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Requirements for Lymphocyte Activation by Unusual Strains of Simian Immunodeficiency Virus

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When residues 17 and 18 in nef of simian immunodeficiency virus strain SIVmac239 were changed from RQ to YE, the resultant virus was able to replicate in peripheral blood mononuclear cell cultures without prior lymphocyte activation and without the addition of exogenous interleukin-2, caused extensive lymphocyte activation in these cultures, and produced an acute disease in rhesus and pigtail macaques (Z. Du, S. M. Lang, V. G. Sasseville, A. A. Lackner, P. O. Ilyinskii, M. D. Daniel, J. U. Jung, and R. C. Desrosiers, Cell 82:665–674, 1995). These properties are similar to those of the acutely lethal pathogen SIVpbj14 but dissimilar to those of the parental SIVmac239. We show here that the single change of R to Y at position 17 in nef of SIVmac239 is sufficient to confer the full, unusual phenotype. Conversely, the lymphocyte-activating properties of SIVpbj14 were lost by the single change of Y to R at position 17 of nef. The change of R17F or Q18E in SIVmac239 nef did not confer the unusual in vitro properties. Since SIVpbj14 has a duplication of the NF-κB binding sequence in the transcriptional control region, we also constructed and tested strains of SIVmac239/R17Y with zero, one, and two NF-κB binding elements. We found no difference in the properties of SIVmac239/R17Y, either in cell culture or in vivo, whether zero, one, or two NF-κB binding sites were present. Thus, tyrosine at position 17 of nef is absolutely necessary for the unusual phenotype of SIVpbj14 and is sufficient to convert SIVmac239 to a virus with a phenotype like that of SIVpbj14. Multiple NF-κB binding sites are not required for the in vitro properties or for acute disease.

We recently reported that the change of amino acids at positions 17 and 18 in nef of simian immunodeficiency virus strain SIVmac239 from RQ to YE created a virus capable of replicating in lymphocytes without prior lymphocyte activation and without the addition of exogenous interleukin-2 (IL-2) (5). This property is very unusual for the human immunodeficiency virus and SIV group of viruses which ordinarily require prior lymphocyte activation for reasonable levels of virus replication (14, 23). Activation of lymphocytes increases the pool size of nucleotides needed for reverse transcription, facilitates efficient nuclear targeting of viral DNA, and induces transcription factors such as NF-κB important for expression of proviral DNA (10, 15, 20, 22). The ability of SIVmac239 with the YE change to replicate in peripheral blood mononuclear cells (PBMC) in the absence of lectin stimulation and in the absence of exogenously added IL-2 results from its ability to cause extensive lymphocyte activation (5). SIVmac239/YE also induces an acute disease characterized by severe diarrhea, rash, and extensive pathology in the gastrointestinal tract. Gastrointestinal lesions are characterized by extensive lymphocyte activation and proliferation, inflammation, blunting of villi, and destruction of architecture.

The specific change of RQ to YE was selected on the basis of sequence comparison to SIV strain pbj14 (SIVpbj14) and the nature of sequence motifs produced by these changes. SIVpbj14 had been described several years earlier as an acutely lethal pathogen capable of causing lymphocyte activation in cell culture (7, 8). The presence of YE at positions 17 and 18 of nef created a series of tyrosine residues with a spacing and context reminiscent of the immune receptor tyrosine-mediated activation motifs (ITAMs) present on the cytoplasmic domains of B- and T-cell antigen receptors and Fc receptors (17, 19). The ITAMs function as SH2 binding domains for the transduction of signals from the cell surface (for reviews, see references 1 and 16).

While most strains of SIVmac and SIVsmm contain a single NF-κB binding element, SIVpbj14 is also unusual in that the NF-κB element is duplicated (2). It has been suggested that the presence of two NF-κB transcriptional control elements may contribute to the unusual properties of SIVpbj14 (3, 4). Here, we describe the effects of several variations at the nef and NF-κB loci. Our results show that tyrosine at position 17 of nef is absolutely necessary for the unusual phenotype of SIVpbj14 in vitro and is sufficient to convert SIVmac239 to a virus with a phenotype like that of SIVpbj14. Not only are two NF-κB binding elements not needed for the unusual phenotype of SIVmac239/YE, even a single NF-κB element is unnecessary.

