

- Sci. Vigne Vin* **29**, 63 (1995)] defined 12 "ecogeogroups." He included in the Noirien group 'Pinot noir', 'Chardonnay', 'Gamay', 'Meunier', 'Melon', 'Troyen', 'Auxerrois', and 'Gouget'.
13. We compared 'Gouais blanc' and 'Heunisch weiss' at 11 microsatellite loci and found them to be identical (J.-M. Boursiquot, C. Roux, P. This, unpublished results). H. Goethe [*Handbuch der Ampelographie* (P. Parey, Berlin, 1887)] indicates that 'Heunisch weiss' is of Croatian origin.
  14. Grape cultivars are highly heterozygous. Although modern cultivars are hermaphroditic and naturally self-pollinate, the viability of selfed progeny declines beyond a few generations. Wild grapes and some primitive cultivars are dioecious and therefore obligate outcrossers.
  15. We found 'Malaga II' to be the selfed progeny of 'Muscat of Alexandria'. It was selected as a seedling in the 1950s and is not cultivated.
  16. The parents of 'Cabernet Sauvignon', 'Cabernet franc', and 'Sauvignon blanc' are also genetically dissimilar, sharing only 12 of 56 alleles at 28 loci.
  17. J. E. Bowers, G. S. Dangl, R. Vignani, C. P. Meredith, *Genome* **39**, 628 (1996).
  18. J. E. Bowers, G. S. Dangl, C. P. Meredith, *Am. J. Enol. Vitic.* **50**, 243 (1999).
  19. M. Thomas, personal communication.
  20. K. Sefc, F. Regner, E. Turetschek, J. Glössl, H. Steinkellner, *Genome* **42**, 367 (1999).
  21. E. Hagelberg, I. C. Gray, A. J. Jeffreys, *Nature* **352**, 427 (1991); B. S. Weir, *Genetic Data Analysis II* (Sinauer, Sunderland, MA, 1996), pp. 209–215.
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# Differential Stimulation of PKC Phosphorylation of Potassium Channels by ZIP1 and ZIP2

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Targeting of protein modification enzymes is a key biochemical step to achieve specific and effective posttranslational modifications. Two alternatively spliced ZIP1 and ZIP2 proteins are described, which bind to both Kvβ2 subunits of potassium channel and protein kinase C (PKC) ζ, thereby acting as a physical link in the assembly of PKCζ-ZIP-potassium channel complexes. ZIP1 and ZIP2 differentially stimulate phosphorylation of Kvβ2 by PKCζ. They also interact to form heteromultimers, which allows for a hybrid stimulatory activity to PKCζ. Finally, ZIP1 and ZIP2 coexist in the same cell type and are elevated differentially by neurotrophic factors. These results provide a mechanism for specificity and regulation of PKCζ-targeted phosphorylation.

Protein phosphorylation is important for the regulation of neuronal excitability and many other non-neuronal processes. Phosphorylation of ion channels, which could change channel expression as well as kinetic properties, is thought to be one of the key regulatory mechanisms of membrane excitability (1). The existence of signaling complexes containing both ion channels and closely associated protein kinases and phosphatases has been inferred from biochemical and electrophysiological studies (2). Such ion channel-kinase/phosphatase complexes are thought to provide the necessary macromolecular organization for signaling specificity and regulation (3, 4). Many ion channels are phosphorylated by serine-threonine kinases such as PKC and PKA (5). The mechanisms for targeting the specificity and regulating the activity of PKC or PKA to ion channel proteins are not well understood.

Potassium channels are important for controlling neuronal excitability (6). Shaker-type

potassium channels have been implicated in associative memory in model systems (7). The auxiliary Kvβ subunits specifically interact with a subset of Kvα subunits, which, in some cases, results in modulatory effects (8–10). The mammalian Kvβ2 is an abundant subunit found in both excitable and nonexcitable cells, and the native neuronal Shaker-type potassium channel has an estimated stoichiometry of (Kvα)<sub>4</sub>(Kvβ)<sub>4</sub> (11).

To identify proteins that functionally interact with potassium channels, a yeast two-hybrid library made from rat hippocampal mRNA was screened against full-length Kvβ2. The interacting clones include multiple partial cDNA fragments of Kvβ2 (12). In addition, we found two cDNA fragments, B20 and B24, encoding two previously unknown Kvβ2-binding proteins. Sequence comparison of full-length cDNAs showed that B20 and B24 are identical except for a stretch of 27 residues missing in B24. Although B24 has never been reported, B20 was identical to a previously reported gene known as ZIP (PKC-zeta interacting protein) that is isolated as a protein interacting with an atypical PKCζ isozyme (13). Human homologs for this protein, known as A170 or p62, have also been identified (14). Thus, B20 and B24 were termed ZIP1 and ZIP2.

We generated primers flanking the insertion site, which allows polymerase chain reaction

(PCR) to amplify DNA covering the putative splicing site, thereby producing DNA fragments of distinct sizes (15). Reverse transcription (RT)-PCR using rat cerebellum mRNA amplified two DNA fragments (Fig. 1C, lane 5). They possessed the same mobility as the PCR products obtained using cloned ZIP1 and ZIP2 as templates and corresponded individually to the coding sequences of ZIP1 and ZIP2 (Fig. 1C, lanes 2 through 4). These data provide evidence that ZIP1 and ZIP2 resulted from alternative splicing, and both transcripts are present in the brain. The two forms contain a number of protein domains suggestive of their biochemical features (Fig. 1, A and B). The NH<sub>2</sub>-terminal acidic motif is a novel domain homologous to a segment of CDC24 in yeast. They were found in diverse proteins of evolutionarily distant species ranging from bacteria to mammal. All share a characteristic sequence pattern of (Y/W)XDXG(L/F) (V/I). The zinc finger domain contains three CXXC motifs and a DYDL signature (16, 17). Both PEST and ubiquitin associated (UBA) domains are involved in regulation of protein turnover by either coding protein stability or directing proteins to degradation pathways. In addition, the PEST sequence is implicated in interaction with calmodulin (18).

