sites were annealed as follows (MRE in italic):

3′ extension:

5′-CTAGAA*TAACGG*AA-3′

3′-TTA*TTGCC*TTTCTAGATCTTCGAACCATGGGCCCTAGGCGG-5′

5′ extension:

5′-GGCGGATCCCGGGTACCAAGCTTCTAGATCTAA*TAACGG*AA-3′

3′-TTA*TTGCC*TTGATC-5′

We labeled both duplexes by a Klenow fill-in reaction with [α-^{32}P] deoxycytidine triphosphate, followed with an excess of unlabeled deoxyribonucleotides to produce duplex probes. We made shortened probes by subsequent digestion with one of the indicated restriction enzymes and then used either a fill-in reaction or treatment with mung bean nuclease to produce blunt-ended probes.

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25. The same mutants have been tested for DNA binding with the 6-bp and 33-bp 3′-extended probes, and all mutants gave results comparable to those in Fig. 2B.
26. Nonspecific DNA binding was monitored by adsorption to magnetic DNA-affinity beads made as described [O. S. Gabrielsen, E. Hornes, L. Korsnes, A. Ruet, T. B. Øyen, *Nucleic Acids Res.* 17, 6253 (1989)]. A 260-bp DNA fragment that harbored the yeast *tRNA^{Tyr} Sup 4-o* gene, which contains no MRE sites, was immobilized on streptavidin-coated magnetic Dynabeads (Dynal As, Oslo, Norway) (with 7 μg of DNA per milligram of beads). No specific complexes were formed with R_2R_3 and the same DNA fragment in mobility-shift assays. Bacterial extracts (3 μg of total protein) were incubated for 10 min at 25°C with magnetic DNA-affinity beads (2 mg) in 25 μl of buffer (20 mM tris-HCl; 1 mM EDTA; 10% glycerol; 100 mM NaCl; 1 mM dithiothreitol; and 0.05% Triton X-100; pH 8). Nonadsorbed proteins were recovered after magnetic separation and analyzed by SDS–polyacrylamide gel electrophoresis in parallel with nontreated extracts.
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29. We thank E. Quemeneur and C. Doira for oligonucleotide synthesis and DNA sequencing, C. Carle for amino acid sequencing and composition analysis, J. Huet for the gift of DNA Dynabeads, and B. Perbal, who encouraged us to initiate this study and for providing the chicken c-*myb* gene.

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Ca^{2+}-Induced Ca^{2+} Release in Sea Urchin Egg Homogenates: Modulation by Cyclic ADP-Ribose

ANTONY GALIONE,* HON CHEUNG LEE, WILLIAM B. BUSA

Calcium-induced calcium release (CICR) may function widely in calcium-mediated cell signaling, but has been most thoroughly characterized in muscle cells. In a homogenate of sea urchin eggs, which display transients in the intracellular free calcium concentration ($[Ca^{2+}]_i$) during fertilization and anaphase, addition of Ca^{2+} triggered CICR. Ca^{2+} release was also induced by the CICR modulators ryanodine and caffeine. Responses to both Ca^{2+} and CICR modulators (but not Ca^{2+} release mediated by inositol 1,4,5-trisphosphate) were inhibited by procaine and ruthenium red, inhibitors of CICR. Intact eggs also displayed transients of $[Ca^{2+}]_i$ when microinjected with ryanodine. Cyclic ADP-ribose, a metabolite with potent Ca^{2+}-releasing properties, appears to act by way of the CICR mechanism and may thus be an endogenous modulator of CICR. A CICR mechanism is present in these nonmuscle cells as is assumed in various models of intracellular Ca^{2+} wave propagation.