Mutations in nef were created by site-specific mutagenesis according to previously described procedures (5, 6). The mutations in nef used for the current studies are shown in Fig. 1. Construction of variant forms of SIVmac239 with zero, one,

SIVmac239	^{10}SRPSGDLRQRLLRARGET**YGRL**LGEVEDGYSQSP^{43}
SIVmac239YE	^{10}SRPSGDL**YERL**LRARGET**YGRL**LGEVEDGYSQSP^{43}
SIVmac239FE	^{10}SRPSGDLFERLLRARGET**YGRL**LGEVEDGYSQSP^{43}
SIVmac239R17Y	^{10}SRPSGDL**YQRL**LRARGET**YGRL**LGEVEDGYSQSP^{43}
SIVmac239Q18E	^{10}SRPSGDLRERLLRARGET**YGRL**LGEVEDGYSQSP^{43}
SIVmac239YE/Y28F	^{10}SRPSGDL**YERL**LRARGETFGRLLGEVEDGYSQSP^{43}
SIVPBj1.9	^{10}RRRGGNL**YERL**LQARGET**YGRL**WEGLEGEYSQSQ^{43}
SIVPBj1.9Y17R	^{10}RRRGGNLRERLLQARGET**YGRL**WEGLEGEYSQSQ^{43}

FIG. 1. Sequences of nef mutants. The amino acid sequences of SIVmac239, SIVpbj14, and nef mutants are shown from residues 10 to 43. The YXXL motifs are indicated in bold.

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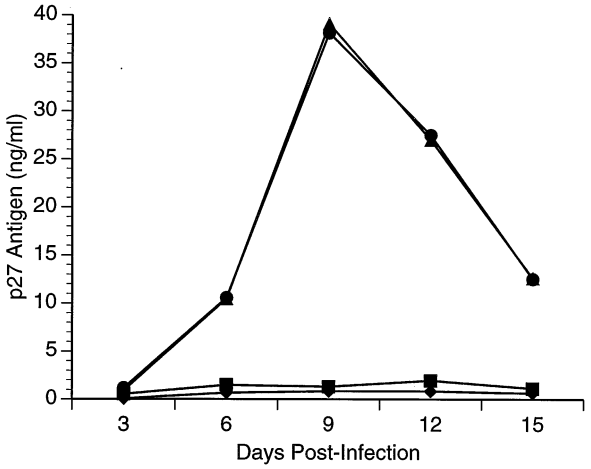


FIG. 2. Replication of SIVmac239 (■) and SIVmac239 nef mutants (YE [●], R17Y [▲], and Q18E [◆]) in resting PBMC. Twenty million resting PBMC in 2 ml were infected with virus containing 20 ng of p27. The cells were cultured in RPMI-1640 medium with 10% fetal calf serum and without the addition of mitogen or IL-2. Virus production was monitored by antigen capture assay (Coulter Corporation, Hialeah, Fla.). PBMC from rhesus monkey Mm187-91 were used for this experiment.

and two NF-κB binding elements has recently been described (11). SIVmac239 mutants with a change in amino acid R to Y at position 17 in nef (SIVmac239/R17Y) with zero, one, and two NF-κB binding elements were constructed by exchange of restriction fragments. The NF-κB variations were introduced into both long terminal repeats. The proper structure of all variants was confirmed by DNA sequencing. Virus stocks were prepared by transfecting cloned DNA into CEMx174 cells by a DEAE dextran procedure and harvesting supernatant virus 10 days after transfection (5). Rhesus monkey PBMC were isolated from healthy, SIV-negative rhesus monkeys. Virus replication in resting and phytohemagglutinin (PHA)-stimulated PBMC was analyzed by monitoring p27 antigen production in the cell-free supernatant. Resting PBMC cultures were incubated in RPMI 1640 medium with 10% fetal calf serum without the addition of mitogen and without IL-2. PHA-activated PBMC were cultured similarly in the presence of IL-2.