ZIP was identified from rat brain cDNA library, but its expression was not restricted to the nervous system. A 69-kD polypeptide was detected in all tissues tested (Fig. 1D). Different from most tissues, regions in the central nervous system gave rise to two bands, 69 and 66 kD, both of which could be completely blocked with purified GST-ZIP1 fusion protein (19). The sizes of the two polypeptides are consistent with those of ZIP1 and ZIP2, although we cannot rule out that the two species result from differential posttranslational modifications. RT-PCR was employed to detect the expression and relative ratio of ZIP1 and ZIP2 transcripts. In nonexcitable tissues, ZIP1 was present in significantly higher amounts than ZIP2, whereas in regions of the central nervous system, the ZIP1/ZIP2 ratio was closer to 1 (Fig. 1D, lower panel). The expression of the ZIP proteins was also temporally regulated. In the cerebellum, an area with a high expression level of ZIP (Fig. 2C), the expression of the ZIP proteins peaked at postnatal day 13 (Fig. 1E). The two ZIP polypeptides differed both in expression level

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and in relative amount during postnatal development. RT-PCR also detected an increase of ZIP1 transcripts (Fig. 1E, lower panel).

The yeast two-hybrid method was employed to further test the interaction among ZIPs and various potassium channel subunits. The formation of the  $\alpha$ - $\beta$  complex of potassium channels is mediated by the T1 domain of Kv1 $\alpha$  subunits and the conserved core regions of Kv $\beta$ -subunits (8, 10). ZIP1 and ZIP2 interacted with both Kv $\beta$ 1 and Kv $\beta$ 2, but not with the NH<sub>2</sub>-terminal domain of the Kv1 $\alpha$  subunits of ShB (NShB) (Fig. 2A), which contains the Kv $\beta$  binding site. This suggests that ZIP binds to potassium channels via the Kv $\beta$  subunits. ZIP1 and ZIP2 interacted both homomerically and heteromerically (Fig. 2A). Nondetergent soluble protein complexes from rat brain containing ZIP migrated with a Stoke's radius larger than  $6.1 \times 10^{-9}$  m which corresponds to a 640-kD globular protein (20). The purified GST-cdc homology domain of ZIP alone behaved as an oligomer (13).

To directly demonstrate the interaction between Kv $\beta$ 2 and ZIP, we tested for binding of GST-ZIPs expressed in *Escherichia coli* to HA-tagged Kv $\beta$ 2 expressed in mammalian tissue culture cells by incubating the GST-ZIP1-Affigel or GST-ZIP2-Affigel resin with an excess

amount of HA-Kv $\beta$ 2 (15). While the control GST-PDZ3-Affigel resin displayed no detectable binding (lanes 6 and 10), both GST-ZIP1-Affigel and GST-ZIP2-Affigel precipitated the HA-tagged Kv $\beta$ 2 (lanes 7 and 11) (Fig. 2B).

The ability of ZIP to interact with both PKC $\zeta$  and Kv $\beta$ 2 led us to hypothesize that ZIPs act as a link in the PKC $\zeta$ -ZIP-Kv $\beta$ 2 complex. Sequential horizontal sections of rat brain were probed by in situ hybridization with either sense control (right panels) or antisense (left panels) probes of Kv $\beta$ 2, ZIP1, and PKC $\zeta$  (Fig. 2C). Their mRNA localization was overlapping, especially among pyramidal cells in the hippocampus and Purkinje cells in the cerebellum, where transcripts for the three proteins were found to be more concentrated than in other parts of the brain.

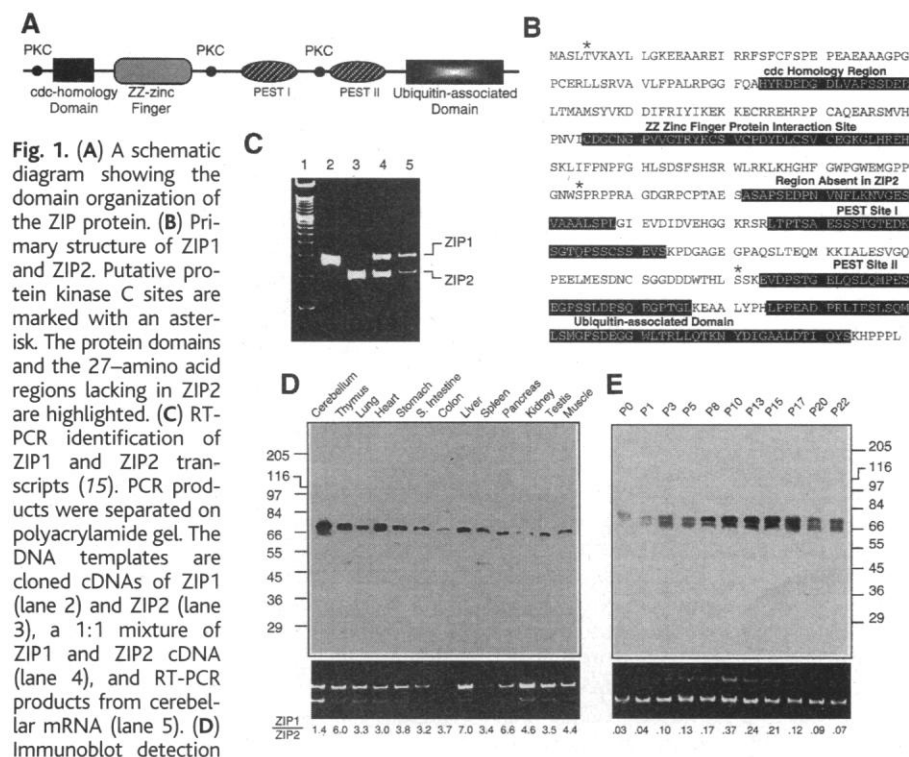
Immunoprecipitation was carried out using detergent-solubilized protein lysate prepared from adult rat cerebellum. Antibodies specific to Kv $\beta$ 2, ZIP, and PKC $\zeta$  were used to precipitate protein complexes. Each antibody was capable of precipitating protein complexes containing Kv $\beta$ 2, ZIP, and PKC $\zeta$ . In contrast, translin, an unrelated protein abundant in the cerebellum, was present in the crude soluble lysate, but none of the antibodies was able to precipitate it. ZIP1 is

