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23. The fact that any cleavage was observed at all without trien probably reflects the limited ability of the proteolytic antibody binding pocket to accommodate a Zn(II) aquo complex.
24. B.L.I. gratefully acknowledges a Junior Postdoctoral Fellowship from the California Division of the American Cancer Society. We thank our colleagues S. Benkovic, S. Brenner, S. Danishefsky, D. Hilvert, K. Janda, A. Tramontano, R. Ghadiri, and P. Wright for helpful discussions and criticisms. The authors also must acknowledge M. Mubaraki, D. S. Pasternak, and D. Schloeder for essential and superior technical assistance.

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Sindbis Virus: An Efficient, Broad Host Range Vector for Gene Expression in Animal Cells

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Sindbis virus, an enveloped virus with a single-stranded RNA genome, was engineered to express a bacterial protein, chloramphenicol acetyltransferase (CAT), in cultured insect, avian, and mammalian cells. The vectors were self-replicating and gene expression was efficient and rapid; up to 10^8 CAT polypeptides were produced per infected cell in 16 to 20 hours. CAT expression could be made temperature-sensitive by means of a derivative that incorporated a temperature-sensitive mutation in viral RNA synthesis. Vector genomic RNAs were packaged into infectious particles when Sindbis helper virus was used to supply virion structural proteins. The vector RNAs were stable to at least seven cycles of infection. The expression of CAT increased about 10^3-fold, despite a 10^{15}-fold dilution during the passaging. Sindbis virus vectors should prove useful for expressing large quantities of gene products in a variety of animal cells.

THE GENOME OF SINDBIS VIRUS, AN alphavirus, consists of a single-stranded RNA molecule that is 11703 nucleotides (nt) in length (*1, 2*). It is capped at the 5′ terminus and polyadenylated at the 3′ terminus. As the genomic RNA is infectious and serves as mRNA it is considered to have (+) polarity. The 5′ two-thirds of the genomic RNA is translated early during infection to produce the nonstructural proteins required for RNA replication and transcription; the 3′ end of the molecule encodes the structural proteins that are expressed at high levels throughout the infection cycle. Replication proceeds by the synthesis of a full-length (−) strand with the genomic RNA as template. The (−) strand then serves as template for the synthesis of new genomic RNA molecules. The three structural proteins are not translated from the genomic RNA, but are expressed via transcription of the (−) strand at an internal site, called the junction region, to produce a capped and polyadenylated subgenomic mRNA of 4100 nt that is colinear with the 3′-terminal one third of the genome. The subgenomic mRNA does not serve as a template for RNA synthesis nor is it packaged into mature virions. Translation of the subgenomic mRNA produces a polyprotein that is cleaved co- and posttranslationally by a combination of viral and host-encoded proteases to produce the capsid protein and the two envelope glycoproteins. The capsid protein complexes with the genomic RNA to form intracellular icosahedral nucleocapsids. These interact with the cytoplasmic domains of the transmembrane envelope proteins, resulting in the budding of virus at the plasma membrane.

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Three features of Sindbis virus suggest that it might be a useful vector for the expression of foreign genes. First, Sindbis virus has a wide host range; it is naturally transmitted by mosquitos to vertebrate hosts, usually a bird or a mammal (*3*). In the laboratory it infects cultured mammalian, avian, reptilian, amphibian, and some insect (mosquito and Drosophila) cells (*4*). Second, Sindbis virus gene expression occurs in the cytoplasm of the host cell, and is rapid and efficient. During the 8 to 12 hours of a typical infection at 37°C, some 10^7 to 10^8 molecules of viral structural proteins are synthesized by each infected cell. Thus a Sindbis virus vector might be useful for production of large amounts of gene products. Third, temperature-sensitive mutations in RNA synthesis are available (*5*) that may be used to modulate the expression of foreign genes by simply shifting cultures to the nonpermissive temperature at various times after infection.

