BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **243,** 113–116 (1998)
ARTICLE NO. RC978070

Tolerance of Diverse Amino Acid Substitutions at Conserved Positions in the Nuclear Export Signal (NES) of HIV-1 Rev

Ming Jie Zhang and Andrew I. Dayton[1]

Laboratory of Molecular Virology, Division of Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852-1448

Received December 8, 1997

The effector domain of the Rev protein is a nuclear export signal (NES) that is responsible for transporting Rev and its bound congeners out of the nucleus and into the cytoplasm. Previous work has identified several critical residues in the NES and has led to the belief that NESs of the Rev type are necessarily leucine rich. Here we present the sequences of a large number of functional Rev molecules with NES mutations. The data indicate a previously unreported diversity in allowable residues at a number of positions, including each of the leucine residues previously considered essential.

The Rev pathway is one of two key HIV autoregulatory pathways and represents a major potential therapeutic target. The Rev protein of HIV-1 interacts with its viral RNA target sequence, the RRE to transport incompletely spliced and unspliced viral mRNA out of the nucleus and into the cytoplasm for expression. (1) The effector domain of Rev is a nuclear export signal that has been reported to bind to various cellular factors (2-7) that are believed to be responsible for exporting Rev and its bound congeners across the nuclear membrane. Towards understanding the biochemical basis of the interaction between the Rev NES and its associated cellular factor(s) we have previously devised a method called NEST (Nuclear Export Signal Trap) for positively selecting functional Rev mutants from pools of Rev clones randomly mutagenized at various positions in the NES (8). Here we implement this method to select a large number of Rev NES mutants with variable activities.

[1] To whom correspondence should be addressed at HFM-315, CBER/FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Fax: 301-827-0998. E-mail: Dayton@A1.cber.fda.gov.

The Rev NES spans approximately positions 74-84. (9, 10) Leucines at positions 81 and 83 have previously been reported to be essential for function and a core region has been described, comprising essentially LXLX (11, 12). Leu75 and Leu78 upstream of the core regions have been reported to be important, though not essential for function (13) and regions upstream of the core region may only have a requirement for general hydrophobicity (12). To examine these issues in more exhaustive detail we have employed the NEST method to isolate large numbers of functional Rev clones with mutations in the NES region.

MATERIALS AND METHODS

Plasmids. pCMV128-CD28 is an HIV-1-rev-dependent, CD28 expression plasmid derived from CMV128(14) by replacing the CAT gene with a CD28 gene excised from pCDM8-CD28 by PCR (15). The pWtL RRE is a Rev dependent gag indicator plasmid (16)

Construction of randomized Rev libraries. The pRev expression plasmid pRev(wt) was used for library formation. The NES positions indicated in Figure 1 were randomly mutagenized by PCR. A silent mutation at amino acid #73 introduced a Pst I site without changing the peptide sequence. This Pst I site was used for subcloning PCR products and for distinguishing mutant from wild type clones.

Cells and transfections. COS-7 cells were grown as previously described (16). One day before transfection they were split 1:2. On the day of transfection, cells were trypsinized and suspended in RPMI 1640 supplemented with 10% fetal bovine serum. After centrifugation cells were resuspended in the same medium at a concentration of 8×10^6 cells per milliliter. To electroporation cuvettes (Bio-Rad, 0.4 cm) containing transfecting DNA (5 μg, in a volume of approximately 5 μl in water) was added 0.5 ml of cells. After 2 minutes incubation at room temperature, the cell/DNA mixtures were electroporated at room temperature using a (Bio-Rad Gene Pulsar) electroporator set at 0.25 kV, 960 μF. The electroporated cells were incubated for an additional 2 minutes at room temperature and then plated in 50 ml of medium in a 150 mm tissue culture dish. Three hours later the medium was changed and 2 days post electroporation the cells were harvested.

Selection of the functional Rev effector domain variants. Cells were harvested for magnetic bead separation by detaching in phosphate buffered saline (PBS) containing 1% NaN_3, 0.5 mM EDTA

0006-291X/98 $25.00

A. Typical effector domains (12, 18, 21)

		-6 -5 -4 -3 -2 -1 1 2 3 4 5	
HIV-1 REV	72	- P L Q L P P L E R L T L D C N -	86
Visna REV	103	- E S N M V G M E M L T L E T Q -	117
HIV-2 REV	70	- D Q T I Q H L Q G L T I Q E L -	84
SIV mac239 REV	70	- D L A I Q Q L Q N L A I E S I -	84
HTLV-I REX	81	- A L S A Q L Y S S L S L D S -	94
HTLV-IIREX	82	- A L S A L L S N T L S L A S -	93
X.L.TFIIIA		T N G S L V L D K L T I Q	
B.A.TFIIIA		A D P L P V L E N L T L K	