thought to bind specifically to PKC $\zeta$  (13). Consistent with the results, PKC $\zeta$ II could not be precipitated by any of the above antibodies (Fig. 2D). Although the stoichiometry of the Kv $\beta$ 2-ZIP-PKC $\zeta$  complexes is not known, within the soluble fraction of cerebellar extracts the 69-kD species was coimmunoprecipitated more efficiently, suggestive of a potential binding difference of the two ZIP polypeptides to Kv $\beta$ 2 and PKC $\zeta$ .

Complementary DNAs encoding Kv $\beta$ 2 and PKC $\zeta$  were expressed as HA-tagged fusion proteins in mammalian COS7 cells cultured in phosphate-free media (21). Soluble protein lysates of either PKC $\zeta$  or Kv $\beta$ 2-transfected COS7 cells were prepared, mixed, and precipitated with anti-HA monoclonal antibody. Activation of PKC $\zeta$  in the presence of [<sup>32</sup>P]- $\gamma$ -ATP induced basal phosphorylation of the Kv $\beta$ 2 subunit. When purified, recombinant GST-ZIP1 was added to the reaction, the Kv $\beta$ 2 phosphorylation was stimulated by more than ninefold, while GST-ZIP2 showed less than twofold stimulation ( $n = 5$ , Fig. 3A). Using myelin basic protein (MBP), a common experimental substrate of the PKC $\zeta$  enzyme, similar levels of MBP phosphorylation were found in the presence or absence of GST-ZIP1, GST-ZIP2, or a control GST fusion protein, GST-PDZ3 (Fig. 3B). Thus, the ZIP-mediated stimulation of PKC $\zeta$  is substrate-selective, presumably restricted to the ZIP interacting proteins such as Kv $\beta$ 2. The ratios of the ZIP1 and ZIP2 messages varied significantly among different tissues, suggestive of a distinct function for ZIP1 and ZIP2. Indeed, GST-ZIP1 is significantly more potent than GST-ZIP2 in its ability to stimulate Kv $\beta$ 2 phosphorylation. The time course of their activity could be best fit with two exponential time constants (Fig. 3C and legends).

Under fixed amounts of Kv $\beta$ 2 and PKC $\zeta$ , the phosphorylation of Kv $\beta$ 2 was measured with increasing concentrations of purified GST-ZIP1 or GST-ZIP2. The EC<sub>50</sub> values of GST-ZIP1 and GST-ZIP2 were comparable:  $1.3 \pm 0.4 \mu\text{M}$  ( $n = 4$ ) and  $2.1 \pm 0.4 \mu\text{M}$  ( $n = 4$ ), respectively. In contrast, the maximal stimulation by GST-ZIP1 was 7.4-fold higher than that by GST-ZIP2. ZIP1 and ZIP2 behaved similarly in binding to Kv $\beta$ 2 and PKC $\zeta$  as tested in the yeast two-hybrid system (Fig. 2A) (13). The differential stimulation could thus result from their potential different binding affinity influenced by differential posttranslational modifications of ZIP1 and ZIP2 as suggested by the coimmunoprecipitation experiments (Fig. 2D).

ZIP-mediated stimulation showed a reproducible decline phase following the peak stimulation at high concentrations of GST-ZIP. The maximal ZIP-mediated stimulation was neither enhanced nor reduced by the addi-



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tion of the same or a higher concentration of a control GST-fusion protein such as GST-PDZ3 of PSD-95, indicating that the decline phase did not result from an excess of GST. Other supportive evidence includes that changes of inputs of PKC $\zeta$  or Kv $\beta$ 2 induced a shift of ZIP concentration for the maximal stimulation of the Kv $\beta$ 2 phosphorylation (22). These data are consistent with the notion that the ZIP-mediated stimulation was achieved through the formation of a stoichiometrically defined reaction complex involving PKC $\zeta$ , ZIP, and Kv $\beta$ 2. Thus, instead of reaching saturation, the higher concentration of ZIP would disrupt the optimal ratio for a functional PKC $\zeta$ -ZIP-Kv $\beta$ 2 complex, for example, by assembly of less active forms of oligomeric ZIP.