The change of nef amino acid Q-18 to E (Q18E) in SIVmac239 did not allow significant viral replication in resting PBMC cultures (Fig. 2). In contrast, the R17Y change in nef imparted the full replicative capacity for resting PBMC cultures observed with the original R17Y and Q18E mutations (Fig. 2). SIVmac239/Q18E and SIVmac239/R17Y replicated in a manner similar to that of SIVmac239 and SIVmac239/YE in activated PBMC cultures (data not shown). Thus, the change of a single residue in nef, R17Y, is fully sufficient for a high level of SIVmac239 replication in resting PBMC cultures.

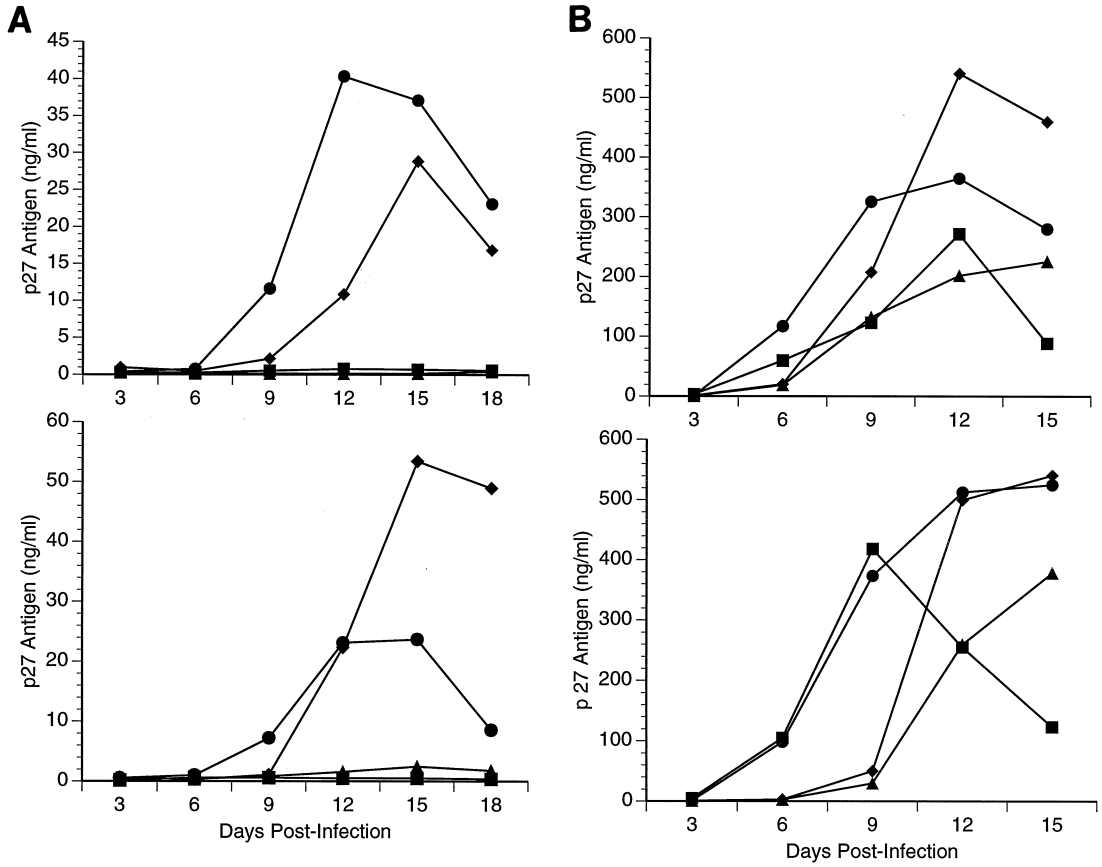


FIG. 3. Effect of tyrosine at residue 17 of nef of SIVpbj14. (A) Resting PBMC were used for virus replication as described in the legend to Fig. 2. (B) PBMC were stimulated with PHA (1 μg/ml) and infected with the viruses at the same time. The cells were maintained in RPMI-1640 medium containing 10% fetal calf serum and 10% IL-2 (Schiapparelli Biosystems, New York, N.Y.). ■, SIVmac239; ●, SIVmac239/YE; ▲, SIVpbj1.9/Y17R; ◆, SIVpbj1.9. Different sources of rhesus monkey PBMC were used for the upper and lower panels.