As it is technically difficult to engineer RNA molecules, a cDNA clone of the Sindbis virus genome was placed immediately downstream of a promoter for phage SP6 DNA-dependent RNA polymerase, such that transcription in vitro with SP6 RNA polymerase produced infectious RNA transcripts (*6*). This approach has been used to map the cis-acting sequences required for replication and packaging of deletion-rear-

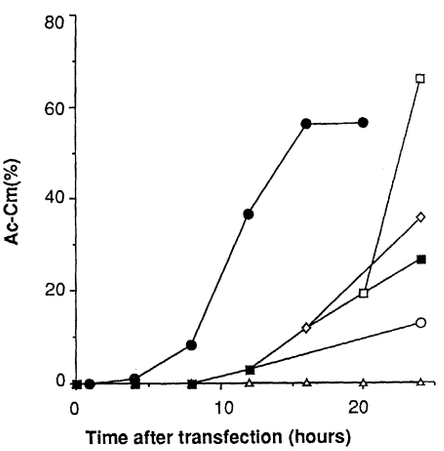

Fig. 1. Time course of CAT expression by TSCAT in chick embryo fibroblasts at 37°C, and by TSCAT-ts-6 at 30° with or without shifting to incubation at 40°C. The time course of CAT expression by TSCAT at 37° (●) in a representative experiment is shown. In an independent experiment parallel cultures were transfected with TSCAT-ts-6 at time 0. One set of cultures was maintained at 30° and sampled at the indicated times (■). At 0 or 8 (△), 12 (○), 16 (◇) and 20 (□) hours after transfection, plates from another set of cultures were shifted to and incubated at 40°C until 24 hours after transfection. Half of each cell extract was assayed for CAT activity, and the percent of chloramphenicol converted to acetylated chloramphenicol (Ac-Cm) is graphed.

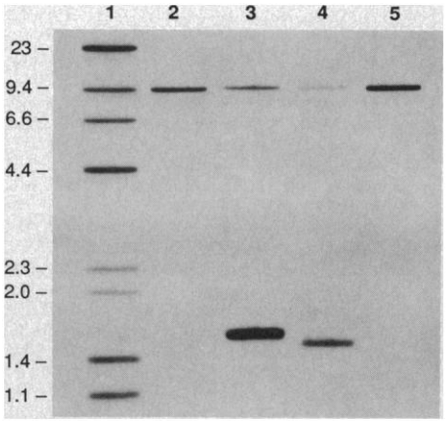

Fig. 2. Nuclease S1 analysis of TRCAT and TSCAT RNA labeled in vivo (*6–8*). Lane 1, molecular mass standards. Lanes 2 and 5, in vitro transcripts, labeled with ^{3}H-UTP, of TSCAT and TRCAT respectively, hybridized to the respective cDNA clones, and treated with S1 nuclease. Lanes 3 and 4, intracellular RNA, labeled in vivo with ^{3}H-uridine, of TSCAT and TRCAT respectively, hybridized to the respective cDNA clones, and treated with S1 nuclease.

rangement mutants of Sindbis virus (*7*), and then to adapt them for the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene (*8*). The same approach was used to engineer tobacco mosaic virus and brome mosaic virus to express CAT in plant cells (*9*).

We replaced the structural protein–encoding region in cDNA clones of Sindbis virus with the bacterial CAT gene. One clone (TSCAT) retains 117 nt downstream of the initiation site for the viral subgenomic mRNA. TSCAT was constructed from the cDNA clone Toto 1000 (*6*) to give an in-frame fusion of 23 codons of the capsid protein, plus 17 codons of polylinker and 5′ untranslated sequences of the CAT gene, followed by the CAT coding sequences. The other clone (TRCAT) was derived from Toto 1002 (*6*), and retains only 14 nt downstream of the initiation site for the viral subgenomic mRNA, such that the first initiation codon is that of the CAT gene. The sequences replaced are nt 7715 through 11087 of Toto 1000 in TSCAT and nt 7612 through 11087 of Toto 1002 in TRCAT; nucleotide positions are as described (*1*). Both TSCAT and TRCAT retain 616 nucleotides, plus the poly(A) sequence, of the 3′ end of the Sindbis genome. The genome sizes of TSCAT and TRCAT are 9066 nt and 8969 nt, respectively.