B. Atypical effector domains (19, 20)

FIV	95LAFKKMMTDLEDRFRKLFGSPSKDEYT121
EIAV	32PQGPLESDQWCRVLRQSLPEEKIP55

C. Natural Rev effector domain variations (21)

MA	L P P I E R L T L D	48%
MN(G)	L P P - Q R L T L D	<1%
U455	L P P I E R L R L D	41%
BCSG3C	L P P L E R L N L G	29%
ANT	L P P L E Q L S I R	48%
CPZ	L P E L D K L S L Q	27%
L75A	A P P L E R L T L D	35%

D. Consensus sequence of Rev/Rex effector domain (18)

```
                F
                I
L-X2-3-L-X2-3-L-X-L
                V       L
                M
```

FIG. 1. Rev effector domain and nuclear export signals. (A) Typical effector domains. The effector domain regions of HIV-1 Rev, visna virus Rev, HIV-2 Rev, SIVmac239 Rev, HTLV-I Rex, HTLV-II Rex, and the nuclear export signals of TFIIIA proteins (12, 18, 21) are shown. (B) Atypical effector domains. The effector domains of FIV and EIAV (19, 20) are shown. (C) Natural Rev effector domain variations. Variant effector domain of five HIV-1 isolates and the closely related CPZ isolate of SIV (20) are shown. (D) Consensus sequence of Rev/Rex effector domain. See Ref. 18 for details.

(15). Antibody treatment was with anti-CD28 monoclonal antibody (BDH) for 30 minutes at 40°C. After wash three times, cells were incubated for 30 minutes with M450 Dynabeads (Dynal), which had been prewashed with medium S, (16). DNA was recovered from CD28 positive, transfected cells by Hirt (17) fractionation as previously described (16).

Analysis of Rev activity and expression. The pWt RRE was co-transfected into COS-7 cells together with pRev (wt) or its mutants and pSVCMV-CAT with lipofectamine (GIBCO BRL). The transfection was normalized with CAT ELISA (Boehringer Mannheim). Gag production was measured by p24 ELISA (DuPont) 48 hours post transfection. The Rev and Rev variant expression levels were checked by Western blot with a rabbit anti-Rev as the first antibody and HRP-goat ant-rabbit IgG (NEN) as second antibody.

RESULTS

Table 1 lists mutants selected from the R1 library which was randomized at amino acid positions 78 and 79. Leu78 can be replaced by other hydrophobic residues such as isoleucine or valine with moderate loss of function (Red 14, 38) or with no loss of function (or even a moderate gain) if Glu79 is replaced by histidine, serine or tyrosine. In a global sense, hydrophobic residues at position 78 are certainly preferred as evidenced by the complete absence of hydrophilic residues in the group

TABLE 1

Activities of R1 Series Rev Variants

Clones tested	77	78	79	80	Activity
Rev WT	Pro	Leu	Glu	Arg	100.00
Red91			His		131.40
Red94		Ile	His		128.69
Red3		Ile	Ser		107.81
Red6			Ser		98.05
Red10		Ile	Tyr		97.26
Red14		Val			77.22
Red38		Ile			71.94
Red133			Tyr		63.85
Red128		Ile	Ala		57.94
Red39		Phe	Gln		56.13
Red93		Met	Asp		55.70
Red24			Ala		49.37
Red84			Asp		45.99
Red22			Phe		35.44
Red21		Val	Ala		30.38
Red86		Val	Trp		14.35
Red136		Ile	Leu		2.15
Red143		Ile	Asn		1.12

of clones selected by the NEST method. The situation with position 79 is less clear. Basic residues are strikingly absent from this position. However, position 79 can well tolerate hydrophobic and charged or uncharged hydrophilic residues. Although a wide variety of residues are allowed at this position, particular residues have noticeably specific and often surprising effects. For instance, Glu79 can be replaced by the similarly acidic residue, aspartate (Red 84), resulting in an activity of 46% of normal, roughly similar to putting a hydrophobic alanine residue at the same position (Red24). In the context of Ile78, however, a tyrosine at position 79 results in a mutant with essentially normal activity (Red10). Thus, with some notable exceptions, hydro-

TABLE 2

Activities of R2 Series Rev Variants

Clones tested	79	80	81	82	Activity
Rev WT	Glu	Arg	Leu	Thr	100.00
Red8		Asp			132.91
Red35		Val	Ser		104.01
Red2		Thr			90.93
Red36		Gln			90.51
Red13		Lys			88.54
Red12		Gly			87.24
Red101		Glu			76.59
Red33		Ser			66.46
Red125		Phe			63.92
Red55		Ala			53.59
Red111		Asn			34.60
Red20		Thr	Met		23.42
Red18		Tyr			16.24
Red23		Lys	Cys		6.96
Red11		Glu	Val		2.11

