rat hippocampal cDNA library in pPC86 [X.-J. Li *et al.*, *Nature* **378**, 398 (1995)] was amplified once in DH10B (Gibco BRL) (*28*) and transformed into yeast containing pBD-NOS(2–377). pAD-PIN was identified as a 0.5-kb clone that activated *lacZ* transcription and conferred histidine protorophy in the presence of pBD-NOS(2–377). Plasmids were sequenced by automated fluorescent sequencing. The PIN sequence has been deposited in GenBank (accession number U66461).

7. pPC97 derivatives containing fragments of RAFT were constructed by PCR and cloned into the Sal I and Sac I sites of pPC97. Truncated NOS fragments comprising amino acids 2 to 163 and 2 to 281 were generated by restriction of the initial NOS(2–377) PCR fragment with Nco I and Ava I, respectively, followed by blunt-end ligation into pPC97. Other truncated NOS fragments were prepared by PCR.
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11. A pBluescript plasmid containing the cDNA for PIN was obtained by screening a rat brain λZAPII cDNA library (Stratagene) with a probe derived from the Sal I–Not I insert in pAD-PIN. Library screening was performed according to the directions of the manufacturer.
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21. Rat cerebella were homogenized in IP buffer [50 mM tris-HCl (pH 7.7), 100 mM NaCl] and clarified by centrifugation. Washes were performed with wash buffer 1 [50 mM tris-HCl (pH 7.7), 500 mM NaCl] and wash buffer 2 [50 mM tris-HCl (pH 7.7), 500 mM LiCl]. The rabbit polyclonal antibody to the hemagglutinin epitope (HA) was from BAbCO (Richmond, CA). A rabbit antiserum to PIN was generated to a hexahisitidine-tagged fusion protein containing the full-length PIN sequence (*23*). The antiserum was used in immunoblots at a dilution of 1:1000 and recognizes a band of the expected molecular size. Preincubation of the antiserum with GST-PIN confirmed the specificity of this antiserum (*10*).
22. A Sal I–Bgl II fragment comprising the entire translated sequence of the insert in pAD-PIN was generated by PCR and subcloned into the Sal I–Bam HI site of the cytomegalovirus (CMV)-driven eukaryotic expression vector pCMV-myc to generate a fusion protein consisting of an NH_2-terminal Myc tag followed by a pentaglycine linker and the PIN insert.
23. Fusion proteins were prepared as in (*20*) except that proteins were eluted from glutathione agarose by cleavage with thrombin in thrombin cleavage buffer [50 mM tris-HCl (pH 7.7), 100 mM NaCl, 2.5 mM $CaCl_2$, and 1% Triton X-100] for 16 hours at 37°C. The eluate was adjusted to 5 mM EGTA, 4 μM leupeptin, and 400 nM aprotinin. Dilutions were made with thrombin cleavage buffer adjusted in this manner. A GST-BIRK fusion consisting of amino acids 347 to 442 of BIRK2 [D. S. Bredt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6753 (1995)] was cleaved with thrombin as above and used as a control protein in NOS assays. A different preparation with a bacterially expressed hexahistidine-tagged PIN fusion protein inhibited nNOS activity and dimerization in a similar concentration-dependent manner (*10*).
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29. Lysates from transfected HEK 293 cells (20 μl) were incubated with fusion protein (50 μl) at 37°C for 1 hour. Enzymatic activity assays were initiated by the addition of $CaCl_2$, NADPH, and [^{3}H]arginine to a final volume of 250 μl. Citrulline accumulation was measured as described [D. S. Bredt and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9030 (1989)]. Background levels were determined in the presence of 2 mM EGTA and no added $CaCl_2$.
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24 April 1996; accepted 22 August 1996

Change of a Catalytic Reaction Carried Out by a DNA Replication Protein

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The RepA protein of plasmid pC194 initiates and terminates rolling circle replication. At initiation, it forms a 5′-phosphotyrosyl DNA link, whereas at termination, a glutamate residue directs hydrolytic cleavage of the newly synthesized origin, and the resulting 3′-hydroxyl group undergoes transesterification with the phosphotyrosine link. The protein is thus released from DNA, and the termination is uncoupled from reinitiation of replication. Replacement of the glutamate with tyrosine in RepA altered this mechanism, so that termination occurred by two successive transesterifications and became coupled to reinitiation. This result suggests that various enzymes involved in DNA cleavage and rejoining may have similar mechanistic and evolutionary roots.