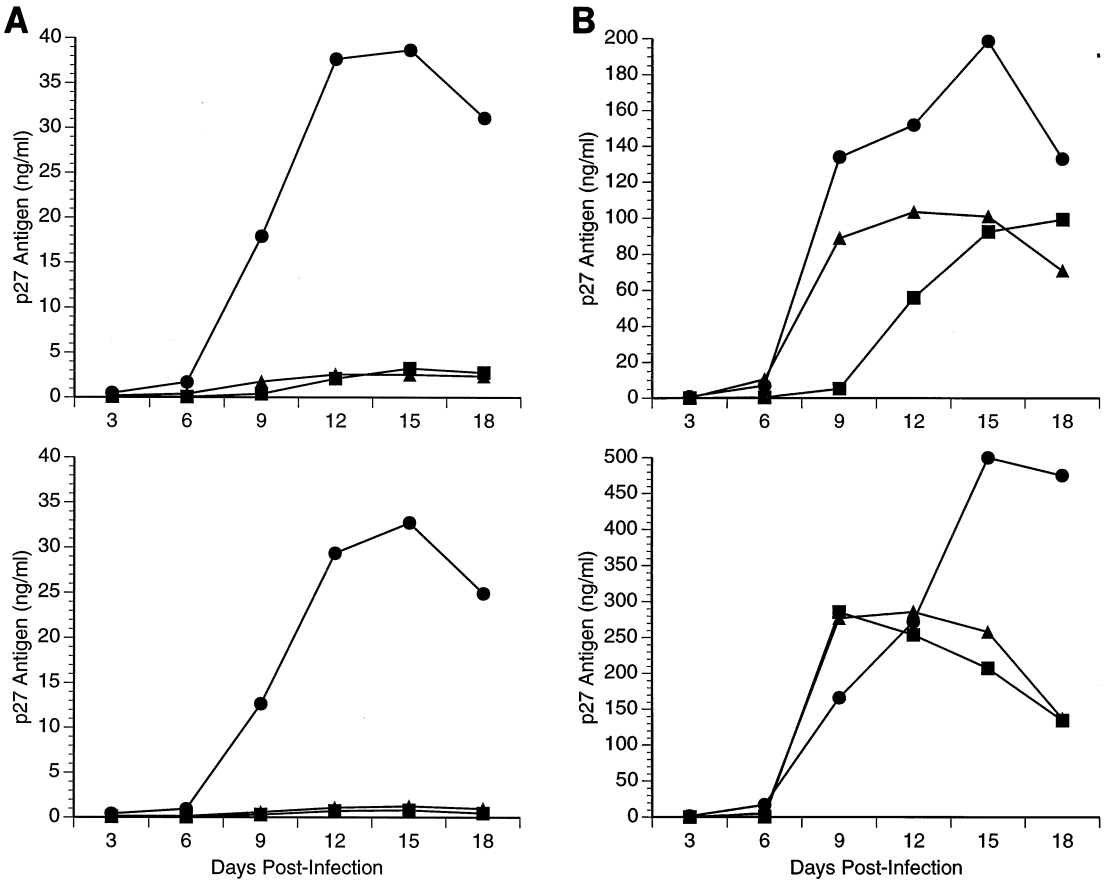


FIG. 4. Effect of phenylalanine at position 17 of nef on virus replication. (A) Resting PBMC were used for virus replication as described in the legend to Fig. 2. (B) PHA-stimulated PBMC were used for virus replication as described in the legend to Fig. 3. ■, SIVmac239; ●, SIVmac239/R17Y; ▲, SIVmac239/FE. PBMC from two different rhesus monkeys, Mm174-91 (upper panels) and Mm175-91 (lower panels), were used.