EXTRACELLULAR SIGNALS INDUCE OSCILLATIONS of $[Ca^{2+}]_i$ or propagated waves of intracellular Ca^{2+} release in various nonmuscle cell types. The classic example of such complex spatiotemporal behavior of $[Ca^{2+}]_i$ during cell signaling is observed during fertilization of the egg. In sea urchin, starfish, fish, and frog eggs, fertilization elicits a single propagated wave of increased $[Ca^{2+}]_i$ starting at the site of sperm entry and sweeping in a regenerative fashion across the egg at about 10 μm/s (*1*). Hamster eggs display both Ca^{2+} waves and a series of periodic transients of $[Ca^{2+}]_i$ after fertilization (*2*). The $[Ca^{2+}]_i$ transient or transients at fertilization regulate the metabolic and developmental activation of the egg (*3*). Other nonmuscle cell types also display oscillatory or propagated $[Ca^{2+}]_i$ transients in response to calcium mobilizing hormones or Ca^{2+} itself (*4, 5*). Calcium-induced Ca^{2+} release (CICR), whereby an increase in the concentration of extravesicular free Ca^{2+} triggers Ca^{2+} release from intracellular stores, has been characterized in muscle fibers and sarcoplasmic reticulum (SR) vesicles (*6, 7*). CICR is mediated by the ryanodine receptor (*8, 9*) and may function in producing both Ca^{2+} waves and oscillations (*10, 11*) by acting in concert with inositol 1,4,5-trisphosphate (IP_3)–mediated Ca^{2+} release. Pharmacological agents that modulate CICR include the stimulators caffeine and ryanodine and the inhibitors procaine and ruthenium red (*6, 12*), but no endogenous modulating compounds (other than adenosine nucleotides) have yet been reported. We now report direct evidence for and characterization of a CICR mechanism (distinct from the IP_3-mediated release mechanism) in sea urchin egg homogenates. Also, we present evidence suggesting that CICR occurs in the intact cell. Further, cyclic adenosine diphosphate (ADP)–ribose (cADPR), which is a metabolite of nicotinamide adenine dinucleotide (NAD^+) and is present in homogenates of urchin eggs (*13*) and various mammalian cells (*14*), was found to modulate the urchin egg CICR mechanism. Like IP_3, cADPR triggers calcium mobilization and egg activation in urchin eggs (*15*).

Homogenates of sea urchin eggs supplemented with an adenosine triphosphate (ATP)–regenerating system, mitochondrial inhibitors, and the calcium-reporting dye fura 2, sequester added Ca^{2+} into vesicular stores in an ATP-dependent manner and release Ca^{2+} in response to nanomolar concentrations of either cADPR or $InsP_3$ (Fig. 1) (*15*). Such homogenates display desensi-

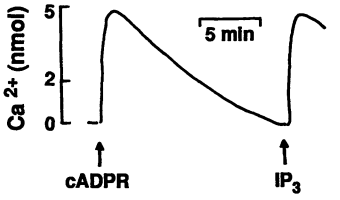

Fig. 1. Ca^{2+} release induced by cADPR and IP_3 in egg homogenates measured fluorometrically with fura 2. Addition of cADPR (20 nM, final concentration) elicited a large rapid Ca^{2+} release, which was then resequestered. IP_3 (100 nM) subsequently triggered a similar release, which was also resequestered. The absolute amount of Ca^{2+} released is indicated on the ordinate. Breaks in the record occurred during additions to the cuvette. Abbreviations: cADPR, cyclic ADP-ribose; IP_3, inositol 1,4,5-trisphosphate. See (*30*) for methods.

A. Galione and W. B. Busa, Department of Biology, The Johns Hopkins University, Baltimore, MD 21218.
H. C. Lee, Department of Physiology, University of Minnesota, Minneapolis, MN 55455.

*To whom correspondence should be addressed.