DNA strand transfer during site-specific recombination can occur by two different mechanisms. In the first, a covalent protein-DNA intermediate is formed, similar to that formed in reactions catalyzed by the DNA topoisomerases (*1*). Strand transfer proceeds through two successive transesterifications as exemplified in reactions catalyzed by the λ integrase and by the γδ resolvase (*2*, *3*). A phosphotyrosyl (*1*, *2*) or phosphoserine linkage (*3*) is formed, and a hydroxyl group is left on the cleaved DNA. This group then initiates a second transesterification reaction, forming a phosphodiester link and resolving the protein-DNA complex. The second mechanism, demonstrated in phage Mu transposition and hu-

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man immunodeficiency virus integration, does not involve a covalent protein-DNA intermediate (4). A phosphodiester bond in the donor DNA is first hydrolyzed to generate a hydroxyl group. Transesterification between this hydroxyl group and a phosphodiester bond in the acceptor DNA then occurs to complete strand transfer.

Several proteins involved in rolling circle replication also form a covalent link with the DNA. They initiate replication by formation of a 5′-phosphotyrosyl bond and by generation of a 3′-hydroxyl group, which then serves as a primer for DNA polymerase. The phage ϕX174 gene A protein couples termination of one replication cycle to initiation of the next, by two successive transesterification reactions that involve a second tyrosine on the same face of a putative α-helix (Fig. 1A). This tyrosine undergoes transesterification with the newly synthesized origin, forming a phosphotyrosyl bond and a 3′-hydroxyl DNA end. The hydroxyl end acts as a nucleophile in the second transesterification to resolve the link between the DNA and the other tyrosine, forming a single-stranded DNA (ssDNA) circle (5). The two catalytic tyrosines, Tyr^{343} and Tyr^{347}, are thus alternatively engaged in a covalent link with the origin during successive replication cycles. In contrast, termination of the replication catalyzed by the RepA protein of the plasmid pC194 is not coupled to reinitiation (6) and involves a hydrolytic as well as a transesterification step (Fig. 1B) (7). There is only one tyrosine, Tyr^{214}, in the active site of the RepA protein. It has been proposed that nearby glutamate, Glu^{210}, participates in the termination step by directing the hydrolysis of a phosphodiester bond within the newly synthesized origin. The 3′-hydroxyl end resulting from this hydrolytic step then undergoes transesterification with the 5′-phosphotyrosyl bond to complete the replication cycle. The RepA protein is thus released from its substrate at the end of each replication cycle.

It appears that the chemical reaction (transesterification or hydrolysis) that mediates DNA cleavage at the termination step is determined primarily by the particular functional group (tyrosine or glutamate) at a key position in the protein active site. If so, replacing the glutamate with a tyrosine in RepA might convert the hydrolytic site to a transesterification site. To test this hypothesis, we generated a RepA $Glu^{210} \rightarrow Tyr^{210}$ mutant. A conservative $Met^{211} \rightarrow Val^{211}$ mutation was introduced concomitantly, which made the novel RepA active site identical to that of the ϕX174 Rep protein (8). The capacity of the mutant protein [designated as Y-Y; the wild-type protein is designated as E-Y (9)] to support replication from the pC194 origin was tested in *Escherichia coli* cells (Fig. 2). The experimental system was composed of two plasmids. One was a RepA donor, carrying the RepA gene under the control of an inducible promoter, and the other was a RepA target, carrying the pC194 replication origin. RepA activity was deduced from the amount of ssDNA rings produced from the target plasmid (6, 7). The results indicate that the initiation activity of the Y-Y derivative is comparable to that of the wild-type protein (Fig. 2). However, its termination activity might be impaired, because, in addition to monomers, slowly migrating ssDNA products were formed, possibly by multiple rounds of target plasmid replication. A possible explanation for the impaired termination of the Rep Y-Y mutant is that the local structure at the protein active site is altered by the mutation. Replication products obtained with a Y-Y mutant derivative carrying an additional change, with Ser^{215} replaced

