JOURNAL OF VIROLOGY, Jan. 1999, p. 814–818 Vol. 73, No. 1
0022-538X/99/$04.00+0

Genetic Selection of Poliovirus 2A^{pro}-Binding Peptides

IVÁN VENTOSO,* ANGEL BARCO, AND LUIS CARRASCO

Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco 28049, Madrid, Spain

Received 20 July 1998/Accepted 23 September 1998

The yeast two-hybrid system has been used to identify mammalian clones that interact with poliovirus 2A proteinase (2A^{pro}). Eight clones which encode previously unidentified human proteins were selected from a HeLa cell cDNA expression library. In addition, five clones encoding short peptides that interact with poliovirus 2A^{pro} were also identified. The lengths of these peptides range from 6 to 30 amino acids, but all of them contain the Leu-X-Thr-Z motif (X represents any amino acid; Z represents a hydrophobic residue). This sequence is invariably located just at the carboxy terminus of each peptide. This approach raises the possibility of designing substrate analogue inhibitors of 2A^{pro}. Thus, two nonhydrolyzable peptides containing the Leu-X-Thr-Z motif prevented cleavage of eukaryotic initiation factor 4G by poliovirus 2A^{pro} in vitro. A more general method for identifying peptides with antiproteinase activity is discussed.

All poliovirus proteins are generated by proteolytic processing of a large polyprotein synthesized upon translation of the genomic RNA (23). All proteolytic cleavages of the polyprotein but one are carried out by the three virus-encoded proteinases, 2A proteinase (2A^{pro}), 3C^{pro}, and 3CD^{pro} (23, 33). 2A^{pro} is a multifunctional enzyme that participates in several processes during the replication cycle of poliovirus. In addition to its basic function as a protease that cleaves the viral polyprotein, poliovirus 2A^{pro} also enhances viral translation, blocks cellular gene expression, and participates in viral RNA replication (6, 11, 14, 21, 28).

2A^{pro}-mediated cleavage of the viral polyprotein occurs at Tyr-Gly bonds. The first cleavage takes place intramolecularly at the P1-2A^{pro} junction, in a reaction where 2A^{pro} cleaves at its own amino terminus. This cleavage separates the capsid protein precursor P1 from the nascent polyprotein (27). Alternative processing of 3CD^{pro} by poliovirus 2A^{pro} generates 3C′ and 3D′ polypeptides (16, 27). Since 2A^{pro} hydrolyzes only 2 of the 10 Tyr-Gly bonds present in the viral polyprotein, additional amino acid residues and/or structural determinants surrounding the cleavage sites determine 2A^{pro} specificity (27). However, little is known about these additional determinants of substrate recognition by 2A^{pro}. Sequence comparison and site-directed mutagenesis of amino acids flanking the scissile bond in VP1-2A^{pro} and 3CD^{pro} showed that Thr at position P2 (nomenclature of Berger and Schechter [4]) is the most important determinant, whereas a variety of substitutions at other positions, including the scissile Tyr-Gly dipeptide, can be tolerated without loss of substrate proteolysis (13, 26, 31). Moreover, data from rhinovirus and coxsackievirus 2A proteinases suggest that the proposed cleavage site for picornavirus 2A^{pro} within eukaryotic initiation factor 4G (eIF-4G) is an Arg-Gly dipeptide instead of Tyr-Gly (15).

The three-dimensional structure of picornavirus 2A^{pro} is not yet available, but both random and site-directed mutagenesis, together with the use of proteinase inhibitors, have given some insights about the structure-function relationships of 2A^{pro} (2, 3, 12, 20, 35). Poliovirus 2A^{pro} activity is blocked by alkylating agents, such as iodoacetamide and *N*-ethylmaleimide, and also by elastase-specific inhibitors, such as elastatinal and methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone, but not by other serine/cysteine protease inhibitors, such as aprotinin, leupeptin, antipain, or E-64 (20). Thus, 2A^{pro} has been classified as a novel subclass of trypsin-like serine proteinases where the catalytic triad of the enzyme is formed by His-20, Asp-38, and Cys-109 (3). In addition, a number of residues outside the predicted catalytic pocket of poliovirus 2A^{pro} have recently been implicated in substrate recognition (2, 35).