ZIP1 and ZIP2 are two alternatively spliced transcripts with distinct ability to stimulate the phosphorylation of Kv $\beta$ 2. The ratio of these two messages may be regulated in response to hormone stimulation and may thus be a way to modulate the intracellular phosphorylation level. Neurotrophins, such as nerve growth factor (NGF), are involved in regulation of synaptic plasticity and ion channel activity (23). PC12 cells respond to NGF stimulation (24), providing a model system to test the hypothesis.

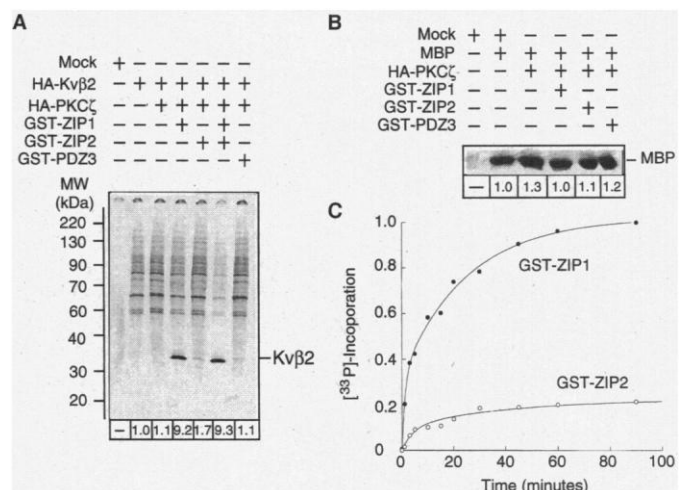
The mRNAs of both ZIP1 and ZIP2 in PC12 cells were detectable in the absence of NGF. In the presence of NGF, the ZIP1/ZIP2 ratio increased from 0.2 to 1.0 within 24 hours

(Fig. 4A). At the protein level, the expression of ZIP was increased by approximately 30-fold. Upon NGF stimulation, the signal for the putative 66-kD ZIP2 intensified, which was accompanied by an increase in the 69-kD putative ZIP1 polypeptide (Fig. 4B). Consistent with the changes in the ZIP1/ZIP2 mRNA ratio, the ra-

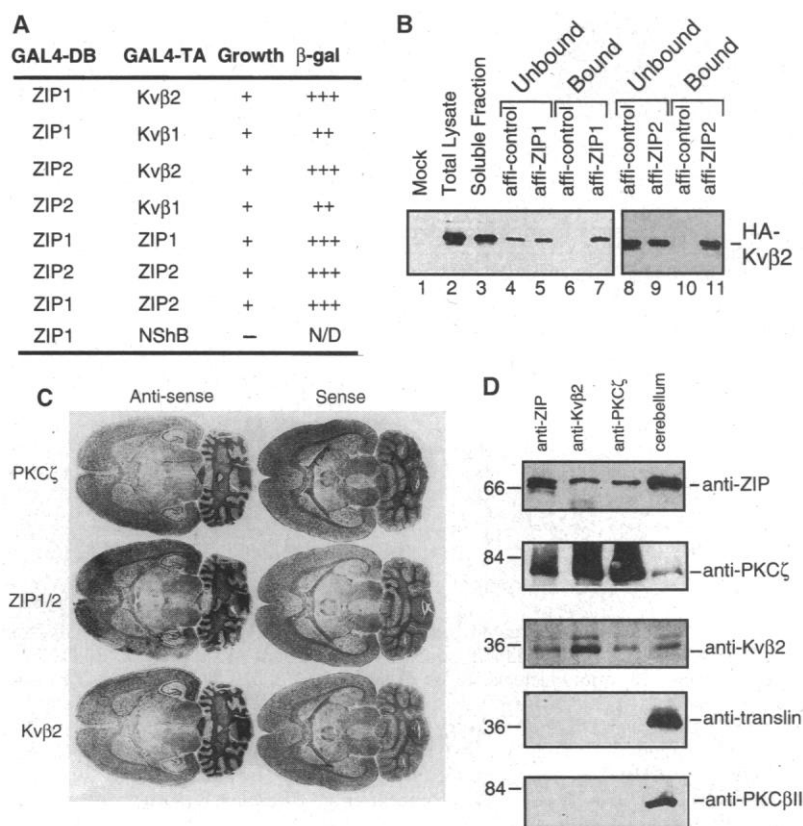
tio of 69 kD to 66 kD increased from 0.1 in the absence of NGF to 0.8 after 72 hours of the stimulation. In contrast, at the protein level Kv $\beta$ 2 showed almost no change and PKC $\zeta$  showed only a modest change in response to NGF stimulation (Fig. 4B). These results show that both ZIP1 and ZIP2 were present in