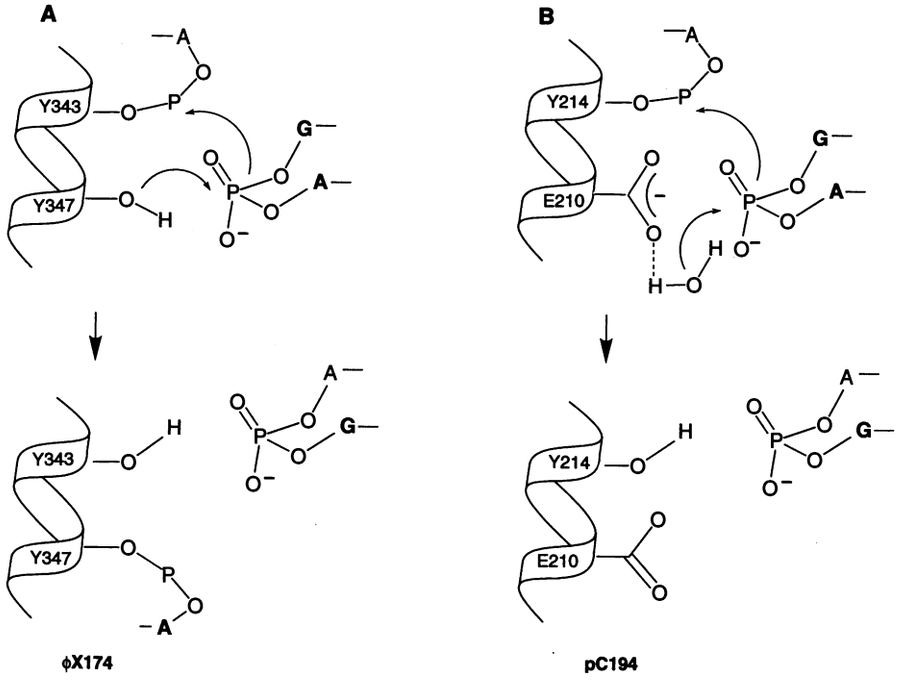

Fig. 1. Comparative mechanisms for termination of replication by the ϕX174 and pC194 initiator proteins. The two catalytic residues are juxtaposed and are separated by one turn of the α-helix in the active site. After initiation of replication, the proteins are engaged in a covalent bond with DNA substrate. Termination is initiated by a nucleophilic attack of the phosphodiester bond in the origin sequence. (**A**) In ϕX174, this reaction involves the OH group of the free tyrosine. It is followed by a series of transesterifications leading to the release of a closed ssDNA molecule product and the transfer of the protein to the new 5′-phosphate. Another round of replication can thus be reinitiated. (**B**) In pC194, the termination step is carried out by the action of the glutamate that coordinates the attacking water molecule. Hydrolysis of the phosphodiester bond creates a 3′-OH end, which carries out the nucleophile attack of the tyrosyl-phosphodiester bond. A subsequent transesterification reaction leads to the formation of a circular ssDNA product, releases the Rep protein from the replicative complex, and leaves a duplex molecule (not represented). Therefore, replication cannot be reinitiated.

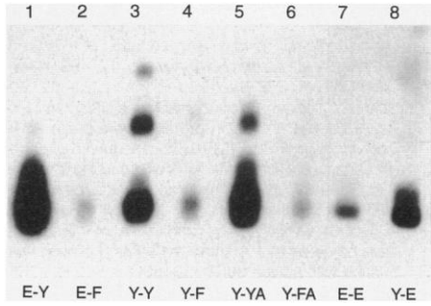

Fig. 2. Activity of the RepA active site mutants. Experiments were carried out in *E. coli*, with the use of a previously described system (*6, 7*), which consists of two plasmids: (i) a RepA donor (pHV1175), which carries RepA under control of the thermoinducible λpL promoter and replicates using ColE1 origin functions and (ii) a RepA target (pHV1180), which contains the pC194 origin and derives from vector pACYC184. Replication from the pC194 origin was induced by shifting of the temperature to 40°C. The resulting ssDNA was detected by hybridization as previously described (*6, 7*), and its amount was measured by phosphorimager analysis (*13*). Mutations of RepA were made either as described (*14*) or by PCR, and were transferred in the donor plasmid by fragment exchange. Lane 1, products of the wild-type RepA (E-Y); lanes 2 through 8, products of the indicated RepA mutants.

by alanine (designated as Y-YA), were mostly monomers (Fig. 2). The $Ser^{215} \rightarrow Ala^{215}$ mutation alone did not affect RepA activity, which indicates that this serine was not involved in catalysis (*7*), but may have made the conformation of the active site more suitable and thus restored correct termination in the Y-Y mutant protein.