A genetic assay, the yeast two-hybrid system, has recently emerged as a powerful tool for the demonstration of protein-protein interactions in intact cells (8, 9). The sensitivity of this method is high enough to detect certain enzyme-substrate interactions (1, 30). More recently, combinatorial peptide libraries have been used to study peptide-protein interactions by the two-hybrid system (34). This method has allowed the identification of short sequences and amino acid residues critical for their interaction with a given protein target. Furthermore, genetic selection of peptides that interact with a target protein with high affinity might constitute the basis for designing inhibitors of protein function.

Our initial aim was to find human proteins that might interact with poliovirus 2A^{pro}. To this end, the *Saccharomyces cerevisiae* two-hybrid system was used (MATCHMAKER; Clontech). The poliovirus 2A^{pro} gene was amplified by PCR and cloned into plasmid pGBT-9 by standard procedures (25) to generate a hybrid protein between the proteinase and GAL-4 DNA-binding domain. Yeast strain Y190 (*MAT*α *ura3 his3 leu2 lys2 ade2 trp1 gal4-542*) was transformed with plasmid pGBT-2A^{pro} by the lithium acetate method (10) and tested for expression of the hybrid gene GAL-4BD-2A^{pro}. Synthesis of this protein neither affected yeast growth nor by itself activated the transcription of the β-galactosidase gene (data not shown).

Our next step was to transform the yeast clone expressing the hybrid GAL-4BD-2A^{pro} gene with a HeLa cell cDNA library. Of $\sim 2.5 \times 10^5$ colonies screened, 40 clones with a His$^+$ β-Gal$^+$ phenotype were obtained. Twenty of these were taken for further analysis. To assay the specificity of the interaction between poliovirus 2A^{pro} and the proteins encoded by pGAD-GH, these plasmids were isolated from positive clones and retransformed with pGBT-2A^{pro} or pGBT-3C^{pro}. Only clones cotransformed with poliovirus 2A^{pro} showed β-galactosidase

* Corresponding author. Mailing address: Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco 28049, Madrid, Spain. Phone: 34-91-3975070. Fax: 34-91-3974799. E-mail: IVENTOSO@TRASTO.CBM.UAM.ES.

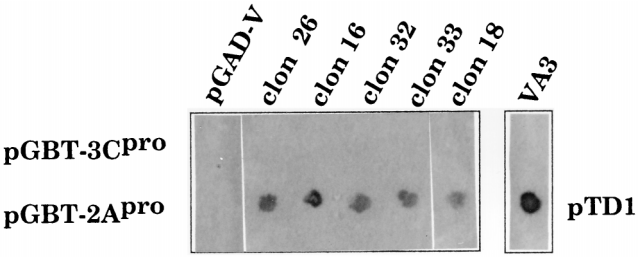

FIG. 1. Interaction of poliovirus $2A^{pro}$ with clones from a human cDNA library tested by the two-hybrid system. Shown are results of a filter β-galactosidase assay of yeast cotransformants bearing the cDNA library clones indicated and plasmid pGBT-$2A^{pro}$ or pGBT-$3C^{pro}$. Colonies were transferred to Whatman 3MM filter paper, lysed, and incubated with an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution. A blue color indicates a positive interaction. pGAD-V is the library vector without an insert, used as a negative control. The VA3 and pTD1 vectors encode p53 and T antigen from simian virus 40, respectively (17), and were used as a positive control of interaction.

activity (data not shown). pGAD-GH-containing inserts isolated from these clones were sequenced. About 60% of these sequences encode unidentified proteins (unpublished data).