We also performed the converse experiment of changing the corresponding tyrosine at position 17 in nef of SIVpbj14 to arginine. SIVpbj14 clone 1.9 was kindly provided by Steven Dewhurst (2). SIVpbj14 clone 1.9 replicated in a manner similar to that of SIVmac239/YE in resting PBMC cultures from rhesus monkeys (Fig. 3A). Mutation of Y17R in nef of SIVpbj14 abrogated this ability to replicate in resting PBMC cultures (Fig. 3A). The Y17R mutant of SIVpbj14 replicated in a manner similar to that of the parental clone in PHA-activated PBMC (Fig. 3B). Thus, tyrosine at position 17 of nef is absolutely essential for the in vitro phenotype of SIVpbj14 and is sufficient to convert SIVmac239 to a virus with a phenotype like that of SIVpbj14.

Next we sought to obtain genetic evidence that the YXXL motif was not functioning as an internalization signal. YXXL and FXXL sequences in the cytoplasmic domain of membrane proteins can function as signals for internalization (21). Mutation of Y17F in nef allowed wild-type replication in PHA-activated PBMC (Fig. 4B), but the Y17F mutant did not replicate more efficiently than SIVmac239 in resting PBMC (Fig. 4A). These results do not support a role for the YXXL sequence as an internalization element.

The R17Y change creates a sequence of 15DLYQRLLRAR GETYGRL31, reminiscent of SH2 binding domains present in ITAMs. A key feature of the ITAMs is two YXXL elements separated by seven amino acids (1, 19). We thus asked whether tyrosine at position 28 was required for the unusual in vitro phenotype. A change of Y28F was created in the context of SIVmac239/YE; thus, this strain contained Y at position 17, E at position 18, and F at position 28. This mutant strain lost its ability to replicate in resting PBMC cultures (Fig. 5). Thus, tyrosine residues at both positions 17 and 28 are required for virus replication in resting PBMC cultures.

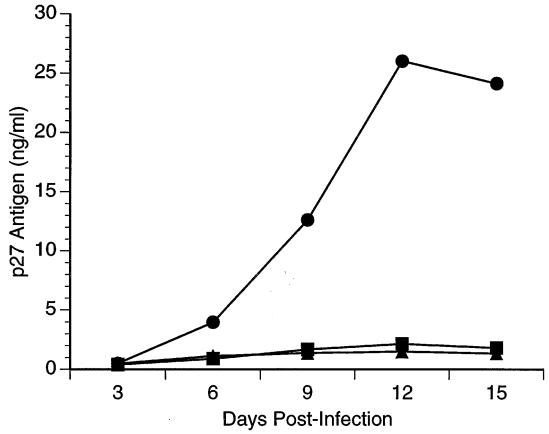
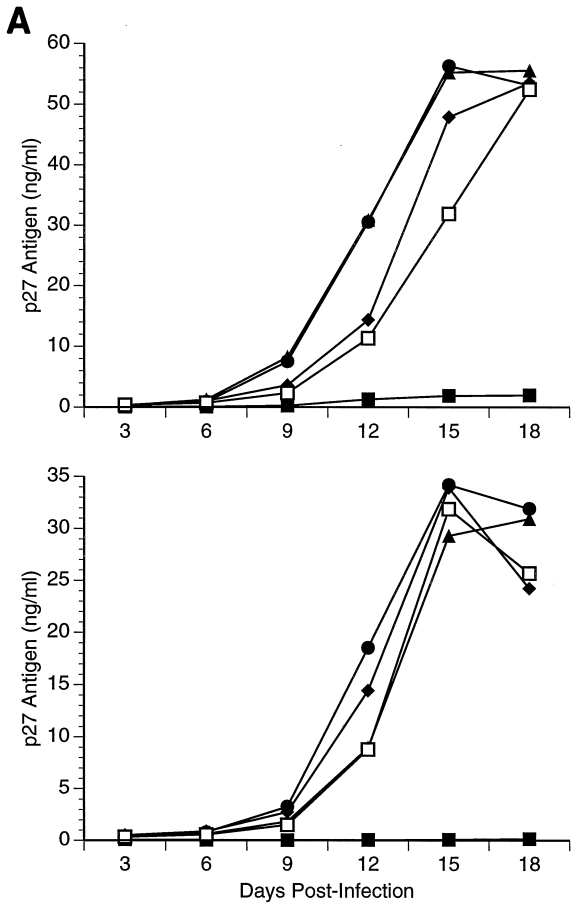