**Fig. 3.** Stimulation of PKC $\zeta$  phosphorylation of the Kv $\beta$ 2 subunit.

(A) In vitro phosphorylation of Kv $\beta$ 2. Anti-HA precipitated Kv $\beta$ 2 and PKC $\zeta$  were tested for phosphorylation in the presence of 1  $\mu$ M purified GST proteins (27). The phosphorylated proteins were visualized by autoradiography. The protein inputs for each reaction are listed on the top of the gel. The Kv $\beta$ 2 signal was quantified by phosphorimaging, normalized against Kv $\beta$ 2 signal in the absence of PKC $\zeta$  and GST fusion proteins, and listed at the bottom. (B) Phosphorylation of myelin basic protein (MBP) was carried out using the procedures (27). (C) ZIP-mediated differential stimulation of the Kv $\beta$ 2 phosphorylation by PKC $\zeta$ . The time course of Kv $\beta$ 2 phosphorylation was performed as described (27) except that the reaction was carried out at 4°C in the presence of 1  $\mu$ M purified GST-ZIP1 and GST-ZIP2. The data were best fit with two time constants  $\tau_1$  and  $\tau_2$  in the equation:  $A[1 - \exp(-t/\tau_1)] + B[1 - \exp(-t/\tau_2)]$ . For GST-ZIP1 ( $n = 3$ ),  $A = 0.33 \pm 0.040$ ,  $B = 0.69 \pm 0.037$ ,  $\tau_1 = 1.3 \pm 0.35$  min and  $\tau_2 = 26 \pm 3.9$  min; for GST-ZIP2 ( $n = 3$ ),  $A = 0.12 \pm 0.037$ ,  $B = 0.10 \pm 0.059$ ,  $\tau_1 = 40 \pm 34$  min and  $\tau_2 = 4.3 \pm 3.0$  min.



**Fig. 2.** (A) Summary of the yeast two-hybrid test. Transformants with both GAL4-DB and GAL4-TA plasmids were selected and allowed to grow in His media. Growth (+) and no growth (-) are indicated. The  $\beta$ -galactosidase activity is tested using X-gal, where blue was observed within the first 30 min (indicated by +++) and within the first 2 hours (++) (B) Affinity binding of GST-ZIP1 or GST-ZIP2 to the HA-Kv $\beta$ 2. Soluble lysates (lane 3) from HA-Kv $\beta$ 2 transfected cells were incubated with Affi-gel cross-linked with GST-ZIP1, GST-ZIP2, or control GST-PDZ3 of PSD95 (27). The unbound (lanes 4, 5, 8, and 9) and bound (lanes 6, 7, 10, and 11) materials were separated on SDS-PAGE and detected with anti-HA antibody. The samples are marked on the top of gel. (C) In situ hybridization detection of mRNAs encoding Kv $\beta$ 2, ZIP1/2, and PKC $\zeta$  (28). Horizontal sections of adult rat brain were hybridized with DIG-labeled antisense (left panels) and sense (right panels) probes. The various ribo-probes were generated using full-length cDNAs as indicated on the left. (D) Antisera against GST fusion proteins of Kv $\beta$ 2 and ZIP1 were generated and affinity-purified. In immunoblot analyses, these two antibodies and commercial anti-PKC $\zeta$  antibody detected polypeptides of Kv $\beta$ 2, ZIP1/ZIP2, and PKC $\zeta$  of predicted sizes. The immunoblot signals could be blocked by the corresponding antigens (29). Coimmunoprecipitation was performed using adult soluble cerebellar extracts (30). The antibody-precipitated materials were separated by SDS-PAGE followed by the immunoblot detection. Each lane is identified by antibody that was used for precipitation. The starting material, soluble cerebellar extract, was also loaded for comparison. (Right) Antibodies used in immunoblot. (Left) Molecular size standards.



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the same cell type, suggesting the potential for the formation of heteromultimers and regulation of stoichiometry of the ZIP1-ZIP2 heteromultimers.

Phosphorylation of Kv $\beta$ 2 was assayed with increasing concentrations of purified GST-ZIP1 supplemented with fixed concentrations of GST-ZIP2. In the presence of GST-ZIP2, the overall EC<sub>50</sub> value gradually shifted to the lower concentration of GST-ZIP1 (Fig. 4C). For example, when a fixed amount of 1  $\mu$ M GST-ZIP2 was included, the EC<sub>50</sub> value of GST-ZIP1 reduced by approximately 10-fold, from  $1.3 \pm 0.4$   $\mu$ M ( $n = 4$ ) to  $0.18 \pm 0.05$   $\mu$ M ( $n = 4$ ) (Fig. 4C). GST-ZIP2, by itself, did show stimulatory activity, although at a considerably lower level. Figure 4D compares experimental data of GST-ZIP1 + 1.0  $\mu$ M GST-ZIP2 with a simulated curve obtained by digital addition of the signal of 1.0  $\mu$ M GST-ZIP2 to signals of GST-ZIP1 at different concentrations. Both the reduction of EC<sub>50</sub> and the shape change of the dose response curve indicate that the stimulatory activity was not a result of linear addition of activities contributed independently by ZIP1 and ZIP2. The heteromultimeric interactions detected between

ZIP1 and ZIP2 by the yeast two-hybrid test (Fig. 2A) were consistent with the notion of a functional interaction between GST-ZIP1 and GST-ZIP2.