The clones were transcribed in vitro with SP6 DNA-dependent RNA polymerase and the transcripts were transfected into chicken embryo fibroblasts by the DEAE-dextran method (*6–8, 10*). About 10^3 cells (0.1% of a semi-confluent monolayer) can be transfected by means of this method (*6*). For each clone, CAT activity (*8, 11*) was first detectable at 4 hours after transfection, increasing to a plateau by 16 to 20 hours at 37°C (Fig. 1). Expression of CAT also occurred upon transfecting TRCAT or TSCAT transcripts into mosquito (*Aedes albopictus* C7-10), *Drosophila* (Schneider 1), quail (QT-6), hamster (BHK), and human (SW13) cell lines.

A derivative of TSCAT (TSCAT-ts-6) was made that carries a mutation that renders RNA synthesis temperature sensitive (*5*). When chick embryo fibroblasts were transfected with TSCAT-ts-6, CAT expression occurred at 30°C, but not at 40°C. The rapid cessation of RNA synthesis due to the ts-6 mutation can be used to modulate the level of gene expression by the Sindbis vector. At various times after transfection with TSCAT-ts-6, cultures of chick embryo fibroblasts at 30° were shifted up to and maintained at 40°C. As expected, early shifts to the nonpermissive temperature gave lower subsequent CAT expression (Fig. 1). If the shift up was relatively late, however, the levels of CAT expression exceeded that at 30°C. Since RNA synthesis is rapidly blocked upon shift up, the results suggest that the CAT mRNA is relatively stable, and is translated more efficiently at 40°C.

The replication of TSCAT and TRCAT was confirmed by S1 nuclease analysis of viral RNA labeled in vivo in the presence of actinomycin D to inhibit host RNA transcription (*12*) (Fig. 2). In each case, two protected RNA species were found, one that corresponds to the genome, and comigrates with the respective in vitro transcripts, and a smaller species that corresponds exactly to the sizes expected of the respective subgenomic mRNA. The presence of the subgenomic RNA suggests that it is the message for CAT protein synthesis.

To quantitate CAT production in transfected chick cells, parallel plates of chick embryo fibroblasts were transfected with the in vitro transcripts and incubated at 37° for 24 hours. Cells from one set of plates were harvested to measure the total CAT enzymatic activity produced by transfected cells; the other set was processed by an immunofluorescence assay for CAT to determine the number of transfected cells (*13*). Each TRCAT-transfected cell produced, on average, about 1×10^8 CAT polypeptides (molecular mass of 25,700 daltons), and each TSCAT-transfected cell produced about 2×10^7 CAT polypeptides. The amount of CAT made by TRCAT corresponds to 3% of the total cell protein (*14*), and is comparable to the level of expression of structural proteins by Sindbis virus itself. For comparison, the levels of gene expression by DNA virus vectors derived from vaccinia virus, cytomegalovirus, and baculovirus are 5×10^5 to 2×10^7 CAT polypeptides, up to 10^9 β-galactosidase molecules, and from 10^7 to 10^{10} polypeptides per cell, respectively (*14*).

One concern with the use of RNA viruses as gene expression vectors is their high mutation rates (*15*). Stability of the CAT-expressing clones was assayed in multiple cycles of infection. TRCAT and TSCAT have their structural genes replaced by the CAT gene, and thus cannot synthesize structural proteins needed to produce infectious particles. However, when the transfected cells were also infected with Sindbis virus, infectious particles containing the vector RNAs were produced. CAT activity was readily detectable when 0.25% to 0.5% of the total media (2 ml), from cell monolayers transfected with TSCAT or TRCAT and coinfected with Sindbis virus was used to infect new monolayers (*16*) (Table 1).