Notably, five clones encoded short peptides, as deduced from the sequences of the cDNA inserts. Although these inserts are quite large (0.12 to 1.3 kbp), the predicted translation frames are halted by the appearance of stop codons. Interestingly, two of these clones contain previously identified DNA sequences. Clone 18 contains the sequence from nucleotide (nt) 927 to nt 1837 of the hnRNP-F gene (GenBank accession no. L28010), but this sequence is out of frame, whereas the insert in clone 16 encompasses nt 1017 to 1137 of the 3′ untranslated region of the RHOA gene (L09159). These results indicate that these peptides are most likely not synthesized by human cells but are selected because expression libraries offer a rich source of diverse protein sequences that can bind to a given protein. Indeed, only one-third of the clones in the cDNA library are in frame.

Western blotting using a monoclonal antibody raised against the activation domain (AD) of GAL-4 indicated that these clones synthesized hybrid polypeptides slightly larger than protein GAL-4AD alone, reinforcing the notion that these clones express short peptides (data not shown). As shown in Fig. 1, the interaction of these clones is specific for poliovirus $2A^{pro}$, whereas neither poliovirus $3C^{pro}$ (Fig. 1) nor poliovirus 2B protein (data not shown) gave a positive signal. The strength of the interaction between the different clones and poliovirus $2A^{pro}$ varied. Thus, clone 16 and, to a lesser extent, clones 26 and 32 showed the highest β-galactosidase activities (Table 1).

The lengths of the selected peptides range from 30 amino acids (clone 16) to 6 amino acids (clones 26 and 33) (Fig. 2A). However, all of them have significant homology at their carboxy termini. A threonine residue at position P2 and a leucine at position P4 are invariably present in all five clones. In four of them, an arginine at position P7 is also present. Furthermore, the carboxy-terminal residue in all clones is a hydrophobic amino acid (Leu, Phe, or Ile). Interestingly, alignments of these sequences with those present surrounding the $2A^{pro}$ cleavage sites in VP1-$2A^{pro}$, $3CD^{pro}$, and eIF-4G reveal an absolute conservation at positions P2 and P4, whereas the rest of the positions are variable. The arginine residue at P7 is also present in eIF-4G, but not in VP1-$2A^{pro}$ and $3CD^{pro}$ substrates. These findings agree well with those reported by Hellen et al. (13), where any change at position P2 in the VP1-$2A^{pro}$ junction abolished cleavage by $2A^{pro}$ in *trans*, pointing to this residue as critical for substrate recognition by this poliovirus protease.

In conclusion, the carboxy terminus of the selected peptides mimics the sequence found at the amino-terminal half of cleavage sites of poliovirus $2A^{pro}$ in physiological substrates. Since none of the selected peptides contains the scissile dipeptide Tyr-Gly, our findings suggest that the amino acid half flanking the cleavage site contains a major determinant for substrate recognition by poliovirus $2A^{pro}$. These findings also help us understand why $2A^{pro}$ cleaves only 2 of the 10 Tyr-Gly dipeptides present in the viral polyprotein (27). Only the two cleavable Tyr-Gly dipeptides are flanked by the recognition sequence of $2A^{pro}$.

Also of interest is the presence of a hydrophobic amino acid at the P1 position of the consensus sequence of these peptides, instead of the tyrosine or arginine residues found in $2A^{pro}$ substrates (Fig. 2B). Perhaps this hydrophobic amino acid serves to stabilize the interaction of peptides with the $2A^{pro}$ catalytic pocket, whereas the presence of a polar amino acid (Tyr or Arg) in natural substrates may induce a more rapid release of the hydrolyzed products.