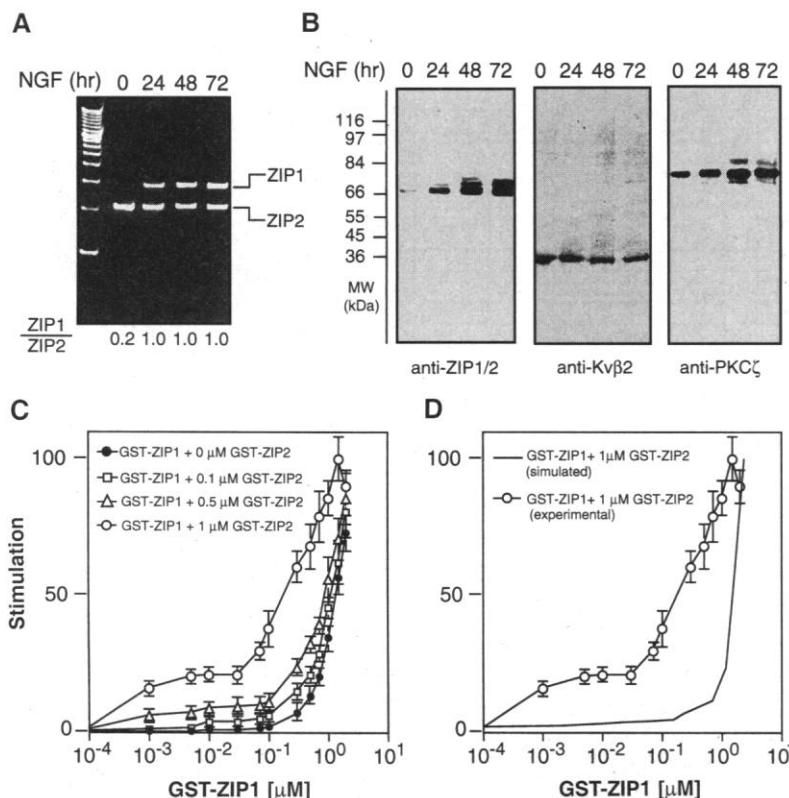
Our results suggest that ZIP acts as a link that targets the activity of PKC $\zeta$  to Kv $\beta$ 2. ZIP1 and ZIP2, two alternatively spliced protein products, possess distinct activities in stimulating PKC $\zeta$  phosphorylation of Kv $\beta$ 2. Their ability to interact with each other to form homo- and heteromultimeric complexes provides an explanation for the synergistic stimulatory activity seen only in the presence of both ZIP1 and ZIP2 (Fig. 4C). The regulation of ZIP activity in various tissues and cell types is not limited to the differential temporal and spatial expression; the ZIP1/ZIP2 ratios are also tissue-specific. Furthermore, the ratio of ZIP1 and ZIP2 within the same cell type is dynamically regulated in response to stimulation by neurotrophic factors.

Redistribution of PKC proteins upon hormone stimulation is a key step involved in specifying and activating PKC activity (25). Our data suggest that one of downstream steps after PKC translocation may involve

specific and dynamic assembly of protein complexes critical for the translation of hormonal signals into targeted covalent modifications. The identification of ZIPs and their biochemical function has provided a mechanism by which the PKC phosphorylation potential to an ion channel protein can be regulated and finely tuned transcriptionally and posttranslationally.

### References and Notes

1. I. B. Levitan, *Annu. Rev. Physiol.* **56**, 193 (1994); E. A. Jonas and L. K. Kaczmarek, *Curr. Opin. Neurobiol.* **6**, 318 (1996).
2. S. K. Chung, P. H. Reinhart, B. L. Martin, D. Brautigam, I. B. Levitan, *Science* **253**, 560 (1991); K. Bielefeldt and M. B. Jackson, *Biophys. J.* **66**, 1904 (1994); C. Rosenmund et al., *Nature* **368**, 853 (1994); S. L. Swope and R. L. Huganir, *J. Biol. Chem.* **269**, 29817 (1994); P. H. Reinhart and I. B. Levitan, *J. Neurosci.* **15**, 4572 (1995); D. Rotin et al., *EMBO J.* **13**, 4440 (1994); R. S. Westphal et al., *Science* **285**, 93 (1999).
3. T. Pawson and J. D. Scott, *Science* **278**, 2075 (1997).
4. T. C. Holmes, D. A. Fadool, R. Ren, I. B. Levitan, *ibid.* **274**, 2089 (1996).
5. M. S. Shearman, K. Sekiguchi, Y. Nishizuka, *Pharmacol. Rev.* **41**, 211 (1989); I. I. Ismailov and D. J. Benos, *Kidney Int.* **48**, 1167 (1995).
6. B. Hille, *Ionic Channels of Excitable Membrane* (Sinauer, Sunderland, MA, 1991).
7. N. Meiri et al., *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4430 (1997).
8. J. Xu and M. Li, *Trends Cardiol. Med.* **8**, 229 (1998); W. F. Yu, J. Xu, M. Li, *Neuron* **16**, 441 (1996).
9. J. Rettig et al., *Nature* **369**, 289 (1994).
10. S. Sewing, J. Roeper, O. Pongs, *Neuron* **16**, 455 (1996).
11. D. N. Parcej, V. E. S. Scott, J. O. Dolly, *Biochemistry* **31**, 11084 (1992).
12. J. Xu and M. Li, *J. Biol. Chem.* **272**, 11728 (1997); J. Xu, W. Yu, J. Wright, R. Raab, M. Li, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1846 (1998); P. M. Chevray and D. Nathans, *ibid.* **89**, 5789 (1992); S. Fields and O. Song, *Nature* **340**, 245 (1989); J. Xu and M. Li, *Methods Enzymol.* **293**, 1 (1998).
13. A. Puls, S. Schmidt, F. Grawe, S. Stabel, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6191 (1997).
14. I. Joung, J. L. Strominger, J. Shin, *ibid.* **93**, 5991 (1996); T. Ishii et al., *Biochem. Biophys. Res. Commun.* **226**, 456 (1996).
15. Expression of GST fusion of ZIP1 and ZIP2 was carried out using pGEX4T2 constructs (Pharmacia Biotech, Indianapolis, IN) and standard procedures. The HA-tagged expression was achieved using pCMV vector containing 12CA5 epitope NH<sub>2</sub>-terminal to the fused cDNA. Unless specified, all constructs expressed complete coding sequence starting from the second amino acid. RT-PCR experiments were performed using tissue or cell sources as indicated. The RNAs were prepared with 50 mg of tissue (or PC12 cells harvested from one 3.5-cm dish) using TRIzol (GIBCO-BRL, Rockville, MD) according to the manufacturer's protocol. The cDNA synthesis was carried out using 2  $\mu$ g of RNA. PCR amplification was performed using the following four primers: ML1654, ML1659, ML1653, and ML1553. All primer sequences are available upon request. The ML1654/ML1659 pair amplified fragments of 303 bp for ZIP1 and 222 bp for ZIP2, whereas the ML1653/ML1553 pair amplified fragments of 473 bp for ZIP1 and 392 bp for ZIP2. Results from both pairs of primers were identical. Data obtained from ML1653 and ML1553 were not shown. The PCR protocol included 20 cycles of 1 min of denaturation at 92°C, 1 min of annealing at 55°C, and 30 s of extension at 68°C.
16. C. P. Ponting, D. J. Blake, K. E. Davies, J. Kendrick-Jones, S. J. Winder, *Trends Biochem. Sci.* **21**, 11 (1996).
17. Single-letter 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;