Table 1. Amplification of TRCAT by passaging in the presence of Sindbis helper virus (*16*).

Passage	Inoculum (μl)*	CAT activity (U)†	Increase in CAT activity‡	Dilution§	Amplification‖
	transfection, no virus	1.1×10^{-5}			
1	transfection, + virus¶	5.4×10^{-6}		1	
2	5	6.7×10^{-5}	12	4.0×10^2	5.0×10^3
3	5	5.1×10^{-4}	94	1.6×10^5	1.5×10^7
4	5	1.2×10^{-3}	217	6.4×10^7	1.4×10^{10}
5	5	1.9×10^{-3}	343	2.6×10^{10}	8.8×10^{12}
6	10	3.4×10^{-3}	624	5.1×10^{12}	3.2×10^{15}
7	10	5.2×10^{-3}	967	1.0×10^{15}	9.9×10^{17}

*From a total volume of 2 ml of media harvested from the previous passage. †Total CAT activity recovered at 12 hours posttransfection from the infected monolayers at each passage. Dilutions of each cell extract were assayed for CAT activity, and the dilution giving an activity in the linear range of the assay was used to calculate the total activity recovered. One U of CAT is defined as the amount of CAT that acetylates 1 μmol of chloramphenicol per minute at 37°C. One U of CAT corresponds to 8 μg CAT protein, or 6×10^{13} CAT trimers (*11*). ‡Net increase in CAT activity = CAT activity at each passage/CAT activity at passage 1. §Net dilution of media from passage 1. ‖The amplification is the net dilution of the media multiplied by the net increase in CAT activity at that passage. ¶Sindbis virus was added at a multiplicity of infection of 0.5 at the time of transfection. The yield of virus at each passage was 5×10^9 to 1.2×10^{10} plaque-forming units, resulting in a multiplicity of infection of 6 to 30 for the subsequent passage.

TRCAT was stable for at least seven passages (10^{15}-fold dilution of the media from the first passage), as judged by the expression of CAT and by analysis of the intracellular viral RNAs. Compared to the transfected cells at the first passage, the monolayer at the seventh passage gave about 1000-fold more CAT expression, and correspondingly about 1000-fold more cells were labeled with antibodies to CAT (anti-CAT). Thus the net amplification of CAT activity was about 10^{18}-fold during seven passages (Table 1). TRCAT was both stable to passaging and efficiently amplified in the presence of Sindbis virus. Similarly, TSCAT could express CAT for at least six passages in the presence of Sindbis virus.

The rapid amplification of TRCAT during passaging makes possible the production of large amounts of gene products with relatively few passages. For example, if the entire sample instead of a small portion had been used for each subsequent passage, about 0.6 mg of CAT could have been produced during passage 3, increasing to about 0.6 g by passage 4, and to over 300 g by passage 5. The stability of TRCAT and its ability to be amplified are thus more than adequate for large scale production of gene products.

