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18. Similar amounts of CAT activities were obtained when cells were infected with 10 μl of the media from the first passage, or when 10 μl of the same media was pretreated with ribonuclease A (0.25 mg/ml) for 30 min at 37°C. In contrast, no CAT activity was obtained if the in vitro transcripts were treated with ribonuclease A before transfection.
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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

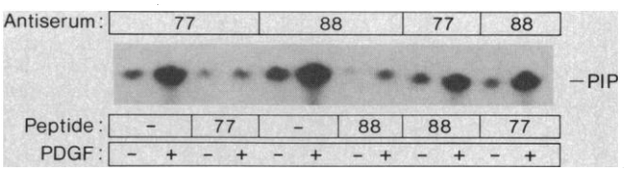

Fig. 1. Phosphatidylinositol kinase activity in PDGF receptor immunoprecipitates. Confluent quiescent cultures of Balb/c 3T3 cells in 150-cm^2 flasks were incubated in the presence (+) or absence (−) of PDGF (10 ng/ml) for 15 min at 37°C. The cells were washed once with cold phosphate-buffered saline 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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from Chinese hamster ovary (CHO) cells transfected with cDNA encoding the mouse PDGF receptor (Fig. 2). No PDGF-sensitive PI kinase activity was coprecipitated from the parent CHO cells, which lack PDGF receptors (Fig. 2). Similar results were obtained by immunoprecipitation with receptor antisera (Fig. 2, A and B) and by immunoaffinity chromatography with a monoclonal antibody to phosphotyrosine (Fig. 2C). The latter purifies the activated tyrosine-phosphorylated form of PDGF receptor as well as other phosphotyrosine-bearing proteins. The increase in the recovery of PI kinase activity obtained with monoclonal antibodies to phosphotyrosine compared to that obtained with receptor antisera correlated with an increase in the recovery of PDGF receptor (*5*). These data confirm that recovery of a PDGF-sensitive PI kinase in receptor immunoprecipitates is a specific phenomenon requiring the PDGF receptor.

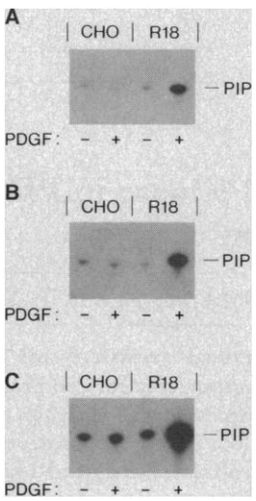

Fig. 2. Phosphatidylinositol kinase activity in PDGF receptor or phosphotyrosine immunoprecipitates from PDGF receptor transfectants. Chinese hamster ovary cells or CHO cells stably transfected with a vector placing mouse PDGF receptor cDNA under the transcriptional control of the SV40 early promoter (R18) (*9*) were incubated in the presence (+) or absence of PDGF (−) and receptor immunoprecipitates were prepared as described in the legend to Fig. 1. Phosphotyrosine immunoprecipitates were prepared by adsorption of clarified cell lysates to phosphotyrosine monoclonal antibody 1G2 immobilized on Sepharose beads (*18*). PI kinase activity in the immunoprecipitates was analyzed as described in the legend to Fig. 1. (**A**) Antiserum 77 (against an extracellular domain) immunoprecipitates; (**B**) antiserum 88 (against a cytoplasmic domain) immunoprecipitates; (**C**) phosphotyrosine antiserum immunoprecipitates. The amount of ^{32}P incorporated into PIP corresponding to the spots shown on the autoradiogram from left to right (in counts per minute) was: (A) 34, 19, 52, 357; (B) 37, 22, 14, 921; (C) 143, 403, 208, 4623. This experiment is representative of five replicate experiments.