**Fig. 4.** (A) RT-PCR results using untreated and NGF-stimulated PC12 cells. PC12 cells were treated with NGF and collected at 24, 48, and 72 hours after treatment (37). Bottom: ZIP1/ZIP2 ratios of corresponding lanes. (B) Immunoblot analyses of Kv $\beta$ 2, ZIP1/2, and PKC $\zeta$  proteins. Identical amounts (100  $\mu$ g) of total protein were loaded from untreated and NGF-stimulated PC12 cells collected after different times as indicated. Bottom: Primary antibodies used for the detection. (C) Stimulation of the Kv $\beta$ 2 phosphorylation by GST-ZIP1 in the presence of fixed concentrations of GST-ZIP2 protein as indicated ( $n = 4$ ). (D) A simulated curve (solid line) was created by adding the signal of GST-ZIP2 at 1  $\mu$ M with the signals of GST-ZIP1 at different concentrations. The curve is plotted with the experimental data under the same conditions.

- P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. X indicates any residue.
18. K. K. Wang, A. Villalobo, B. D. Roufogalis, *Biochem. J.* **262**, 693 (1989).
  19. J. Gong *et al.*, unpublished data.
  20. Preparation of nondetergent cell lysate was carried out using buffer conditions similar to procedures described in (30) except no detergent was included. Gel filtration chromatography was done according to the published protocol in X.-D. Li *et al.* [*J. Biol. Chem.* **272**, 705 (1997)].
  21. Phosphorylation in vitro: COS 7 cells were transfected with mammalian expression plasmids, changed with phosphate-free media after 24 hours, and harvested after 48 hours. The cells were lysed in Hepes-buffered saline (140 mM NaCl, 2.7 mM KCl, and 15 mM Hepes), pH 7.4 and 1% Triton X-100 and centrifuged at 10,000g for 5 min. The extract was added to 10  $\mu$ l of protein G-agarose beads with cross-linked anti-HA antibody. The beads were collected and washed three times with Hepes-buffered saline. The pellet was then resuspended in 20  $\mu$ l kinase buffer (35 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 0.1 mM CaCl<sub>2</sub>, and 1 mM phenylphosphate). After supplementing the reaction with the indicated amounts of GST ZIPs or control protein, phosphorylation was activated by adding 5  $\mu$ l of phosphatidylserine (1 mg/ml) and 0.5  $\mu$ l of [<sup>32</sup>P]- $\gamma$ -ATP (10 mCi/ml). Unless specified, all phosphorylation reactions were incubated for 30 min at 30°C. The reaction was stopped by addition of SDS loading buffer. After boiling for 5 min, the samples were separated by SDS-PAGE. Protein phosphorylation was detected by autoradiography using Kodak scientific imaging films. Quantification of the relative amount of radioactivity was performed with a phosphor imager (Fujix BAS 1000, Fuji, Tokyo).
  22. J. Gong *et al.*, unpublished data.
  23. M. Bothwell, *Annu. Rev. Neurosci.* **18**, 223 (1995).
  24. L. A. Greene and A. S. Tischler, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2424 (1976).
  25. D. Mochly-Rosen, *Science* **268**, 247 (1995).
  26. Antibodies for ZIP and Kv $\beta$ 2 were generated by immunizing rabbits with GST-fusion proteins corresponding to the full-length cDNAs of rat ZIP1 and Kv $\beta$ 2. The PKC $\zeta$  and PKC $\beta$ II antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
  27. The HA-tagged Kv $\beta$ 2 was expressed by transient transfection and cell lysates from HA-Kv $\beta$ 2 and mock-transfected cells were prepared according to the protocol described in Yu *et al.* (10). Affi-gel 10 (Bio-Rad, CA) was used to conjugate GST control, GST-ZIP1 and GST-ZIP2. The binding reactions were performed using 20  $\mu$ l beads and 100  $\mu$ l of cell extracts in a buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol. After incubation on ice for 1 hour, the beads were washed three times, and the bound material was released by boiling the beads in SDS-sample buffer. The Kv $\beta$ 2 binding was tested by fractionating the bound material on SDS gel followed by immunoblot with anti-HA antibody.
  28. In situ hybridization was performed using a DIG-RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Briefly, DNA templates for riboprobe synthesis were prepared by digesting plasmid clones containing the full-length rat Kv $\beta$ 2, ZIP1, and PKC $\zeta$  cDNAs. After linearization, DIG-labeled RNA probes were prepared by in vitro transcription. Adult Sprague-Dawley rats were used for analysis of Kv $\beta$ 2, ZIP1/2 and PKC $\zeta$  mRNA expression. Animals were anesthetized and decapitated, and their brains were removed immediately and fixed in 4% paraformaldehyde for 2 hours. Frozen sections (12  $\mu$ m) were cut on a cryostat, thaw mounted onto SuperfrostPlus slides, and air-dried. All solutions were prepared in deionized H<sub>2</sub>O treated with 0.1% (V/V) diethylpyrocarbonate and autoclaved. Sections were fixed by immersion in 4% paraformaldehyde in PBS, pH 7.4, then briefly rinsed twice with PBS. After treatment with Proteinase K, sections were refixed in 