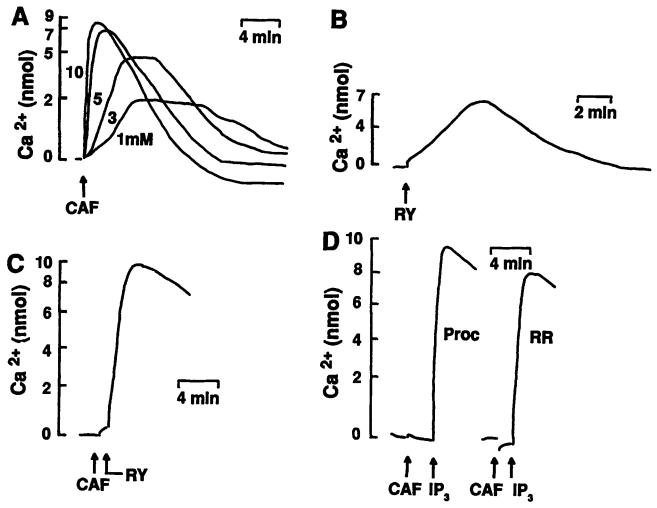

Fig. 2. Characterization of caffeine- and ryanodine-induced Ca^{2+} release. (**A**) The rate and magnitude of caffeine-induced release was dose dependent over the range 1–10 mM (final concentration) in homogenates previously treated with 1 μM IP_3. (**B**) Ryanodine (100 μM) induced releases of similar magnitude but with much slower kinetics in homogenate previously treated with 1 μM IP_3. (**C**) After addition of a submaximal (1 mM) dose of caffeine, this same dose of ryanodine triggered rapid Ca^{2+} release. (**D**) Ca^{2+} release in response to caffeine (10 mM) was completely inhibited by either procaine (1 mM) or ruthenium red (30 μM), without significant effect on IP_3-induced (1 μM) release. The two traces shown are from two different portions of the same homogenate. Abbreviations: CAF, caffeine; RY, ryanodine; Proc, procaine; RR, ruthenium red.

tization to subsequent additions of the same agent (that may be due to lack of breakdown of the agents added) but neither agent cross-desensitizes the homogenate to the other agent, suggesting that the cADPR- and $InsP_3$-sensitive release mechanisms are distinct (*15, 16*).

Pharmacological modulators of CICR triggered large Ca^{2+} releases in homogenates of sea urchin eggs at concentrations similar to those effective in muscle cells and preparations of SR. The kinetics of Ca^{2+} release were also similar to those seen in muscle cells (Fig. 2, A and B). As observed in cells expressing the cloned skeletal muscle ryanodine receptor (*17*), caffeine-induced release (10 mM) was fast (time to 90% of full response was 20 ± 3 s; average ± SEM, N = 5) while ryanodine-induced release (100 μM) was significantly slower (270 ± 30 s, N = 5). However, when ryanodine was added after a submaximal dose of caffeine (which, alone, caused only a small slow Ca^{2+} release, Fig. 2A) the rate of release was enhanced (to 60 ± 8 s, N = 4; Fig. 2C); This result is consistent with experiments on SR indicating that caffeine can open CICR channels in SR and that ryanodine binds preferentially to the already open channel and locks it in this conformation (*9*). The pharmacological inhibitors of CICR in SR, procaine and ruthenium red, also inhibited the response of egg homogenates to caffeine while the response to IP_3 was unaffected (Fig. 2D). Thus, the putative CICR and IP_3-mediated release mechanisms appear to be functionally distinct, as was suggested by the observation of ryanodine- and caffeine-induced release in homogenates desensitized to IP_3 (Fig. 2, A and B).

Direct evidence for a CICR mechanism (that is, Ca^{2+} release in response to addition of Ca^{2+}, rather than pharmacological agents) was obtained under two conditions: (i) In the presence of a submaximal dose of caffeine, addition of 0.2 to 3.0 nmol of Ca^{2+} triggered Ca^{2+} release of 3.3 ± 0.6 nmol, (N = 6) (Fig. 3, A and B). This effect was abolished by addition of either procaine (1 mM) or ruthenium red (30 μM) (*18*). Release of Ca^{2+} in SR in response to caffeine may be due to sensitization of CICR to low concentrations of Ca^{2+} (*6*). (ii) In the presence of high concentrations of phosphate, microsomes that had been treated with five (10-nmol) applications of Ca^{2+} displayed a large slow release about 1 min after sequestration of the last addition (Fig. 3C). Such spontaneous Ca^{2+} release has been observed in both cardiac muscle and in SR vesicles treated with Ca^{2+} in the presence of phosphate and may be triggered by a suprathreshold concentration of Ca^{2+} within the lumen, which occurred because phosphate, a precipitating anion, increases the capacity of the SR to store Ca^{2+} (*19*).