The mechanism of action of this newly introduced tyrosine was tested by replacement of the original active site tyrosine, Tyr^{214}, with phenylalanine (Y-F and Y-FA derivatives, Fig. 2). The Y-F and Y-FA mutants displayed about 5% of the wild-type RepA activity. A similar small amount of residual activity was observed previously with a $Tyr^{214} \rightarrow Phe^{214}$ single mutant (*7*); thus, Tyr^{210} by itself appears incapable of supporting initiation. However, when a carboxylic functional group was introduced at position 214 of the Y-Y mutant (giving Y-E, Fig. 2), the activity of the protein was enhanced, rising to about 40% of the wild-type RepA activity. RepA containing two glutamates in the active site (E-E) displayed only a small amount of residual activity. These results indicate that initiation of replication by RepA requires a tyrosine as one catalytic center, most likely for the formation of a covalent linkage with DNA, whereas the second catalytic center can be either a tyrosine or a glutamate. The positions of the glutamate and tyrosine residues can be exchanged with only a limited effect on RepA activity, suggesting an essentially symmetric arrangement of the catalytic centers relative to the DNA phosphodiester bond at the cleavage site.

To determine whether the RepA Y-Y or Y-YA mutant can promote reinitiation, we used a plasmid that contains one complete and one truncated pC194 origin sequence (Fig. 3A). The full origin (I) allows both initiation and termination of replication, whereas the truncated origin (T) supports termination (with an efficiency of about 20%) but not initiation (*6*). Initiation at I followed by termination at T or I generated small- or full-sized ssDNA rings, respectively. If the termination at T is coupled to reinitiation and is followed in turn by termination at I, then a medium-sized DNA circle is expected.

From the T-I plasmid, two types of ssDNA rings, full- and small-sized, were generated by wild-type RepA (E-Y, Fig. 3B), which is in agreement with previous work (*6*). The Y-Y mutant generates the same two DNA types, albeit at a different ratio. The smaller amount of the small-sized product is consistent with the impaired termination activity of this mutant. The Y-YA mutant, which had an improved termination capacity and generated amounts of the small-sized rings similar to those in the wild type, formed ssDNA rings of intermediate size, which is indicative of reinitiation (Fig. 3B, lanes 5 and 6). Specific hybridization experiments and analysis by polymerase chain reaction (PCR) confirmed that this product is created as expected from a reinitiation event (*10*). The Y-YA mutant was unable to initiate replication at T without previous initiation at I, because it gave no replication product with a target plasmid carrying the truncated origin only (Fig. 3B, lane 8). These results indicate that the Y-YA mutant acquired the capacity to reinitiate replication upon termination. This implies that the tyrosine introduced at position 210 can be transferred to the DNA 5′-phosphoryl end at the termination step and therefore indicates a mechanistic switch from hydrolysis to transesterification. From the amounts of the small- and medium-sized DNA rings formed by the Y-YA mutant, it is estimated that about 12% of the termination events at T are followed by reinitiation. Thus, termination usually occurs without reinitiation. The simultaneous catalysis of termination with and without reinitiation suggests that Tyr^{210} may attack the phosphodiester bond at the newly synthesized origin either directly or through a coordinated water molecule.

By altering a key amino acid side chain in the active site of RepA, we changed the mechanism of RepA-mediated termination of replication from hydrolysis to transesterification. These results imply an evolutionary link between RepA and the ϕX174 gene A protein. Through evolution, the two proteins have acquired different mechanisms of action to match the requirements of their respective replicons. The phage gene A protein ensures efficient replication of the viral genome without regard to its bacterial host, by linking termination and reinitiation. In contrast, the pC194 RepA protein does not couple termination to reinitiation and thus allows regulation of the plasmid copy. For another plasmid, pT181, that replicates by the rolling circle mechanism, reinitiation is prevented by inactivation of its initiator protein, through the formation of a covalent adduct with a short oligonucleotide (*11*). No such modification was found for pC194 RepA (*10*) and the question of its subsequent inactivation remains to be answered.