To test whether the peptides described above are able to interact in vitro with poliovirus $2A^{pro}$, two peptides were designed and synthesized. In principle, the selected peptides cannot be cleaved by poliovirus $2A^{pro}$, because they lack Tyr-Gly bonds. Thus, we hypothesized that these peptides could behave as potential nonhydrolyzable inhibitors of poliovirus $2A^{pro}$. Such substrate analogues have been described for other viral and cellular proteinases (7, 18, 24). To test this hypothesis, two peptides of 8 amino acids were synthesized containing the invariable residues found with the two-hybrid system. Peptide 1 contains the sequence found in clone 32, whereas peptide 2 was designed by modifying the sequence present in clone 16: the Val at position P3 was replaced by a Ser, and an additional Arg was placed at position P8 (Fig. 2A and 3A). These changes were introduced to increase the solubility of the peptide and to make it more similar to the cleavage site present in eIF-4G (Fig. 2B and 3A).

The effects of these peptides on poliovirus $2A^{pro}$ activity were tested by an in vitro cleavage assay of eIF-4G. Poliovirus $2A^{pro}$ was purified as a fusion protein with maltose binding protein (MBP) as described previously (22). Previous results in vitro showed that recombinant MBP-$2A^{pro}$ is able to bisect human eIF-4G in cell-free systems (22). Addition of 1 μg of

TABLE 1. β-Galactosidase activities in liquid medium

Plasmid	β-Galactosidase activity[a] with:						
	pGAD-V	Clone 26	Clone 16	Clone 32	Clone 33	Clone 18	VA3
pGBT-$3C^{pro}$	<1	<1	<1	<1	<1	<1	<1
pGBT-$2A^{pro}$	<1	6.8 ± 0.18	28.4 ± 2.36	6.78 ± 1.59	3.54 ± 0.42	4.66 ± 1.47	ND
pTD1	ND	ND	ND	ND	ND	ND	138.4 ± 46.8

[a] Expressed in Miller units (19). Standard deviations obtained from three independent measurements are given. ND, not determined.