The CICR mechanism also appears to be active in the intact egg. Microinjection of 10 pl (about 1.6% of the egg volume) of ryanodine (10 mM) into the unfertilized egg elicited large Ca^{2+} transients (Fig. 4). As detected with the fluorescent Ca^{2+} indicator indo-1, the emission ratio (405 to 485 nm) increased from 0.32

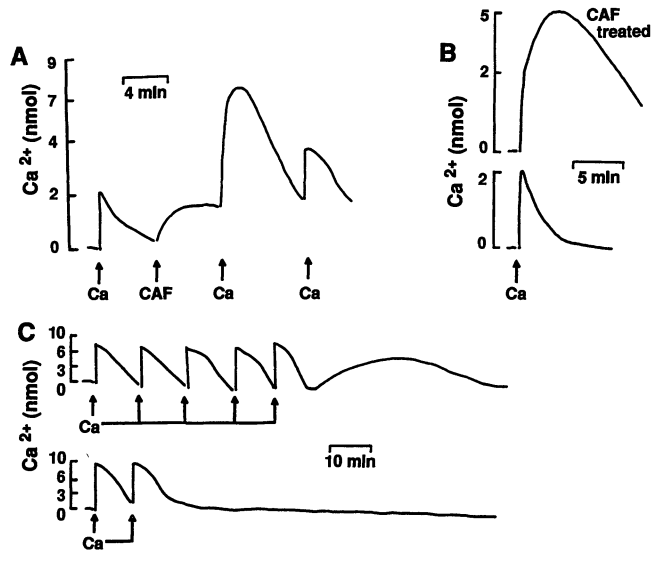

Fig. 3. CICR in egg homogenates. (**A**) Addition of Ca^{2+} (2 nmol) elicited characteristic instantaneous increase in concentration of free Ca^{2+}; that Ca^{2+} was rapidly sequestered. Caffeine (1 mM) then triggered a small release, after which one addition of Ca^{2+} (2 nmol) elicited a large Ca^{2+} release, while a further addition (2 nmol) was without effect. (**B**) The kinetics and magnitude of increase in concentration of free Ca^{2+} after Ca^{2+} (2 nmol) was added differed in normal homogenates (bottom trace) and homogenates previously treated with caffeine (1 mM) (top trace). (**C**) In the presence of phosphate (120 mM, substituted for an equal amount of potassium gluconate), five (top trace) but not two (bottom trace) sequential additions of Ca^{2+} (2 nmol) elicited a slow Ca^{2+} release about 1 min after sequestration of the final addition.

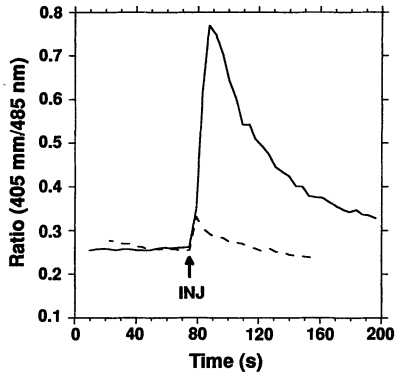

Fig. 4. A large $[Ca^{2+}]_i$ transient in intact sea urchin eggs elicited by microinjection of ryanodine. Ryanodine (10 mM) was dissolved in a buffer containing 0.5 M KCl, 0.1 mM EGTA, and 10 mM Hepes (pH 6.7). An injection volume of about 1.5% of egg volume triggered a large $[Ca^{2+}]_i$ transient (solid line) like that seen at fertilization, while an equal volume of vehicle alone yielded a small artifact (dashed line). These records are representative of seven experiments on the eggs of two urchins. The techniques were described (*15*).