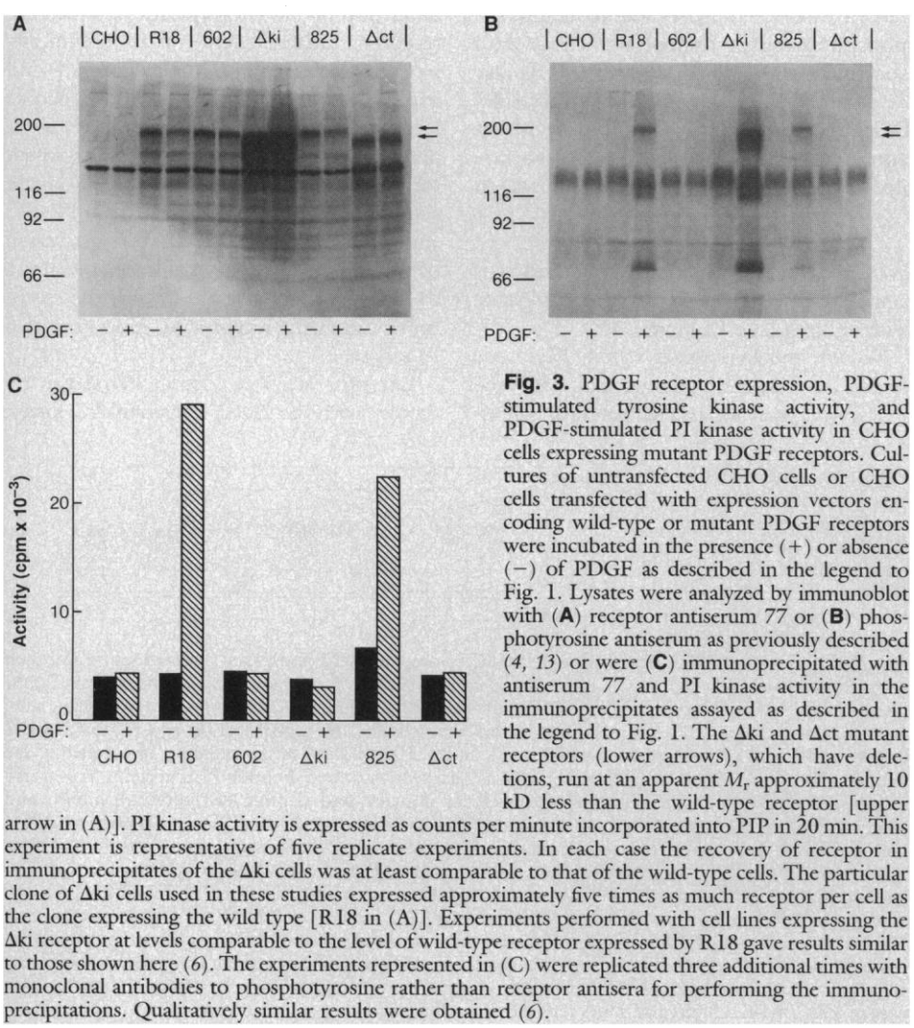

Fig. 3. PDGF receptor expression, PDGF-stimulated tyrosine kinase activity, and PDGF-stimulated PI kinase activity in CHO cells expressing mutant PDGF receptors. Cultures of untransfected CHO cells or CHO cells transfected with expression vectors encoding wild-type or mutant PDGF receptors were incubated in the presence (+) or absence (−) of PDGF as described in the legend to Fig. 1. Lysates were analyzed by immunoblot with (**A**) receptor antiserum 77 or (**B**) phosphotyrosine antiserum as previously described (*4, 13*) or were (**C**) immunoprecipitated with antiserum 77 and PI kinase activity in the immunoprecipitates assayed as described in the legend to Fig. 1. The Δki and Δct mutant receptors (lower arrows), which have deletions, run at an apparent M_r approximately 10 kD less than the wild-type receptor [upper arrow in (A)]. PI kinase activity is expressed as counts per minute incorporated into PIP in 20 min. This experiment is representative of five replicate experiments. In each case the recovery of receptor in immunoprecipitates of the Δki cells was at least comparable to that of the wild-type cells. The particular clone of Δki cells used in these studies expressed approximately five times as much receptor per cell as the clone expressing the wild type [R18 in (A)]. Experiments performed with cell lines expressing the Δki receptor at levels comparable to the level of wild-type receptor expressed by R18 gave results similar to those shown here (*6*). The experiments represented in (C) were replicated three additional times with monoclonal antibodies to phosphotyrosine rather than receptor antisera for performing the immunoprecipitations. Qualitatively similar results were obtained (*6*).