Although these Sindbis vectors are quite stable, it is still advantageous to minimize the number of passages. One way of accomplishing this is to maximize the number of infectious particles produced per cell. Given the icosahedal structure of Sindbis virus (*17*), the size of the genome may affect the packaging efficiency. The TRCAT genomes (76% of the size of the Sindbis genome) could be packaged into infectious particles with structural proteins provided by coninfecting Sindbis virus, since the CAT activity obtained in the subsequent infection was undiminished if the media were pretreated with ribonuclease A (*18*). By comparing the CAT activity obtained for consecutive passages, we estimate that from about 300 to as many as 5000 infectious TRCAT particles were produced per cell under these conditions; much of the variability is attributable to the varying number of Sindbis helper virions that coinfect each TRCAT-infected cell, since they can compete with (as well as help) TRCAT in replication and packaging. In comparison, about 10^4 infectious virus particles were produced per cell by Sindbis virus infection under similar conditions. If the competition due to coinfecting Sindbis virus is taken into account, this implies that TRCAT is packaged with an efficiency of at least 10% that of Sindbis virus. Even smaller genomes, 72 to 73% of the size of the Sindbis genome, are packageable; derivatives of TRCAT and TSCAT, called TRCAT62 (8421 nt) and TSCAT62 (8518 nt), were made that retain only 62 nucleotides rather than 616 nt of the Sindbis 3′ terminus. Cells transfected with these RNAs expressed CAT, and the RNAs were packaged into infectious particles. Thus Sindbis virus can package RNA molecules of a range of sizes. The exact dependence of packaging efficiency on the genome size remains to be determined. However, if Sindbis genome-sized RNA proves to be packaged more efficiently, the size of Sindbis clones containing small foreign inserts can be increased quite easily by retaining more of the 3′ region of the Sindbis structural genes.

The cloning capacity of Sindbis vectors depends on how much of the structural region can be replaced while retaining efficient gene expression and packaging. TRCAT retains only 14 nt and 616 nt of 5′ and 3′ termini of the subgenomic mRNA. The cloning capacity of a self-replicating and packageable vector is therefore at least 3475 nt, sufficient to encode a polypeptide of 127 kD. The results with TRCAT62 suggest that the cloning capacity is as much as 4029 nt (corresponding to a 148-kD polypeptide). Even larger cloning capacities may be possible if transfected cells are to be studied directly, without the need for packaging to generate infectious particles.

At present, the Sindbis vectors can produce 10^{11} molecules of a gene product, with about 1000 cells transfected. Passaging in the presence of helper virus can be used to enhance the yields. The analysis of the foreign gene product can be relatively simple, since actinomycin D may be used to inhibit host transcription and infection with Sindbis virus inhibits host protein synthesis. The vectors are therefore suitable for analytical scale studies of, for example, post-translational modifications of a given protein in a variety of cell types from a number of animal species. Furthermore, the ability to modulate gene expression can be useful, for example, to study the in vivo effects of regulatory proteins needed at specific concentrations in the cell. For large-scale production, it would be ideal to develop "suicide" vectors, that do not produce infectious particles during the final production stage. The Sindbis vectors are suitable for this as they lack structural protein genes and any free RNA genomes are unlikely to survive the usual protein purification procedures. One approach would be to use helper virus with multiple temperature-sensitive mutations in the structural genes to inhibit packaging during the production step. The alternative and general approach is to develop virus-free packaging systems to provide the structural proteins, analogous to the helper cell lines developed for retrovirus vectors (*19*).

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12. The in vivo labeling of viral RNA, isolation of viral RNAs, and S1 nuclease analysis of viral RNA have been described (*6–8*). S1 nuclease analysis of TSCAT and TRCAT viral RNA used the respective cDNA clones as unlabeled probe.
13. Quantitation of the level of gene expression in various systems has classically relied on the assay of CAT enzyme activity produced. We measured CAT enzyme activities (*11*) assuming 100% recovery of CAT, and used the specific activity of CAT (*11*) to convert enzyme activities to number of CAT polypeptides. We also assumed that the CAT fusion protein made by TSCAT has the same specific activity as CAT. For immunofluorescence labeling of cells making CAT protein, the cells were fixed with 4% freshly prepared paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at 4°C. The monolayers were washed and excess paraformaldehyde was neutralized with glycine (1 mg/ml) for 10 min. Cells were permeabilized with 0.5% Triton X-100 in PBS, and nonspecific protein binding was blocked with 2% bovine serum albumin and 2% horse serum. Cells were then incubated for 1 hour at room temperature with a 1:500 dilution of an affinity-purified rabbit anti-CAT (5 Prime-3 Prime Inc.). After washing, the cells were treated with a 1:100 dilution of a fluorescein-labeled goat anti-rabbit IgG antibody (Boehringer Mannheim Biochemicals).
14. Each TRCAT-infected cell produces approximately 10^8 CAT polypeptides of 25,700 daltons, or 4 pg of CAT. The protein content of chicken embryo fibroblasts is approximately 130 pg per cell (measured by means of a protein assay kit (Bio-Rad) with bovine serum albumin as standard). This corresponds to 31 μg of CAT per milligram of total protein. In comparison, pBR328 in *E. coli* gives 34 μg of CAT per milligram of total protein [T. J. Close and R. L. Rodriguez, *Gene* **20**, 305 (1982)]. Expression levels