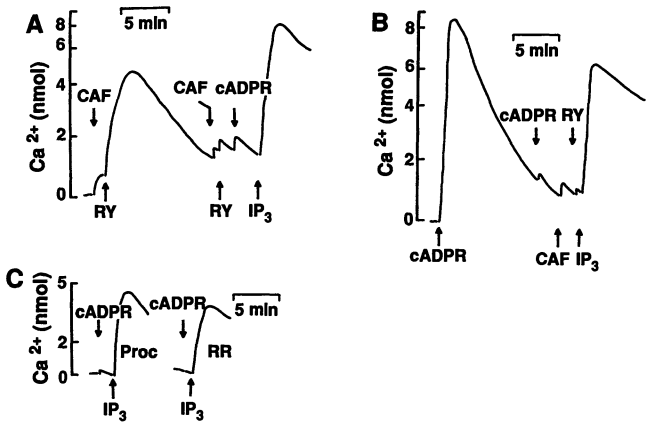

Fig. 5. Effects of cADPR on the same Ca^{2+} release mechanism modulated by caffeine and ryanodine. (**A**) After Ca^{2+} release stimulated by caffeine and ryanodine (1 mM and 100 μM, respectively), the homogenate was desensitized to further additions of caffeine (10 mM) and cADPR (1 μM), but not to IP_3 (1 μM). (**B**) Identical response to a reversed order of additions was also observed. (**C**) Both procaine and ruthenium red (1 mM and 30 μM, respectively) inhibited cADPR- but not IP_3-induced releases at concentrations approximating their median effective concentrations (EC_{50}) (20 nM or 100 nM, respectively). The control (without inhibitors) for this experiment is shown in Fig. 1. The two traces shown here and the trace in Fig. 1 were from three different portions of one preparation of homogenate.

± 0.02 to 0.78 ± 0.05, (N = 3), immediately after injection of ryanodine, and from 0.31 ± 0.03 to 0.36 ± 0.02 (N = 3) after injection of vehicle alone. Ratios of 0.2 and 0.8 correspond to free Ca^{2+} concentrations of 200 nM and 1 μM, respectively, in Ca^{2+}-EGTA calibration buffers. Microinjection of 10 pl of ryanodine (10 mM) triggered egg activation (as judged by cortical granule exocytosis) in five of nine eggs injected, and 10 pl of 20 mM ryanodine activated all eggs tested (N = 8). None of ten eggs injected with vehicle alone were activated. Thus, the intact cell possesses a ryanodine-sensitive release mechanism like that observed in homogenates. Injections of caffeine (10 pl of 0.1 M, final concentration in the egg ~1.6 mM) activated only two of ten eggs. However, caffeine concentrations ≥3 mM are required to elicit release of large amounts of Ca^{2+} in egg homogenates (Fig. 2A) and in muscle (*12*). Such doses were not achieved in these studies because of solubility limitations.

Whereas the CICR mechanism is functionally distinct from that activated by $InsP_3$, cADPR-induced release appeared to act by the CICR mechanism. Homogenates treated with ryanodine and caffeine were desensitized not only to subsequent additions of these agents but also to cADPR, although IP_3 could still trigger a large Ca^{2+} release (Fig. 5A). Similarly, cADPR desensitized the homogenates not only to itself but also to caffeine and ryanodine, without affecting the response to IP_3 (Fig. 5B). Inhibitors of CICR abolished the response to cADPR but had little effect on IP_3-mediated release (Fig. 5C). Finally, treatment of the homogenate with a subthreshold dose of cADPR (5 nM, which alone caused no detectable Ca^{2+} release) increased the rate of Ca^{2+} release in response to ryanodine (100 μM) (to 53 ± 4 s, N = 4), as did subthreshold concentrations of caffeine (see above). These observations suggest that caffeine, ryanodine, and cADPR all activate the same mechanism, CICR, which is distinct from that activated by IP_3. However, high concentrations (up to 10 μM) of cADPR appeared not to mobilize Ca^{2+} from SR in caffeine-sensitive, glycerinated skeletal muscle fibers from rabbit, because these fibers failed to contract in response to cADPR (*20*). These cells may have different isoforms or modes of regulation of ryanodine receptors; the skeletal muscle ryanodine receptor may interact with a voltage sensor to effect excitation-contraction coupling (*8*), but depolarization does not trigger Ca^{2+} release in the sea urchin egg.