We used two methods to determine whether the PI kinase activity is actually intrinsic to the PDGF receptor or resides in an associated molecule. First, phosphotyrosine-bearing proteins were purified from lysates of PDGF-stimulated cultures by anti-phosphotyrosine (APT) affinity chromatography. Subsequent adsorption of the APT column eluates to wheat germ agglutinin–Sepharose removed more than 90% of the PDGF receptor but less than half of the PDGF-stimulated PI kinase activity. Second, PI kinase activity was separated from the PDGF receptor when APT eluates were analyzed by anion-exchange chromatography (Mono Q). The PI kinase eluted from the column at a lower salt concentration than the receptor (*6*). These data suggest that most of the PI kinase activity present in APT eluates is not intrinsic to the PDGF receptor molecule, but resides in an associated molecule. Consistent with this interpretation is the observation that a mutant PDGF receptor (Δki) with an active PDGF-sensitive tyrosine kinase does not exhibit receptor-associated PI kinase (Fig. 3) (see below). Moreover, published studies have shown that a PI kinase associated with affinity-purified epidermal growth factor (EGF) receptor could be separated from the receptor by gel filtration (*7*) and that the PI kinase activity copurified with v-*src* and v-*ros* is not a property of the oncogene products themselves (*8*).

We examined a number of CHO cell lines that express PDGF-receptor mutants for PDGF-sensitive PI kinase activity in order to correlate the presence or absence of PDGF-sensitive PI kinase with other receptor-mediated responses such as autophosphorylation at tyrosine, PI turnover, and mitogenesis. The preparation of the cell lines expressing mutated PDGF receptor cDNAs and their characterization have been described in detail (*9–12*). Six cell lines were selected for study: the parent CHO cells, which are devoid of PDGF receptors; R18, which express the wild-type mouse PDGF receptor (*9*); Δki, which express a PDGF receptor in which the kinase insert region has been deleted to yield a receptor with a continuous rather than split tyrosine kinase domain (*10*); K602A, which express a PDGF receptor in which lysine at the adenosine triphosphate (ATP) binding site has

been converted to alanine (*11*); Y825F, which express a receptor in which the tyrosine at putative autophosphorylation site 825 has been converted to phenylalanine (*12*); and Δct, which express a receptor with a carboxyl-terminus deletion (*11*). All of these receptor forms were expressed at the cell surface and were capable of binding PDGF with high affinity (*9–12*).

The levels of receptor expression, PDGF-stimulated receptor autophosphorylation at tyrosine, and PDGF-stimulated PI kinase activity for each of the cells lines are shown in Fig. 3. PDGF-stimulated autophosphorylation of the PDGF receptor at tyrosine was detected in cells expressing the wild-type receptor (R18) or the single putative autophosphorylation site mutant (Y825F) (Fig. 3B), and PDGF-stimulated PI kinase activity was detected in receptor immunoprecipitates from these cells (Fig. 3C). No PDGF-stimulated receptor autophosphorylation was detected in cells expressing the ATP-binding site mutant (K602A) or the carboxyl-terminus deletion mutant (Δct) (Fig. 3B); these mutants also lacked detectable tyrosine kinase activity in vitro (*11*). Receptor immunoprecipitates from cells expressing these mutant receptors had no detectable PDGF-stimulated PI kinase activity (Fig. 3C), suggesting that an intact tyrosine kinase may be necessary for PDGF-stimulated PI kinase.