TABLE 3

Activities of R3 Series Rev Variants

Clones tested	80	81	82	83	84	85	Activity
Wild type	Arg	Leu	Thr	Leu	Asp	Cys	100.00
Red46			Ala	Ile			133.97
Red79, 81			Arg				83.97
Red58			Val	Ile			66.76
Red34				Met			56.12
Red26			Leu	Met			55.27
Red27			Glu	Ile			47.05
Red44, 80			Arg	Ile			40.30
Red77			Lys	Ile			38.82
Red45			Asn				29.32
Red85			Leu	Ile			28.48
Red43			Arg	Met			24.68
Red83			Arg	Val			17.09
Red9			Gly				8.02

philic, non-basic residues seem to be preferred at position 79, particularly in the context of a wild type Leu78.

Table 2 lists mutants selected from the R2 library which was randomized at positions 80 and 81. Although almost all of the highly active mutants retain the wild type Leu81, one mutant, Red35, has serine at position 81 (in the context of a Val80) and retains full function. Position 80 generally tolerates a wide variety of substitutions with a clear preference for hydrophilic (uncharged, acidic or basic) residues. It is curious that the one selected mutant with a substitution of Leu81 by serine (Red 35) involves substitution of the hydrophilic Arg80 by the hydrophobic valine. Possibly it is important to have a hydrophilic residue paired with a hydrophobic residue at positions 80-81, although the lack of order dependence would be hard to interpret.

Table 3 lists mutants selected from the R3 library which was randomized at positions 82 and 83. Surprisingly, when Thr82-Leu83 is substituted by Ala82-Ile83, the resulting mutant (Red46) has an activity somewhat greater than wild type. In the context of an Ile83, even substitution of leucine or valine into position 82 results in substantial reduction of activity with respect to Red46 and moderate to substantial reduction of activity with respect to wild type .In general, there was a strong preference to select from this library hydrophobic residues at position 83. Although a large variety of residues were tolerated at position 82, all of the substitutions excepting those in Red46 were significantly detrimental to function.

Table 4 lists several selected mutants with aberrant, unintended mutations in the NES. Most notable amongst these is Red 59, with respectable function and a substitution of Pro-Asp-Ser-Ser (78-81) for the wild type Leu-Glu-Arg-Leu (78-81). To determine whether or not the observed effects of various mutations on measured activity were due to alterations in steady state Rev levels, we performed Western blot analysis of every Rev mutant. In Figure 2 are presented the steady state levels beside the activity of the corresponding mutants. In general there was little correlation between steady state levels and levels of activity, confirming that the effects of mutation were mediated primarily by effects on intrinsic Rev activity.

DISCUSSION

The NES of Rev lies in the approximate region of positions 74-84. This region is leucine rich and is similar to the other NESs listed in Figure 1. Leucine richness has generally been considered a hallmark of NESs of the Rev class, and previous studies have suggested that the leucines at positions 81 through 83 are essential for Rev NES function (9-12).Contrary to the conventional wisdom, our data clearly indicates that the leucines at positions 81 and 83 can be substituted without loss of NES activity. Leu 81 can be substituted by the hydrophilic residue, serine (in the context of a valine substitution for Arg80 as in Red35 of Table 2) with no change from wild type in activity. Leu83 can be substituted by isoleucine with no loss of activity (in the context of an alanine substituted for Thr82 as in Red46 of Table 3) or by methionine alone with moderate loss of activity (as in Red 34 of Table 4) or by phenylalanine in the context of a cystine substitution for Arg 80 with moderate loss of activity (as in Red 113 of Table 4). Consistent with other reports (18) Leu78 can be substituted by isoleucine, valine, methionine, and phenylalanine with appropriate activity.

TABLE 4

Activities of R4 Series Rev Variants

Clones tested	76	77	78	79	80	81	82	83	84	85	Activity
Wild type	Pro	Pro	Leu	Glu	Arg	Leu	Thr	Leu	Asp	Cys	100.00
Red41									Thr	Ile	114.97
Red59			Pro	Asp	Ser	Ser					85.23
Red115									Val		65.40
Red113					Cys			Phe			61.39
Red48		Leu		Val				Thr			25.11
Red28				Ser	Phe						1.69

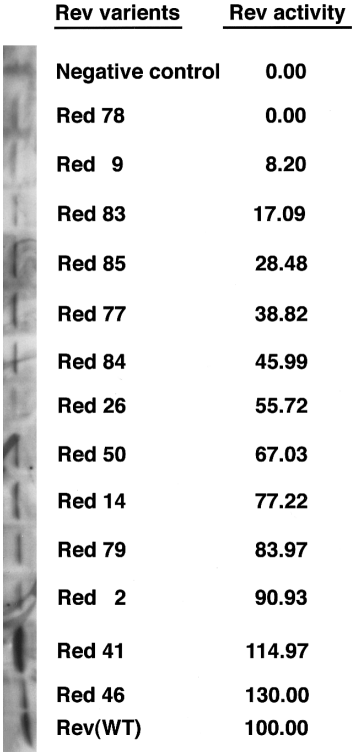

FIG. 2. Western blot analysis of the expression levels of the selected Rev effector domain mutants. The Rev effector domain mutants with a wide range of different activities have equivalent expression levels.