FIG. 5. Tyrosines in tandem at residues 17 and 28 are required. Virus replication was monitored in resting PBMC cultures as described in the legend to Fig. 2. ■, SIVmac239; ●, SIVmac239/YE; ▲, SIVmac239/YE/Y28F.



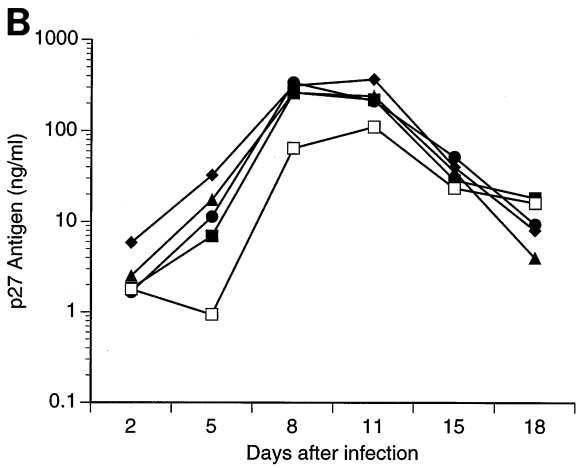


FIG. 6. Effect of number of NF-κB binding sites on replication of SIVmac239/R17Y. (A) SIVmac239/R17Y with zero (▲), one (◆), or two (□) NF-κB binding sites was used to infect resting PBMC. Virus replication was monitored as described in the legend to Fig. 2. ■, SIVmac239. Different sources of rhesus monkey PBMC were used in the upper and lower panels. (B) PHA-stimulated PBMC were used for virus replication as described in the legend to Fig. 3. ■, ●, and ▲, SIVmac239/R17Y with zero, one, and two NF-κB binding sites, respectively; ◆, SIVmac239/YE; □, SIVmac239.

Most strains of SIVmac, SIVsmm, and human immunodeficiency virus type 2 contain a single binding site for the NF-κB transcription factor. SIVpbj14 is also unusual in that it contains a duplication of the NF-κB binding elements (2). We thus investigated the relative contribution of the number of NF-κB binding elements to the phenotype of SIVmac239/R17Y. Derivatives of SIVmac239/R17Y with zero, one, and two NF-κB binding elements were constructed for this purpose. Amazingly, SIVmac239/R17Y mutants with zero, one, and two NF-κB binding sites all replicated similarly in both resting and stimulated PBMC (Fig. 6). Thus, not only are two NF-κB elements not needed for the unusual phenotype, even a single NF-κB element is not needed.

While surprising, the high level of replication of virus containing no NF-κB elements in this study is consistent with recent observations of the enhancer elements of SIVmac239 (11). SIVmac239 variants completely missing all Sp1 and NF-κB elements can still replicate in a manner similar to that of the parental virus in PHA-activated PBMC (11). This is very different from the situation with human immunodeficiency virus type 1 and is due principally to an enhancer element immediately upstream of the NF-κB binding site in SIVmac239 that is not present in human immunodeficiency virus type 1. SIVmac239/YE missing all NF-κB and Sp1 elements also replicated very well in resting PBMC cultures (data not shown).