Our study of the mechanism of action of a rolling circle replication initiator protein provides evidence of a relation between enzymes catalyzing DNA cleavage by a reaction that includes DNA hydrolysis and enzymes that proceed through formation of a covalent intermediate. The results suggest that gain or loss, through evolution, of as few as three, and possibly of only one residue properly positioned within the active site, may be sufficient to promote the shift from one type of mechanism to the other. As shown also by similarities between endonuclease and site-specific recombinases (*12*), enzymes involved in nucleic acid breakage and rejoining may have similar mechanistic and evolutionary roots.

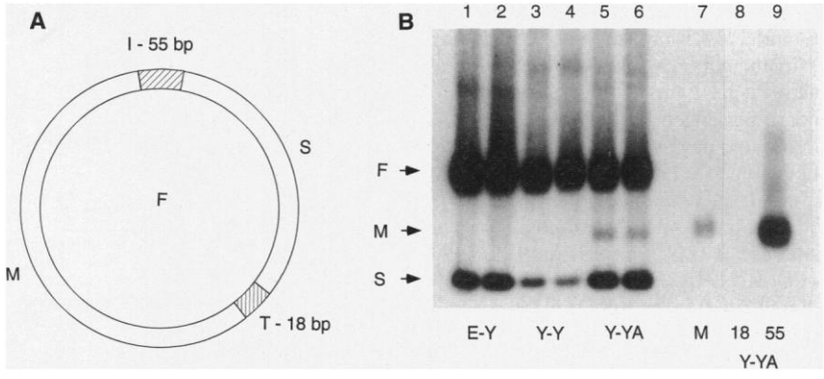

Fig. 3. Reinitiation by the RepA mutants. (**A**) The experimental system consists of a 5.6-kb plasmid, derived from pHV1180, that carries a complete 55-base pair (bp) origin sequence (denoted I) and a 1.5-kb distant termination sequence (denoted T), corresponding to 18 bp of the origin. Initiation of replication at I and termination at T would generate a small-sized product (S) of 1.5 kb. Reinitiation at T followed by termination at I would form a medium-sized product (M), corresponding precisely to the 4.1-kb plasmid pHV1180. The full-sized parental plasmid is referred to as F. This plasmid was introduced in cells harboring the RepA donor plasmid pHV1175 or a mutated derivative. We detected ssDNA as previously described (*6*, *7*). (**B**) Single-stranded DNA products. Lanes 1 and 2 display the activity profile of the wild-type RepA (E-Y), lanes 3 and 4 and lanes 5 and 6 display that of the mutated derivatives Y-Y and Y-YA, respectively. Lane 7 corresponds to circular ssDNA of plasmid pHV1180 used as a size control. We also extracted ssDNA from cells expressing RepA Y-YA and harboring either the plasmid pHV1180 carrying the full 55-bp origin (lane 9) or a control derivative in which only the 18-bp sequence of the origin was present (lane 8).

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8. The ϕX174 and pC194 Rep active site sequences are ^{343}YVAKY and ^{210}EMAKY (*9*), respectively. However, valine instead of methionine is present in several Rep proteins of the pC194 family (*7*), and the two residues are therefore functionally equivalent.
9. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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23 April 1996; accepted 21 August 1996

A Role for Phosphoinositide 3-Kinase in Bacterial Invasion

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Listeria monocytogenes is a bacterial pathogen that invades cultured nonphagocytic cells. Inhibitors and a dominant negative mutation were used to demonstrate that efficient entry requires the phosphoinositide (PI) 3-kinase p85α-p110. Infection with *L. monocytogenes* caused rapid increases in cellular amounts of PI(3,4)P_2 and PI(3,4,5)P_3, indicating that invading bacteria stimulated PI 3-kinase activity. This stimulation required the bacterial protein InlB, host cell tyrosine phosphorylation, and association of p85α with one or more tyrosine-phosphorylated proteins. This role for PI 3-kinase in bacterial entry may have parallels in some endocytic events.

Listeria monocytogenes, a bacterium that causes severe food-borne infections in humans, penetrates or invades nonphagocytic cells in the intestine and other organs (*1*). Invasion into the intestinal epithelial cell line Caco-2 is mediated by interaction between a bacterial surface protein and a cellular receptor, the adhesion molecule E-cadherin (*2*). Little is known, however, about host proteins that affect the entry process after the initial interaction between the bacterium and its receptor. Inhibition of tyrosine phosphorylation (*3*) or actin polymerization (*1*) impairs invasion, but proteins controlling phosphorylation or the actin cytoskeleton during entry have not been identified.