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16. For the first passage, a semi-confluent monolayer (1×10^6 cells) of chicken embryo fibroblasts was transfected with 1 μg TRCAT transcripts by the DEAE-dextran method (*6–8*), and coinfected at the same time with Sindbis virus at a multiplicity of infection of 0.5, in a total volume of 0.2 ml. After 1 hour at 37°C, 2 ml of fresh media was added and the cultures were incubated for 12 hours. The media was harvested for determination of virus yields and for further passaging. The cells were harvested at the same time, and were processed for CAT assays (*11*). Subsequent passages were identical to the first, except that the cells were not treated with DEAE-dextran, but were infected with 5 μl (passages 2 to 5) or 10 μl (passages 6 to 7) of media (diluted with 0.2 ml PBS) from the previous passage. After 1 hour at 37°C, 2 ml of fresh media was added, the cells were incubated for 12 hours at 37°C, when the media and cells were harvested.
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20. We thank D. Berg for helpful comments on the text. Supported by a Biomedical Research Support Grant, grants AI24134, AI11377, and AG05681 from the National Institutes of Health, a grant from the Pew Memorial Trust, and the Monsanto/Washington University Biomedical Research Contract. C.M.R. is a Pew Scholar in the Biomedical Sciences.

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Role of Phosphatidylinositol Kinase in PDGF Receptor Signal Transduction

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The molecules with which the platelet-derived growth factor (PDGF) receptor interacts to elicit the biochemical reactions responsible for cell proliferation have not been identified. Antisera directed against specific PDGF receptor peptides coprecipitated a phosphatidylinositol (PI) kinase and the PDGF receptor. Immunoprecipitates from PDGF-stimulated cells contained 10 to 50 times as much PI kinase as those from unstimulated cells. Mutation of the PDGF receptor by deletion of its kinase insert region resulted in a receptor markedly less effective than the wild type in eliciting cell proliferation and defective in PDGF-stimulated PI kinase, but still capable of PDGF-induced receptor autophosphorylation and phosphoinositide hydrolysis. These data show that the PDGF receptor is physically associated with a PDGF-sensitive PI kinase that is distinct from tyrosine kinase and is not required for PDGF-induced PI hydrolysis. The finding that the mutant PDGF receptor missing the kinase insert domain elicited known early biochemical responses to PDGF, but did not associate with or regulate PI kinase, suggests a novel role for the receptor-associated PI kinase in the transmission of mitogenic signals.

THE BINDING OF PDGF TO ITS RECEPTOR rapidly activates the receptor's tyrosine kinase and elicits a number of biochemical responses that culminate in cell division. Although the mechanism by which the PDGF receptor is coupled to early responses is unknown, the receptor must act by associating with and modifying other effector molecules. Recently, Kaplan and collegues (*1*) reported that stimulation of intact cells with PDGF led to the appearance of PI kinase activity in phosphotyrosine immunoprecipitates, suggesting that either the PI kinase is a substrate of PDGF-stimulated tyrosine kinase or that it is physically associated with the tyrosine-phosphorylated PDGF receptor. A PI kinase is known to associate with v-*src* in Rous sarcoma virus–transformed cells and with middle T–c-*src* complex in polyoma-transformed cells (*1, 2*). Analysis of mutants suggested that the ability of these oncogene products to associate with PI kinase is required for transforming activity (*2, 3*). For these reasons, we examined the ability of wild-type and mutant PDGF receptors to physically associate with and regulate PI kinase.