The other positions examined have more flexible restrictions. Basic amino acids are strikingly absent from position 79 in the mutants selected by the NEST procedure. Non-basic hydrophilic amino acids and hydrophobic amino acids are moderately well tolerated at this position, with histidine and serine mutants retaining full activity. Position 80 tolerates a wide variety of substitutions with a clear preference for hydrophilic residues (either uncharged, acidic or basic). Position 82 also tolerates a wide variety of substitutions, but almost all of them have a moderately deleterious effect. Only substitution of alanine into position 82 allows full activity (in the context of Ile83).

The wide variety of amino acids tolerated at various positions, coupled with the variable and dispensable requirements for individual leucines and the realization that many of the observed effects of mutations are context dependent suggests two conclusions. First, many of the mutations may be causing complex structural changes which are not easily interpretable. Second, there are probably multiple, closely related binding targets for the NESs. The widely divergent NES sequences of EIAV Rev and FIV Rev (19, 20; see Fig 1) strongly support this concept.

ACKNOWLEDGMENTS

M.J.Z. was supported by a senior associate award from the National Research Council. The work was supported by a grant from the AIDS Targeted Anti-Viral Program of the Office of the Director, National Institutes of Health.

REFERENCES

1. Hope T. J. (1997) *Chem. Biol.* **4,** 335–344.
2. Bogerd, H. P., Fridell, R. A., Madore, S., and Cullen, B. R. (1995) *Cell* **82,** 485–494.
3. Fritz, C. C., Zapp, M. L., and Green, M. R. (1995) *Nature* **367,** 530–533.
4. Ruhl, M., Himmelspach, M., Bahr, G. M., Hammerschmid, F., Jaksche, H., Wolff, B., Aschauer, H., Farrington, G. K., Probst, H., and Bevec, D. (1993) J. *Cell Biol.* **123,** 1309–1320.
5. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) *Cell* **90,** 1041–1050.
6. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) *Cell* **90,** 1051–1060.
7. Kutay, U., Bischoff, F. R., Kostka, S., Kraft, R., and Gorlich, D. (1997) *Cell* **90,** 1061–1071.
8. Zhang, M. J., and Dayton, A. I. *J. Biomed. Sci.,* in press.
9. Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995) *Cell* **82,** 475–483.
10. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995) *Cell* **82,** 463–473.
11. Hope, T. J., Bond, B. L., McDonald, D., Klein, N. P., and Parslow, T. G. (1991) *J. Virol.* **65,** 6001–6007.
12. Kim, F. J., Beeche, A. A., Hunter, J. J., Cin, D. J., and Hope, T. J. (1996) *Mol. Cell. Biol.,* 5147–5155.
13. Malim, M. H., McCarn, D. F., Tiley, L. S., and Cullen, B. R. (1991) *J. Virol.* **65,** 4248–4254.
14. Hope, T. J., McDonald, D., Huang, X. J., Low, J., and Parslow, T. G. (1990) *J. Virol.* **64,** 5360–5366.
15. Aruffo, A., and Seed, B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8573–9577.
16. Zhang, M. J., and Dayton, A. I. (1996) *J. AIDAS HR* **13,** 403–407.
17. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369.
18. Bogerd, H. P., Fridell, R. A., Benson, R. E., Hua, J., and Cullen, B. R. (1996) *Mol Cell. Biol.* **16,** 4207–4214.
19. Fridell, R. A., Partin, K. M., Carpenter, S., and Cullen, B. R. (1993) *J. Virol.* **67,** 7317–7323.
20. Mancuso, V. A., Hope, T. J., Zhou, L., Derse, D., Phillips, T., and Parslow, T. G. (1994) *J. Virol.* **68,** 1998–2001.
21. Fridell, R. A., Fischer, U., Luhrmann, R., Meyer, B. E., Meinkoth, J. L., Malim, M. H., and Cullen, B. R. (1996) *Proc Natl. Acad. Sci. USA* **93,** 2936–2940.
22. Hua, J., Caffrey, J. J., and Cullen, B. R. *Virology* **222,** 423–429.