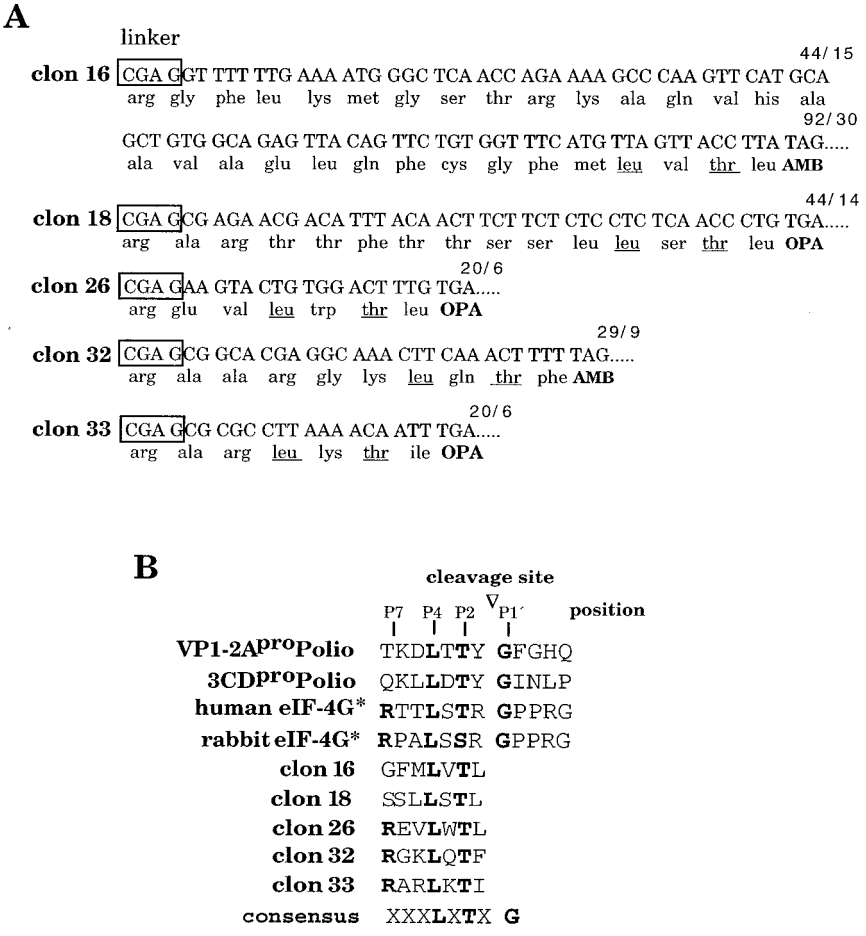

FIG. 2. Sequences of poliovirus 2A^{pro}-binding clones. (A) Nucleotide sequences and derived amino acid sequences of clones selected by the two-hybrid system. Only cDNAs encoding regions are indicated. Boxed nucleotides, artificial linkers used to construct the library (see the Clontech catalog for additional information). Underlined residues identical amino acids found at the same positions. AMB and OPA, stop codons. (B) Amino acid sequence alignments of the carboxy termini of 2A^{pro}-binding peptides and cleavage sites present in all known 2A^{pro} substrates. Conserved residues are boldfaced. The positions of amino acids with respect to the cleavage site are numbered according to the system of Berger and Schechter (4). Asterisks indicate that the cleavage site of poliovirus 2A^{pro} within eIF-4G has not been formally demonstrated. This putative site is predicted on the basis of studies using rhinovirus and coxsackievirus 2A^{pro}.

MBP-2A^{pro} to HeLa S10 extract is sufficient to cleave endogenous eIF-4G almost completely (Fig. 3A, lanes 2 and 13). This cleavage is abolished by the addition of 0.5 mM elastatinal, the most powerful inhibitor of picornavirus 2A^{pro} (Fig. 3A, lane 11). Addition of increasing concentrations of peptide 1 induced a significant inhibition of eIF-4G cleavage (Fig. 3A, lanes 3 through 6). However, upon incubation of MBP-2A^{pro} with peptide 2, a stronger blockade of eIF-4G cleavage was obtained with 1 mM peptide 2, whereas at 2 to 3 mM the cleavage of eIF-4G was almost completely abolished. The 50% inhibitory concentrations (IC_{50}) were 1.6 mM for peptide 1 and 0.6 mM for peptide 2 (Fig. 3B).

To demonstrate if the inhibition of eIF-4G cleavage is specific for these peptides, a parallel incubation with an unrelated peptide containing an irrelevant sequence was carried out (Fig. 3A, control peptide). This peptide did not show any significant effect on eIF-4G cleavage at the highest concentration tested (3 mM) (Fig. 3A, lane 12).

In conclusion, the results shown in Fig. 3A indicate that synthetic peptides derived from the sequences selected in vivo inhibit poliovirus 2A^{pro} activity in vitro in a dose-dependent manner. Furthermore, the degree of interaction observed in vivo correlates well with the extent of inhibition of eIF-4G cleavage exerted by these two peptides in cell-free systems.

Understanding viral proteinase specificity is of key importance, not only for basic research, but also for designing antiviral drugs that block proteinase activity. Potent inhibitors of cellular and viral proteases based on synthetic peptide analogues have been developed (18, 24, 32). Usually, the scissile dipeptide in these pseudosubstrates is replaced by a nonhydrolyzable bond (7). In contrast, peptides used here to block poliovirus 2A^{pro} activity in vitro lack a nonscissile Tyr-Gly pair. In spite of this, these peptides are able to interact with 2A^{pro}, as determined by the two-hybrid system, and to block its proteolytic activity on an eIF-4G substrate. These results suggest that the cleavable dipeptide (Tyr-Gly) is dispensable for recognition and interaction with 2A^{pro}. On the other hand, these findings provide additional support for the notion that poliovirus 2A^{pro} recognizes and cleaves eIF-4G directly (5, 29).