One signaling protein implicated in actin polymerization and activated upon receptor stimulation and tyrosine phosphorylation is the phosphoinositide (PI) 3-kinase p85-p110 (*4*). This lipid kinase is composed of an 85-kD regulatory subunit (p85) and a 110-kD catalytic subunit (p110), each of which exists in at least two isoforms (α and β). The p110 protein phosphorylates the D-3 position of the inositol ring of PI, PI 4-phosphate, and PI 4,5-bisphosphate, which are found in membranes. Two of the resulting products, PI(3,4)P_2 and PI(3,4,5)P_3, may act as second messengers; these lipids are virtually absent in quiescent cells, rapidly increase in amount upon stimulation, and appear to regulate protein kinases or other targets (*4*). In addition to p85-p110, mammalian cells contain at least two other classes of PI 3-kinases (*5*). Recently, PI-3 kinase activity has been implicated in protein trafficking and some endocytic events (*4*, *5*), raising the possibility that a PI 3-kinase may participate in processes resembling endocytosis, such as the induced uptake of pathogens into nonphagocytic cells.

The structurally unrelated compounds wortmannin and LY294002 inhibit PI 3-kinase activity by different mechanisms (*6*, *7*). We examined these inhibitors for effects on entry of *L. monocytogenes* into several mammalian cell lines, including the human epithelial cell lines Caco-2 and HeLa, and the African green monkey kidney cell line Vero. Vero cells were the most sensitive, with half-maximal inhibition of entry occurring at concentrations close to the median inhibitory concentrations (IC_{50}) of these compounds in vitro—about 5 nM for wortmannin and 5 μM for LY294002 (Fig. 1, A and B). At 50 nM, wortmannin reduced entry to about 1 to 2% of that into control dimethyl sulfoxide (DMSO)–treated cells, whereas 50 μM LY294002 reduced entry to about 10 to 20% of the control value. The more efficient inhibition by wortmannin is consistent with the relative effects of these inhibitors on PI 3-kinase activity in other systems (*8*). Entry into Caco-2 (Fig. 1C) and HeLa cells (*9*) was also impaired by wortmannin and LY294002, although the IC_{50} values were higher and the magnitude of inhibition lower than that for Vero cells. Inhibition appeared relatively specific to entry of *L. monocytogenes*, because wortmannin (50 nM) and LY294002 (50 μM) mildly stimulated entry of the unrelated invasive bacterium *Salmonella typhimurium* (strain SL1344) into Vero cells (*9*).

Wortmannin inhibits all three known classes of mammalian PI-3 kinases (*5*, *6*). To examine specifically whether p85α-p110 is needed for invasion, we used a dominant negative form of p85α (Δp85α) that inhibits PI-3 kinase activation by insulin and other agonists (*10*). Entry of *L. monocytogenes* into Chinese hamster ovary (CHO) cells stably overexpressing Δp85α (*11*) (0.012 ± 0.005% invasion) was about

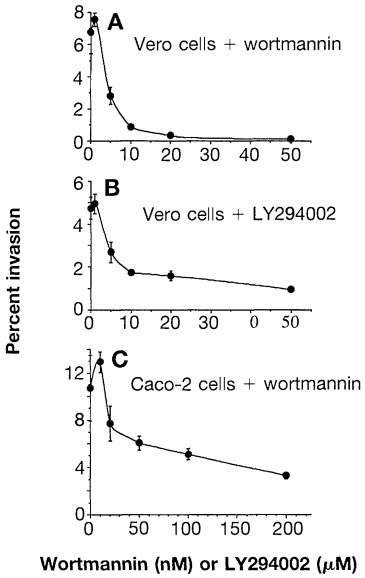

Fig. 1. Dose-dependent inhibition of entry of *L. monocytogenes* strain EGD by wortmannin and LY294002. Invasion tests into Vero or Caco-2 cells were done as described (*2*, *18*), and percent invasion values are the mean ± SD of triplicate (**A** and **B**) or duplicate (**C**) samples from a single experiment. Similar results were obtained in at least two other experiments.

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