4% paraformaldehyde. The sections then were acetylated by immersion in 0.1 M triethanolamine containing 0.25% acetic anhydride, permeabilized by 1% Triton X-100, and rinsed twice with PBS. Prehybridization was carried out at 4°C overnight with prehybridization solution (50% formamide, 5 $\times$  SSC, 5 $\times$  Denhardt's solution, 250  $\mu$ g/ml yeast tRNA, and 500  $\mu$ g/ml salmon sperm DNA). For hybridization, the sections on each slide were covered by a prehybridization solution containing 1  $\mu$ g/ml of cRNA probe, incubated at 65°C overnight in a humid chamber. Sections were immersed sequentially in 0.2 $\times$  SSC twice and buffer 1 (0.1 M Tris pH 7.5, 0.15 M NaCl) twice. The sections were covered by 1:2000 anti-Digoxin antibody in buffer 2 (1% inactivated normal goat serum in buffer 1) and incubated at 4°C overnight. After rinsing with buffer 1 and buffer 3 (0.1 M Tris pH 9.5, 0.1 M NaCl, and 50 mM MgCl<sub>2</sub>), the sections were developed with a solution containing 1.2 mg levanisole and 300  $\mu$ l NBT/BCIP in 5 ml development buffer containing 0.1 M Tris (pH 9.5), 0.1 M NaCl, and 50 mM MgCl<sub>2</sub>.
  29. J. Gong *et al.*, unpublished data.
  30. Immunoprecipitation experiments were performed using cerebellum extracts from adult rat brain. The extracts were prepared by homogenizing rat cerebellum in a lysis buffer containing 10 mM Tris HCl (pH 8.0), 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 10 mM Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin). Homogenization was carried out at 4°C using a Dounce homogenizer with a typical tissue to buffer ratio of 8 ml buffer per gram of tissue. After 20 strokes of homogenization, homogenates were centrifuged at 10,000g for 5 min and the supernatants were collected. To conjugate antibodies to Protein A-agarose, 40  $\mu$ l of Protein A beads (Sigma Chemical, St. Louis, MO) in 1 ml of PBS were first incubated overnight with 20  $\mu$ l of antiserum at 4°C. After removal of unbound material, the agarose was washed three times with PBS, resuspended in 500  $\mu$ l of 0.2 M sodium borate (pH 9.0). The cross-linking was initiated by adding dimethylolmelimidate to a final concentration of 20 mM.
  31. The PC12 culture and NGF stimulation were carried out essentially as described (24). Briefly, 10<sup>5</sup> cells were seeded in a 3.5-cm dish and allowed to grow for 24 hours in the presence of 10% fetal bovine serum and 5% heat inactivated horse serum. The NGF stimulation was initiated by adding purified NGF to a final concentration of 0.1  $\mu$ g per ml. The cells were allowed to grow for 72 hours and harvested for analyses.
  32. We thank D. Ginty for providing purified NGF, A. Lanahan and P. Worley for cDNA libraries, J. Baraban for anti-translin antibody, Y. Ono for rat PKC $\zeta$  cDNA, L. Roman for in situ hybridization technique, M. Regan for RT-PCR technique, S. Wang for the control GST fusion protein and R. Butzner for help with manuscript preparation. We also thank C. Montell, P. Gillespie, and members of the Li Lab for comments on this manuscript. M.B. was a NSF predoctoral fellow. R.H. is supported in part by a postdoctoral fellowship from American Heart Association. M.L. is supported by a grant from NIH (NS33324) and an American Heart Association-Pfizer award.

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## In Vivo Protein Transduction: Delivery of a Biologically Active Protein into the Mouse

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Delivery of therapeutic proteins into tissues and across the blood-brain barrier is severely limited by the size and biochemical properties of the proteins. Here it is shown that intraperitoneal injection of the 120-kilodalton  $\beta$ -galactosidase protein, fused to the protein transduction domain from the human immunodeficiency virus TAT protein, results in delivery of the biologically active fusion protein to all tissues in mice, including the brain. These results open new possibilities for direct delivery of proteins into patients in the context of protein therapy, as well as for epigenetic experimentation with model organisms.

Currently, efficient delivery of therapeutic compounds, peptidyl mimetics, and proteins into cells in vivo can be achieved only when the molecules are small—typically less than 600 daltons (1). Delivery of bioactive peptides across the blood-brain barrier, for ex-

ample, is generally restricted to small (six amino acids or less), highly lipophilic peptides (1). Gene therapy (2) is one promising method for circumventing this problem, but conditions for high-efficiency targeting and long-term protein expression have yet to be discovered.

We have focused on an alternative approach of "protein transduction," or protein therapy, to address this problem. In this method (3), full-length fusion proteins are generated that contain an NH<sub>2</sub>-terminal 11-amino acid

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