Two rhesus monkeys were experimentally infected with each SIVmac239/R17Y variant containing zero, one, and two NF-κB elements. Viral inocula were normalized to contain 300 ng of p27 antigen and were administered by the intravenous route. All animals were negative for antibodies to SIV, type D retrovirus, and simian T-cell leukemia virus type 1. Animals were housed in accordance with standards of the American Association for Accreditation of Laboratory Animal Care. We adhered to the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. The original plan to sacrifice one monkey in each group on day 9 and on day 10 for pathological examination had to be modified when one of the monkeys (205-84) became moribund and had to be sacrificed on day 8 postinoculation. This monkey was infected with the Ynef variant containing no NF-κB binding sites. Subsequent pathologic examination revealed the presence of severe gastrointestinal disease in this animal (Table 1). Animal Mm205-84 showed a clinical disease pattern and pathologic lesions typical of those recently described in detail for SIVmac239/YE (18) and similar to previous descriptions for SIVpbj14 (9, 12, 13). There were no significant differences in either the severity of the disease or the nature of the pathologic lesions whether the Ynef variant contained zero, one, or two NF-κB binding elements (Table 1).

We have shown unambiguously that tyrosine at position 17 of nef is absolutely essential for the unusual phenotype of SIVpbj14 and is fully sufficient to convert SIVmac239 to a virus with a phenotype like that of SIVpbj14. The key feature of this unusual phenotype, both in cell culture and in animals, is the ability to cause lymphocyte activation. An increasing body of evidence indicates that the YXXL motifs in this strain are functioning as SH2 binding domains in the activation of signal transduction pathways for this lymphocyte activation. YEnef can bind src in cotransfected cells and is phosphorylated on tyrosine in these transfected cells (5). YEnef strongly transforms NIH 3T3 cells in culture (5) and can overcome the block to cell growth caused by the N17 ras transdominant inhibitor (12a). The nature of the YXXL sequences is very reminiscent of the ITAMs, a particular type of SH2 binding domain present on B-cell antigen, T-cell antigen, and Fc receptors. Here we have shown that tyrosine at nef position 17 cannot be substituted with phenylalanine, which is more consistent with a role

TABLE 1. Histologic changes in integument, axillary lymph nodes and intestines following infection with SIVmac239/Ynef

Animal no.	No. of NF-κB binding elements	Day p.i.[a]	Degree of histologic change in[b]:								
			Skin (rash)	Axillary lymph node		Intestine					
						Lymphoid nodules			Villi (VA/F)	Cellular infiltrates	Dilated lacteals
				PH	FI	GH	FI	FH			
205-84	0	8	4	4	4	4	4		4	4	4
163-91	0	10	3	2	4	1		2	1	1	2
195-84	1	9	0	2	2	1	1		0	1	0
168-91	1	10	4	3	3	4		4	4	4	4
182-91	2	9	4	3	3	3	3		2	3	3
336-84	2	10	4	3	2	3		1	1	3	2

[a] p.i., postinfection.
[b] PH, paracortical hyperplasia; GH, hyperplasia of T-cell area of gut-associated lymphoid tissue; FH, follicular hyperplasia; FI, follicular involution; VA/F, villous atrophy and fusion. All tissues were examined microscopically and subjectively quantified on a scale of 0 to 4 as follows: 0, within normal limits; 1, mild; 2, moderate; 3, marked; 4, severe.

as an SH2 binding element than as an internalization signal. We have also shown that the presence of tyrosines in tandem at residues 17 and 28 is required for the unusual in vitro phenotype. While Du et al. (5) have shown an association with src in cotransfected cells, the true cellular partners, as well as the pathways activated, remain to be elucidated.

The first two authors contributed equally to this work. We thank Susan Czajak and Dong Ling Xia for technical assistance and Joanne Newton for preparation of the manuscript.