Although the concentrations of peptides that inhibit 2A^{pro} activity are relatively high, the sequences of these peptides suggest a consensus necessary for binding. A genetic approach similar to that described here may help further identification of new peptides with increased affinity for 2A^{pro} and higher in-

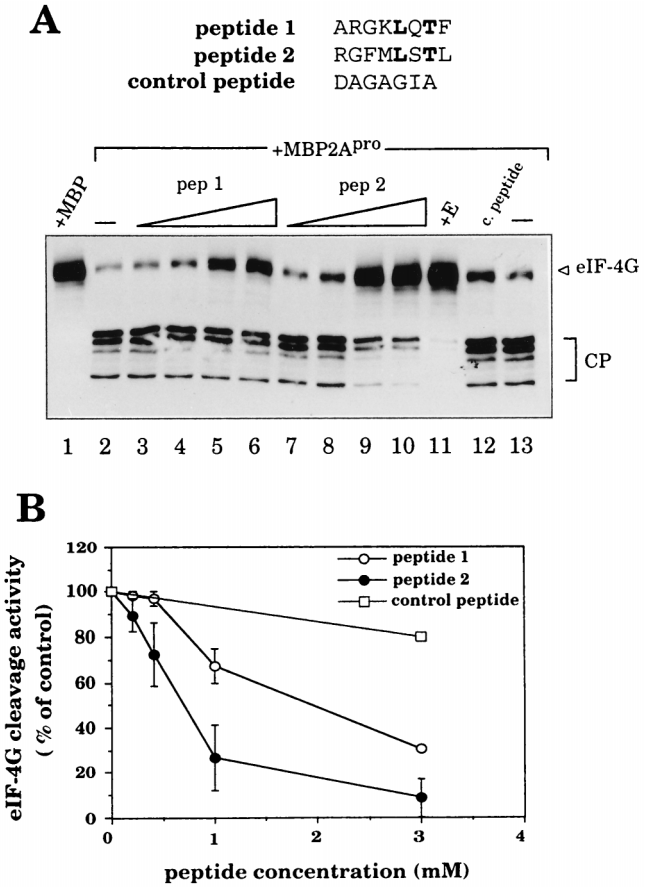

FIG. 3. Effects of synthetic peptides on cleavage of eIF-4G by poliovirus $2A^{pro}$. (A) Sequences of synthetic peptides designed on the basis of peptides obtained with the two-hybrid system and effects of these peptides on in vitro cleavage of eIF-4G by purified MBP-$2A^{pro}$. MBP-$2A^{pro}$ (1 μg) was preincubated for 5 min at room temperature with no peptide (lanes 2 and 13) or with increasing concentrations of peptide 1 (0.2 mM [lane 3], 0.4 mM lane 4], 1 mM [lane 5], and 3 mM [lane 6]) or peptide 2 (0.2 mM [lane 7], 0.4 mM [lane 8], 1 mM [lane 9], and 3 mM [lane 10]). In lane 12, 3 mM control peptide was added, whereas in lane 11, 0.5 mM elastatinal (Sigma) was present. Lane 1, incubation with MBP alone. Samples were analyzed by Western blotting using an antiserum against human eIF-4G. Intact protein and protein cleavage products (CP) are indicated. (B) Inhibition of $2A^{pro}$ proteinase activity by synthetic peptides. The percentage of inhibition was estimated by densitometric quantitation of the eIF-4G that remained intact after incubation. Data are expressed as means of three independent experiments, and standard deviations are indicated. The calculated IC_{50} were 1.6 mM for peptide 1 and 0.6 mM for peptide 2.

hibitory potency. Moreover, the use of a peptide library instead of a cDNA library might increase the number of positive clones selected (34). Alternatively, mutagenesis of selected peptides and rapid screening in yeast could improve the affinity of the peptides selected. This may constitute the basis for designing nonpeptidic analogues able to permeate cells in order to block poliovirus replication. The approach described in the present work may be extended to other proteinases or enzymes in general, of either viral or cellular origin.