The sea urchin egg has a CICR mechanism like that in muscle cells that is functionally distinct from the IP_3-sensitive mechanism of Ca^{2+} release. This mechanism may function in the normal physiology of the cell. Both procaine and ruthenium red block activation of sea urchin eggs (*21*) and an IP_3-independent Ca^{2+} release mechanism in intact *Lytechinus* eggs appears to function in fertilization (*22*). An alternative model in which an autocatalytic cycle of Ca^{2+}-induced IP_3 production and IP_3-mediated Ca^{2+} release might solely propagate the wave was based in part on the ability of neomycin to inhibit both IP_3 production and wave propagation (*23*). However, in the SR neomycin also inhibits CICR (*19*). CICR was suggested not to exist in sea urchin eggs (*22*), on the basis of studies employing Ca^{2+} microinjection or artificially stimulated influx of Ca^{2+} across the plasma membrane. However, CICR can display both Ca^{2+}-dependent activation and inactivation, giving rise to a complex dependence of CICR on both the amount of Ca^{2+} used in triggering the response and the rate of change of the extravesicular concentration of Ca^{2+} (*24*).

CICR might function in producing calcium waves and oscillations seen in numerous nonmuscle cell types (*10, 11*). Such Ca^{2+} release may be modulated by cADPR, an endogenous metabolite in numerous tissues. In the sea urchin egg homogenate, cADPR was effective at concentrations 10^6 times lower than the pharmacological modulator caffeine. Caffeine releases Ca^{2+} in neurons (*25*), chromaffin cells (*26*), and pancreatic (*5*) cells, and opens an IP_3-insensitive Ca^{2+} channel in pancreatic ER vesicles (*27*). Ryanodine-binding proteins occur in liver and brain (*28*), and ruthenium red blocks a Ca^{2+} release mechanism in lacrimal gland (*29*). Thus, CICR may occur in many nonmuscle cells and function (perhaps in concert with IP_3-mediated Ca^{2+} release) in producing complex spatiotemporal patterns of calcium signals.

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30. Homogenates (2.5%) of unfertilized *Lytechinus pictus* eggs (Marinus, Inc., Long Beach, CA) were prepared (*15*) in a buffer consisting of 250 mM potassium gluconate, 250 mM *N*-methylglucamine, 20 mM Hepes (pH 7.2), 1 mM $MgCl_2$, 0.5 mM ATP, creatine kinase (2 U/ml), 4 mM phosphocreatine, oligomycin (1 μg/ml), 1 mM sodium azide, and 2 μM fura-2 pentapotassium salt. The concentration of free Ca^{2+} in 1-ml portions was measured in a spectrofluorometer with 340-nm excitation, 510-nm emission, and 5-nm bandpasses; as appropriate, ratios of the 340 nm to 380 nm fluorescence were checked to ensure that intensity changes monitored at 340 nm accurately reflected changes in the concentration of Ca^{2+}. Basal concentrations of Ca^{2+} were typically 100 nM. All measurements were conducted at 24° ± 0.5°C; similar results were observed in experiments at 17°C. Test agents were added in volumes not exceeding 10 μL with vigorous mixing. Calcium release was calibrated by sequential additions of known amounts of $CaCl_2$ to comparable portions without allowing time for resequestration between additions. The total available sequestered Ca^{2+} was measured by release with ionomycin (5 μM) and was nearly constant in similar portions of homogenate (averaging 1.3 μM). IP_3 was from Calbiochem, ryanodine was from Calbiochem or a gift of Merck, Sharp & Dohme, and cADPR was prepared from mammalian brain homogenates (*13*). Addition of vehicle (DMSO or water for ryanodine, ethanol for ionomycin, and water for other agents) had no effect on the fura-2 signal in homogenates. All figures are representative of experiments repeated at least three times.
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Technical Comment