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REFERENCES

1. **Cambier, J. C.** 1995. Antigen and Fc receptor signaling: the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). J. Immunol. **155:**3281–3285.
2. **Dewhurst, S., J. E. Embretson, D. C. Anderson, J. I. Mullins, and P. N. Fultz.** 1990. Sequence analysis and acute pathogenicity of molecularly cloned $SIV_{smmPBj14}$. Nature (London) **345:**636–640.
3. **Dittmar, M. T., K. Cichutek, P. N. Fultz, and R. Kurth.** 1995. The U3 promoter region of the acutely lethal simian immunodeficiency virus clone smmPBj1.9 confers related biological activity on the apathogenic clone agm3mc. Proc. Natl. Acad. Sci. USA **92:**1362–1366.
4. **Dollard, S. C., S. Gummuluru, S. Tsang, P. N. Fultz, and S. Dewhurst.** 1994. Enhanced responsiveness to nuclear factor κB contributes to the unique phenotype of simian immunodeficiency virus variant $SIV_{smmPBj14}$. J. Virol. **68:**7800–7809.
5. **Du, Z., S. M. Lang, V. G. Sasseville, A. A. Lackner, P. O. Ilyinskii, M. D. Daniel, J. U. Jung, and R. C. Desrosiers.** 1995. Identification of a *nef* allele that causes lymphocyte activation and acute disease in macaque monkeys. Cell **82:**665–674.
6. **Du, Z., D. A. Regier, and R. C. Desrosiers.** 1995. An improved recombinant PCR mutagenesis procedure that uses alkaline denatured plasmid template. BioTechniques **18:**376–378.
7. **Fultz, P. N.** 1991. Replication of an acutely lethal simian immunodeficiency virus activates and induces proliferation of lymphocytes. J. Virol. **65:**4902–4909.
8. **Fultz, P. N., H. M. McClure, D. C. Anderson, and W. M. Switzer.** 1989. Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). AIDS Res. Hum. Retroviruses **5:**397–409.
9. **Fultz, P. N., and P. M. Zack.** 1994. Unique lentivirus-host interactions: SIVsmmPBj14 infection of macaques. Virus Res. **32:**205–225.
10. **Gao, W.-Y., A. Cara, R. Gallo, and F. Lori.** 1996. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. USA **90:**8925–8928.
11. **Ilyinskii, P. O., and R. C. Desrosiers.** 1996. Efficient transcription and replication of simian immunodeficiency virus in the absence of NF-κB and Sp1 binding elements. J. Virol. **70:**3118–3126.
12. **Israel, Z. R., G. A. Dean, D. H. Maul, S. P. O'Neil, M. J. Dreitz, J. I. Mullins, P. N. Fultz, and E. A. Hoover.** 1993. Early pathogenesis of disease caused by SIV_{smm}PBj14 molecular clone 1.9 in macaques. AIDS Res. Hum. Retroviruses **9:**277–286.

12a. **Lang, S., J. Jung, and R. Desrosiers.** Unpublished data.

13. **Lewis, M. G., P. M. Zack, W. R. Elkins, and P. B. Jahrling.** 1992. Infection of rhesus and cynomolgus macaques with a rapidly fatal SIV ($SIV_{SMM/PBj}$) isolate from sooty mangabeys. AIDS Res. Hum. Retroviruses **8:**1631–1639.
14. **McDougal, J. S., A. Mawle, S. P. Cort, J. K. A. Nicholson, G. D. Cross, J. A. Scheppler-Campbell, D. Hicks, and J. Sligh.** 1985. Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. J. Immunol. **135:**3151–3162.
15. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–712.
16. **Pawson, T.** 1995. Protein modules and signalling networks. Nature (London) **373:**573–580.
17. **Reth, M.** 1989. Antigen receptor tail clue. Nature (London) **338:**383–384.
18. **Sasseville, V. G., Z. Du, L. V. Chalifoux, D. R. Pauley, H. L. Young, P. K. Sehgal, R. C. Desrosiers, and A. A. Lackner.** Induction of lymphocyte proliferation and severe gastrointestinal disease in macaques by a nef gene variant of SIVmac239. Am. J. Pathol., in press.
19. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767–778.
20. **Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica.** 1996. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. **9:**1551–1560.
21. **Trowbridge, I. S., J. F. Collawn, and C. R. Hopkins.** 1993. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. **9:**129–161.
22. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61:**213–222.
23. **Zagury, D., J. Bernard, R. Leonard, R. Cheynier, M. Feldman, P. S. Sarin, and R. C. Gallo.** 1986. Long-term cultures of HTLV III infected T cells: a model of cytopathology of T cell depletion in AIDS. Science **231:**850–853.