In contrast to the mutants described above, deletion of the kinase insert region resulted in a receptor mutant, Δki, that had tyrosine kinase activity but lacked the receptor-associated PI kinase. Stimulation of cells expressing the Δki mutant with PDGF led to autophosphorylation of the PDGF receptor at tyrosine (Fig. 3B). In addition to its autokinase activity, the receptor Δki mutant could function as a phosphotransferase, as judged by its ability to tyrosine-phosphorylate a 32-kD substrate in response to PDGF (*10*). However, no PDGF-stimulated PI kinase was detected in PDGF receptor immunoprecipitates of Δki cells (Fig. 3C). Similarly, no PDGF-stimulated PI kinase was detected in PDGF receptor preparations purified from Δki cells by lectin-affinity chromatography or by antiphosphotyrosine chromatography (*6*). These data show that a PDGF-stimulable tyrosine kinase is not sufficient for expression of PDGF-stimulated PI kinase activity. Whether failure to detect PDGF-stimulated PI kinase activity in receptor immunoprecipitates from Δki cells stimulated in vivo with PDGF represents inability of the Δki mutant to associate with or to activate PI kinase is unclear. It does not appear that a tyrosine-phosphorylated active PI kinase is generated in cells expressing the kinase insert deletion mutant, in that no PDGF-stimulated PI kinase could be detected with monoclonal antibodies to phosphotyrosine in these cells (*6*). However, it is still possible that the Δki mutant does associate with and activate PI kinase in vivo by means other than tyrosine phosphorylation, but the association or activation is lost during the sample processing. Regardless, the interaction of the Δki mutant with PDGF-stimulated PI kinase is clearly different from that of the wild-type PDGF receptor, suggesting a role for the kinase insert region of the receptor in signal transduction.

The mitogenic response to PDGF stimulation in CHO cells expressing the Δki mutant PDGF receptor was less than 20% of that seen in CHO cells expressing wild-type PDGF receptor (*10*). This critical role for the kinase insert region in PDGF receptor function is consistent with the high degree of sequence conservation of this region between mouse and human PDGF receptors (*13*). That the Δki mutant does not associate with or regulate a PDGF-sensitive PI kinase suggests that the receptor-associated PDGF-stimulated PI kinase may be necessary for PDGF-induced mitogenesis or may reflect an inability of the mutant receptor to modify other substrates necessary for mitogenesis.

Other early biochemical responses to PDGF, including PDGF-induced phosphoinositide hydrolysis and calcium influx, were intact in cells expressing the Δki mutant (*10*). Thus PDGF-stimulated PI kinase is not necessary for PDGF-induced release of tritiated inositol 1-monophosphate, inositol 1,4-bisphosphate, or inositol 1,4,5-trisphosphate from metabolically labeled cells (*10*). This finding is consistent with the recent report that type I PI kinase converts PI to phosphatidylinositol-3-monophosphate (PIP), a novel product for which no role in PI turnover is currently known (*14*). The PDGF-stimulated PI kinase found in receptor immunoprecipitates converted to PIP only when PI was presented in micellar form; detergent-solubilized PI was not utilized as a substrate. The PDGF-stimulated PI kinase found in receptor immunoprecipitates was also insensitive to inhibition by adenosine (*6*). On the basis of these properties, the PDGF-stimulated PI kinase can be classified as a type I PI kinase (*15*). The finding that the PDGF receptor associates with and activates a novel PI kinase suggests that PDGF-induced PI turnover may be qualitatively different from that induced by other receptors not coupled to PI kinase. Specifically, the PDGF-stimulated PI kinase may alter the distribution and types of PI phosphates available for hydrolysis by phospholipase C, and thus change the pattern of inositol phosphate second messengers produced. Such a role for type I PI kinase is supported by the recent identification of phosphatidylinositol 3,4,5-phosphate in activated neutrophils (*16*).

Like other individual PDGF-induced postreceptor events (*17*), activation of the PDGF receptor-associated PI kinase may be necessary for PDGF-induced mitogenesis, but is not sufficient. The mitogenic response to PDGF was markedly decreased in CHO cells that express the Y825F mutant PDGF receptor compared to that of cells expressing the wild-type receptor (*12*). However, the level of receptor-associated PI kinase in cells expressing the Y825F mutant was not significantly different from that found in cells expressing the wild-type receptor (Fig. 3C). Thus, activation of the PI kinase alone cannot account for the mitogenic effect of PDGF.