Conserved Sequence and Structural Elements in the HIV-1 Principal Neutralizing Determinant: Further Clarifications

It has come to our attention that some researchers are attempting to use nucleotide sequences encoding the HIV-1 principal neutralization determinant (PND) (*1*) and related information submitted by us to GenBank and to the Human Retroviruses and AIDS Database to measure variability among HIV-1 viruses infecting a single individual (intra-individual variation). Such analyses presume the generation of, through molecular cloning and nucleotide sequencing, an accurate representation of the genes encoding different viruses present in the individual at the time the sample is taken. For several reasons, the nucleotide sequence data supporting the conclusions of our analysis could be misleading if they are used to reach conclusions about intra-individual HIV-1 variation. These reasons are as follows:

1) It is possible that a subset of the infectious virus genomes present in the infected PBMCs were preferentially amplified during culturing.

2) The oligonucleotide primers used for PCR amplification in our study may have hybridized more efficiently to the envelope gene of a subset of viral genomes.

3) The list of amino acid sequences shown in our figure 1 includes only amino acid sequences that differed in each PCR product and amino acid sequences occurring more than once from each PCR product (sibling sequences) that were not included or identified in the list of sibling sequences and sibling relationships submitted to the Human Retroviruses and AIDS Database.

4) Because our study involved working with samples from a large number of infected individuals, many viral samples were cultured and the DNA was extracted and PCR amplified in parallel. It is possible that contamination occurred between such samples from different individuals or from exogenous sources such as the HIV-1 IIIB isolate that was being used for other projects in several laboratories involved in our study. For example, there are nine sequences (numbers 166, 169, 170–175, and 225 in our figure 1) that are identical or similar to IIIB-like sequences. In addition, there are instances in which sibling sequences differed significantly (for example, sequences 115, 174, and 201; 151 and 225; and 175, 224, and 229). These sequences are therefore either from individuals that are infected with significantly different HIV-1 viruses or there was contamination of these virus or DNA preparations. In view of these uncertainties, samples that gave rise to IIIB like sequences or sibling sets with significant sequence heterogeneity are being re-examined. We therefore do not recommend the use of the LaRosa sequence set for analyzing intra-individual variation until the conclusion of this re-examination.

The focus and conclusions of our study were the identification of conserved elements (both sequences and predicted secondary structures) in the PND to aid in the design of immunogens able to elicit antibodies that neutralize a large percentage of HIV-1 virus isolates. There are at least three ways to compile the amino acid sequences we obtained. The first includes all duplicate sequences obtained from PCR products from different individuals and does not include duplicate sequences obtained from the same PCR product. The second is that in which all sequences are included, and the third is that in which no duplicate sequences are included. We chose the first method so that we would not overestimate the occurrence of conserved PND sequences by including the large number of identical sibling sequences we had obtained. Regardless of the method used to compile the sequences, and therefore regardless of whether any possible contamination occurred, the conclusions of our study are unchanged; the consensus PND sequence is unchanged; a single amino acid occurred at PND positions 11, 14, 16, 19, 20, 21, 22, 23, and 25 in 80% or more of the sequences, and the frequency of occurrence of each of the sequences listed in our table 1 was not significantly altered.

GREGORY J. LAROSA,* KENT WEINHOLD,† ALBERT T. PROFY,* ALPHONSE J. LANGLOIS,† GORDON R. DREESMAN,‡ R. NEAL BOSWELL,§ PHILLIP SHADDUCK,† DANI P. BOLOGNESI,† THOMAS J. MATTHEWS,† EMILIO A. EMINI,‖ SCOTT D. PUTNEY*

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*Repligen Corporation, Cambridge, MA 02139. †Department of Surgery, Duke University Medical School, Durham, NC 27710. ‡BioTech Resources, Inc., San Antonio, TX 98249. §Department of Medicine, Wilford Hall, U.S. Air Force Medical Center, Lackland Air Force Base, TX 78236. ‖Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.