To test the hypothesis that a PI kinase might be physically associated with the PDGF receptor, we assayed receptor immunoprecipitates of PDGF-stimulated Balb/c 3T3 cells for PI kinase activity (Fig. 1). PI kinase activity was specifically immunoprecipitated with antisera to peptides representing either extracellular ("77") or intracellular ("88") portions of the PDGF receptor (Fig. 1). Immunoprecipitates from PDGF-stimulated cultures contained 10 to 50 times as much PI kinase activity as those from unstimulated cultures. In parallel experiments, the recovery of PDGF receptors in immunoprecipitates of [^{35}S]methionine-labeled cultures was unaffected by PDGF treatment (*4, 5*). The peptides to which the receptor antisera were raised specifically blocked precipitation of the receptor (*4*) and coprecipitation of PDGF-sensitive PI kinase (Fig. 1). These data show that a PI kinase is physically associated with the PDGF receptor. The increased recovery of PI kinase activity in immunoprecipitates from PDGF-stimulated cultures suggests that activation of the receptor by ligand either stimulates a PI kinase already associated with the receptor or causes the receptor to associate with a PI kinase.

Receptor antisera coprecipitated the receptor with a PDGF-sensitive PI kinase

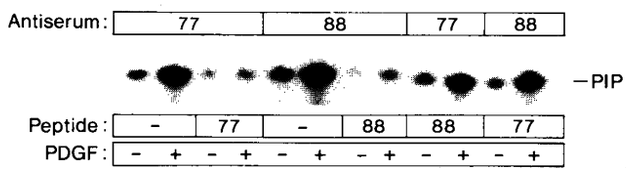

Fig. 1. Phosphatidylinositol kinase activity in PDGF receptor immunoprecipitates. Confluent quiescent cultures of Balb/c 3T3 cells in 150-cm^2 flasks were incubated in the presence (+) or absence (−) of PDGF (10 ng/ml) for 15 min at 37°C. The cells were washed once with cold phosphate-buffered saline and then lysed in situ with 50 m*M* NaCl, 20 m*M* tris HCl, *p*H 7.4, 1 m*M* sodium orthovanadate, 1 m*M* phenylmethylsulfonyl fluoride, 5 m*M* EDTA, 50 m*M* sodium fluoride, 20 m*M* sodium pyrophosphate, and 1% Triton X-100 (RIPA) (18). The lysates were clarified by centrifugation and incubated for 2 hours at 4°C with receptor antiserum 77 or 88 at a 1:500 dilution in the presence or absence of the peptide (50 μg/ml) to which each antiserum was raised, as indicated. Peptide 77 represents amino acid residues 425 to 446 in the receptor's extracellular domain, and peptide 88 represents amino acid residues 738 to 760 in the cytoplasmic domain (*4, 19*). After an additional 20-min incubation at 4°C with protein A–Sepharose, the immunoprecipitates were recovered by centrifugation and washed three times with 1 ml of RIPA, then three times with 1 ml of 100 m*M* NaCl and 10 m*M* tris, *p*H 7.4. The washed immunoprecipitates were resuspended in 100 μl of 20 m*M* Hepes, *p*H 7.2, 5 m*M* $MnCl_2$, sonicated PI (0.2 mg/ml), with 10 μCi of [γ-^{32}P]ATP as described (*2, 3*). The kinase reaction was allowed to continue for 20 min at room temperature; incorporation of ^{32}P into phosphatidylinositol-monophosphate (PIP) was linear over this time. Labeled phospholipids were extracted, separated by thin-layer chromatography, and detected by autoradiography as previously described (*2, 3*). This experiment is representative of three replicate experiments.

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