In conclusion, a PI kinase is physically associated with the PDGF receptor, and the activity of this enzyme or its association with the receptor is regulated by PDGF. Analysis of mutant PDGF receptors showed that all mutants lacking PDGF-stimulated PI kinase were markedly deficient in PDGF-induced mitogenesis. The loss of PDGF-stimulated PI kinase and PDGF-induced mitogenesis with preservation of PDGF-induced PI turnover in cells expressing the kinase insert deletion mutant shows that the receptor-associated PI kinase is not necessary for PDGF-induced PI hydrolysis, but may be necessary for producing specific second messengers mediating the mitogenic action of PDGF.

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Cloning and Expression of a *Xenopus* Embryonic Gap Junction Protein

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Gap junctions in the early amphibian embryo may play a fundamental role in the regulation of differentiation by mediating the cell-to-cell transfer of chemical signals. A complementary DNA encoding a gap junction present in *Xenopus* oocytes and early embryos has now been cloned and sequenced. This protein sequence is homologous to the well-characterized gap junction structural proteins rat connexin32 and connexin43. RNA blot analysis of total *Xenopus* oocyte RNA showed hybridization to a single 1.6-kilobase band. This messenger RNA is abundant in oocytes, decreases to levels below the sensitivity of our assay by stage 15 (18 hours), and is not detectable in RNA from a number of adult organs. To confirm that the oocyte cDNA encodes a gap junction channel, the protein was over expressed in *Xenopus* oocytes by injection of RNA synthesized in vitro. Pairs of RNA-injected oocytes formed many more time- and voltage-sensitive cell-cell channels than water-injected pairs.

CELLS OF THE EARLY AMPHIBIAN embryo are coupled by low-resistance pathways that are permeable to ions and small molecules. Gap junctions are thought to be responsible for this coupling. The precise roles of junctional coupling in the oocyte and early embryo are not known. Gap junctions couple follicular cells to the oocyte and may help control oocyte maturation (*1*). In embryonic tissues, it has been suggested that gap junctions transmit regulatory factors involved in the determination of regional patterns of differentiation (*2, 3*). Some similarity between gap junction proteins from amphibians and mammals is suggested by the report that antibodies generated against adult rat liver gap junctions cross-react with proteins in *Xenopus* blastulae (*2*). On the other hand, unlike mammalian gap junctions, voltage-clamp studies of pairs of amphibian blastomeres show that the steady-state junctional conductance is steeply voltage dependent (*4–6*).

To elucidate the molecular basis for the observed differences between amphibian and mammalian gap junction proteins, we have cloned and sequenced cDNA for a *Xenopus* gap junction protein. A cDNA library in λgt11 constructed from *Xenopus* oocytes was screened with a cDNA coding for rat connexin43 under low-stringency conditions (*7*). One positive clone, X01A, was obtained. The clone was subcloned into the plasmid vector Bluescript (Stratagene) and sequenced on both strands by means of the dideoxy chain termination method (*8*). The cDNA was 1301 nucleotides in length. The cDNA contained a single open reading frame of 1002 bases and portions of 5′ and 3′ untranslated regions. No polyadenylated tail or consensus polyadenylation signal was observed.

The DNA has been entered into the GenBank data base (access no. J03091). The open reading frame encodes a protein of 334 amino acids with a molecular mass of 38 kD. In accordance with the nomenclature suggested by Beyer *et al.* (*7*), this protein was named *Xenopus* connexin38. For brevity, we will refer to the predicted protein as connexin38. The sequence of connexin38 was compared with two rat gap junction proteins (*7, 9*), connexin32 and connexin43 (Fig. 1). Many amino acid residues are identical in all three proteins. The overall amino acid homology is 32% between connexins 38 and 32 and 41% between connexins 38 and 43 with regions of higher and lower homologies. Comparison of connexin38 with a rat lens gap junction protein, connexin46 (*10*), and a human liver gap junction protein (*11*) gives similar results. A search of the National Biomedical Research Foundation protein database and the GenBank and European Molecular Biology Laboratory DNA database identified no other sequences with significant homology.

