractile (myosin heavy and light chains) and regulatory proteins (troponin). Records of tension redevelopment were best fit by a first-order exponential equation: $F_t = F_o(1 - e^{-kt})$, where F_t is force at time t, F_o is maximum force, and k is k_{tr}. Sarcomere length (obtained by laser diffraction) is held isometric to eliminate effects on k_{tr} caused by end compliance (*6*). Complete details of the experimental protocol, curve-fitting procedure, and mechanical set-up have been published (*9*). Relaxing and activating solutions consisted of 7 m*M* EGTA, 1 m*M* free Mg^{2+}, 4.42 m*M* total adenosine triphosphate, 14.5 m*M* creatine phosphate, 20 m*M* imidazole, and sufficient KCl to yield an ionic strength of 180 m*M*. The temperature was set at 15°C, and the solution *p*H was 7.00 (*9*); hs, half sarcomere.

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