We thank John Lewis for helpful suggestions and for critical reading of the manuscript. The technical assistance of M. A. Sanz is also acknowledged.

I.V. holds a fellowship from Comunidad Autónoma de Madrid. A. Barco holds a CSIC postdoctoral fellowship. This work was supported by grants from DGICT project PB94-0148 and C.A.M. The Fundación Ramón Areces is also acknowledged for its financial support of the Centro de Biología Molecular through an institutional grant.

REFERENCES

1. **Aelst, L. V. M. Barr, S. Marcus, A. Polverino, and M. Wigler.** 1993. Complex formation between Ras and Raf and other protein kinases. Proc. Natl. Acad. Sci. USA **90:**6213–6217.
2. **Barco, A., I. Ventoso, and L. Carrasco.** 1997. The yeast *Saccharomyces cerevisiae* as a genetic system for obtaining variants of poliovirus protease 2A*. J. Biol. Chem. **272:**12683–12691.
3. **Bazan, J. F., and R. J. Fletterick.** 1988. Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: structural and functional implications. Proc. Natl. Acad. Sci. USA **85:**7872–7876.
4. **Berger, A., and I. Schechter.** 1970. Mapping the active site of papain with the aid of peptide substrates and inhibitors. Phil. Trans. R. Soc. Lond. B **257:** 249–264.
5. **Bovee, L. M., B. J. Lamphear, R. E. Rhoads, and R. Lloyd.** 1998. Direct cleavage of eIF-4G by poliovirus 2A protease is inefficient *in vitro*. Virology **245:**241–249.
6. **Davies, M. V., J. Pelletier, K. Meerovitch, N. Sonenberg, and R. J. Kaufman.** 1991. The effect of poliovirus proteinase $2A^{pro}$ on cellular metabolism. J. Biol. Chem. **266:**14714–14720.
7. **Dreyer, G. B., B. W. Metcalf, T. A. Tamaszek, Jr., T. J. Carr, A. C. Chandler III, L. Hyland, S. A. Fakhoury, V. W. Magaard, M. L. Moore, J. E. Strickler, C. Debouck, and T. D. Meek.** 1989. Inhibition of human immunodeficiency virus 1 protease in vitro: rational design of substrate analogue inhibitors. Proc. Natl. Acad. Sci. USA **86:**9752–9756.
8. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245–246.
9. **Fields, S., and R. Sternglanz.** 1994. The two-hybrid system: an assay for protein-protein interactions. Trends Genet. **10:**286–291.
10. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Shiestl.** Improved method for high efficiency transformation of intact yeast cells. 1992. Nucleic Acids Res. **20:**1425.
11. **Hambidge, S. J., and P. Sarnow.** 1992. Translational enhancement of the poliovirus 5′ noncoding region mediated by virus-encoded polypeptide 2A. Proc. Natl. Acad. Sci. USA **89:**10272–10276.
12. **Hellen, C. U. T., M. Fäcke, H. Kräusslich, C. Lee, and E. Wimmer.** 1991. Characterization of poliovirus 2A proteinase by mutational analysis: residues required for autocatalytic activity are essential for induction of cleavage of eukaryotic initiation factor 4F polypeptide p220. J. Virol. **65:**4226–4231.
13. **Hellen, C. U. T., C.-K. Lee, and E. Wimmer.** 1992. Determinants of substrate recognition by poliovirus 2A proteinase. J. Virol. **66:**3330–3338.
14. **Krausslich, H. G., M. J. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer.** 1987. Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. J. Virol. **61:**2711–2718.
15. **Lamphear, B. J., R. Yan, F. Yang, D. Waters, H.-D. Liebig, H. Klump, E. Kuechler, T. Skern, and R. E. Rhoads.** 1993. Mapping the cleavage site in protein synthesis initiation factor eIF-4G gamma of the 2A proteases from human coxsackievirus and rhinovirus. J. Biol. Chem. **268:**19200–19203.
16. **Lee, C. K., and E. Wimmer.** 1988. Proteolytic processing of poliovirus polyprotein: elimination of $2A^{pro}$-mediated, alternative cleavage of polypeptide 3CD by in vitro mutagenesis. Virology **166:**405–414.
17. **Li, B., and S. Fields.** 1993. Identification of mutations in p53 that affect its binding to SV40 large T antigen by using the yeast two-hybrid system. FASEB J. **7:**957–963.
18. **Meek, T. D., D. M. Lambert, G. B. Dreyer, T. J. Carr, T. A. Tomaszek, Jr., M. L. Moore, J. E. Strickler, C. Debouck, L. J. Hyland, T. J. Matthews, B. W. Metcalf, and S. R. Petteway.** 1990. Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature **343:**90–92.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Molla, A., C. U. T. Hellen, and E. Wimmer.** 1993. Inhibition of proteolytic activity of poliovirus and rhinovirus 2A proteinases by elastase-specific inhibitors. J. Virol. **67:**4688–4695.
21. **Molla, A., A. V. Paul, M. Schmid, S. K. Jang, and E. Wimmer.** 1993. Studies on dicistronic polioviruses implicate viral proteinase $2A^{pro}$ in RNA replication. Virology **196:**739–747.
22. **Novoa, I., F. Martínez-Abarca, P. Fortes, J. Ortín, and L. Carrasco.** 1997. Cleavage of p220 by purified poliovirus $2A^{pro}$ in cell-free systems. Effects on translation of capped and uncapped mRNAs. Biochemistry **36:**7802–7809.
23. **Palmenberg, A.** 1990. Proteolytic processing of picornaviral polyprotein. Annu. Rev. Microbiol. **44:**603–623.
24. **Roberts, L. B., W. Markland, C. A. Ley, B. R. Kent, W. D. White, K. S. Guterman, and C. R. Ladner.** 1992. Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M18 fusion phase. Proc. Natl. Acad. Sci. USA **89:**2429–2433.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Sommergruber, W., H. Ahorn, A. Zöphel, I. Maurer-Fogy, F. Fessl, G. Schnorrenberg, H.-D. Liebig, D. Blaas, E. Kuechler, and T. Skern.** 1992.