A hydropathicity profile (*12*) of connexin38 predicts the same four potential transmembrane domains described by Beyer *et al.* (*7*). Unlike the other gap junction proteins, connexin38 was predicted to have a fifth hydrophobic domain close to the COOH-terminus. Lacking in the sequence for connexin38 is a transmembrane segment corresponding to the S4 region proposed to be involved in the voltage-dependent gating of Na^+ (*13*), dihydropyridine-sensitive Ca^{2+} (*14*), and fast, transient K^+ (*15*) channels.

An RNA blot analysis (Fig. 2) of *Xenopus* total RNA from different developmental stages demonstrated the presence of connexin38 RNA in eggs. The signal declined by early gastrula and was below the sensitivity of our assay by early neurula. Total RNA from adult tissues was also tested for the presence of connexin38 mRNA; a signal was detected in ovary but not in liver, heart, intestine, testes, and kidney. Connexin38 appears to have a distribution thus far detected only in the oocyte and early embryo. It does not have a distribution similar to connexin43 in the rat, which is found not only in the ovary, principally between ovari-

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XEN CXN38   1  MAGWELLKLLLDDVQEHSTLIGKVWLTVLFIFRIFILSVAGESVWTDEQSDFICNTQQPGCTNVCYDQAFPISHV  75
RAT CXN43   1  *GD*SA*GK***K**AY**AG*****S*******LL*GT*V**A*G****A*R********E*****KS******  75
RAT CXN32   1  *N-*TG*YT**SG*NR***A**R***S*I*****MVLV**A****G**K*S*I***L****NS****HF******  74

XEN CXN38  76  RYWVLQFLFVSTPTLTYLGHMVYLSKKEEKERQKENE----SRILVANE---AQTE----VY-SSATKKIRIQGP 138
RAT CXN43  76  *F****II***V***L**A*VF*VMR****LNK**E*LKVAQTDG*NV*MHLK*I*IKKFK*GIEEHG*VKMR*G 150
RAT CXN32  75  *L*S**LIL****A*LVAM*VAHQQHI*K*M------LRL---EGHGDPLHLE--*VKR-------H-*VH*S*T 130

XEN CXN38 139  LMCTYTTSVVFKSIFEAGFL-LG-QWYI-YGFVMSPIFVCERIPCKHKVECFVSRPMEKTIFIIFMLVVSLISLL 210
RAT CXN43 151  *LR**II*IL***V**VA**-*I-****-***SL*AVYT*K*D**P*Q*D**L***T*****I********V**A 222
RAT CXN32 131  *WW**VI****RLL***V*MYVFYLI*PG*AM-VRLV-K**AF**PNT*D******T***V*TV***AA*G*CII 203

XEN CXN38 211  LNLMELIHLSFKCFQHGIKEGATCPP-TGIPFNGAGNRMPPQE-YTNPPSSNQDIDLPAYNKMSGGHNWSSIQME 284
RAT CXN43 223  **II**FYVFF*GVKDRVKGRSDPYHA*TG*LSPSKDCGS*KYA*F*GC**PTAPLS*MSPPGYKLVTGDRNNSS 297
RAT CXN32 204  **VA*VVY*IIRACARRAQRRSNP*SRKGSGFGHRLSPEYKQN-EI*KLL*E**GS*KDILRRSP*TGAGLAEKS 277

XEN CXN38 285  QQVNGLVKPKCQCDCWSQSAISVVVSGAPGIISNMDAVKRNHQTSSKQQYV                          334
RAT CXN43 298  CRNYNKQASEQNWANY*AEQNRMGQA*STISN*HAQPFDFPDDNQNAKKVAAGHELQPLAIVDQRPSSRASSRAS 372
RAT CXN32 278  DRCSAC                                                                    283

RAT CXN43 373  SRPRPDDLEI                                                                382
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Fig. 1. Alignment of the amino acid sequence of *Xenopus* connexin38 (XEN CXN38) with rat connexin43 and connexin32 (RAT CXN43 and RAT CXN32). Identical residues are indicated by asterisks and gaps by dashes. Gaps are inserted manually to optimize the alignment.

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