Cleavage specificity on synthetic peptide substrates of human rhinovirus 2 proteinase 2A. J. Biol. Chem. **267:**22639–22644.
27. **Toyoda, H., M. J. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45:**761–770.
28. **Ventoso, I. and L. Carrasco.** 1995. A poliovirus $2A^{pro}$ mutant unable to cleave 3CD shows inefficient viral protein synthesis and transactivation defects. J. Virol. **69:**6280–6288.
29. **Ventoso, I., S. E. McMillan, J. W. B. Hershey, and L. Carrasco.** 1998. Poliovirus 2A proteinase cleaves directly the eIF-4G subunit of eIF-4F complex. FEBS Lett. **435:**79–83.
30. **Vojtek, A., S. Holleberg, and J. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
31. **Wang, Q. M., W. Sommergruber, and R. B. Johnson.** 1997. Cleavage specificity of human rhinovirus-2 2A protease for peptide substrates. Biochem. Biophys. Res. Commun. **235:**562–566.
32. **West, M. L., and D. P. Fairlie.** 1995. Targeting HIV-1 protease: a test of drug-design methodologies. Trends Pharmacol. Sci. **16:**67–75.
33. **Wimmer, E., C. U. T. Hellen, and X. Cao.** 1993. Genetics of poliovirus. Annu. Rev. Genet. **27:**353–436.
34. **Yang, M., Z. Wu, and S. Fields.** 1995. Protein-peptide interactions analyzed with the yeast two-hybrid system. Nucleic Acids Res. **23:**1152–1156.
35. **Yu, S. F., and R. E. Lloyd.** 1991. Identification of essential amino acid residues in the functional activity of poliovirus 2A protease. Virology **182:** 615–625.