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Syntaxin: A Synaptic Protein Implicated in Docking of Synaptic Vesicles at Presynaptic Active Zones

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Synaptic vesicles store neurotransmitters that are released during calcium-regulated exocytosis. The specificity of neurotransmitter release requires the localization of both synaptic vesicles and calcium channels to the presynaptic active zone. Two 35-kilodalton proteins (p35 or syntaxins) were identified that interact with the synaptic vesicle protein p65 (synaptotagmin). The p35 proteins are expressed only in the nervous system, are 84 percent identical, include carboxyl-terminal membrane anchors, and are concentrated on the plasma membrane at synaptic sites. An antibody to p35 immunoprecipitated solubilized N-type calcium channels. The p35 proteins may function in docking synaptic vesicles near calcium channels at presynaptic active zones.

Chemical neurotransmitters are stored within the nerve terminal in synaptic vesicles that are often found associated with cytoskeletal components or the presynaptic plasma membrane (*1, 2*). Upon nerve stimulation, activation of voltage-gated Ca^{2+} channels in the nerve terminal plasma membrane results in an influx of Ca^{2+}. The increase in cytosolic Ca^{2+} concentration triggers the fusion of a portion of the synaptic vesicle population with the presynaptic plasma membrane, resulting in neurotransmitter release. The docking and subsequent fusion of synaptic vesicles with the presynaptic plasma membrane occur at a restricted, morphologically distinct domain known as the active zone (*2*). Because membrane fusion is initiated within 200 μs of the influx of Ca^{2+} (*3*), the cellular machinery mediating fusion is likely to be preassembled at sites of neurotransmitter release. The process of synaptic vesicle docking with the presynaptic membrane may represent the assembly of a prefusion complex that is likely to include components of each membrane. Three synaptic vesicle membrane proteins, p65 [synaptotagmin (*4–6*)], synaptophysin (*7, 8*), and synapsin I (*9*), exhibit properties suggestive of a role in synaptic vesicle docking or fusion.

We have focused on the biochemical characterization of p65, including the identification of proteins with which it interacts (*10*). p65 is a transmembrane protein with two repeats in the cytoplasmic domain homologous to the C2 regulatory domain of protein kinase C (*5*), a domain that may be important for interactions with the plasma membrane (*11*). To identify proteins that interact with p65, we solubilized a synaptic vesicle–enriched fraction from rat brain with different detergents and subjected it to immunoprecipitation with a monoclonal antibody to p65 (anti-p65) (*4*). The crude vesicle fraction used for these studies (*10, 12*) contains, in addition to synaptic vesicles, cytoskeletal and plasma membrane components that might interact with p65. Anti-p65 coprecipitated several synaptic vesicle proteins (*10*) and a set of 35-kD proteins (p35) (Fig. 1A). Anti-p65 also precipitated a protein kinase that phosphorylated both p65 and p35 in vitro (Fig. 1B). The protein kinase responsible for this phosphorylation is casein kinase II (*13*).

An oligonucleotide probe, based on an amino acid sequence obtained from the p35 proteins, was used to isolate seven cDNA clones from a rat brain cDNA library (*14*). On the basis of restriction enzyme mapping and nucleotide sequencing, these clones were divided into two classes, p35A and p35B (*15*). The predicted protein sequences encoded by the two classes of cDNA clones are 84% identical (Fig. 2). The sequence obtained by direct microsequencing of the p35 proteins was identical to that of p35A. No similarity with any protein sequence in the SWISS-PROT database was observed. Both p35A and p35B are highly charged (~38% charged amino acids) with an overall acidic isoelectric point (pI) (~5.1). However, the COOH-terminal 23 (p35A) or 24 (p35B) amino acids are very hydrophobic [average Kyte-Doolittle hydropathy scores (*16*) of 2.67 and 2.47, respectively], suggesting that this domain might serve as a membrane anchor. Because the p35 proteins lack an NH_2-terminal signal sequence, they may be membrane-anchored proteins with their NH_2-termini on the cytoplasmic side of the bilayer. This orientation would be similar to that of the synaptic vesicle protein VAMP (*17*). Northern (RNA) blot analysis (*13*) revealed that the p35A and p35B cDNA clones hybridized to 2.2- and 4.4-kb nervous system–specific transcripts, respectively.

The coimmunoprecipitation of p35 with p65 (Fig. 1A) suggested that these two proteins might interact directly. To investigate this possibility, we expressed full-length p35A and the cytoplasmic domain of p65 (p65-IIs) in bacteria (*18*) and mixed the two purified proteins in vitro. The mixture was immunoprecipitated with anti-p65 and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). Anti-p65 coprecipitated p65-IIs and p35A but not bovine serum albumin (BSA) included in the incubation as a nonspecific blocking agent (Fig. 3). A similar result was obtained with p35B (*13*). In the absence of p65-IIs, no p35A was immunoprecipitated. These results indicate that p65 and p35 interact directly.

To further characterize the biochemical

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properties of the p35 proteins, we generated a polyclonal antibody to bacterially expressed p35A [anti-p35 (*19*)]. By protein immunoblot analysis, the antiserum recognized both the p35A and p35B proteins. To determine if p35 is an integral membrane protein, as predicted from its sequence, we extracted a synaptic vesicle–enriched fraction under different conditions and separated it into soluble and particulate fractions by centrifugation at 100,000*g* for 1 hour. The extraction of p35 and three integral membrane proteins of the synaptic vesicle (p65, SV2, and synaptophysin) was monitored by protein immunoblotting (Fig. 4A). High concentrations of salt (0.5 M NaCl) and high pH [0.2 M Na_2CO_3 (pH 11.0)] did not extract p35 from the particulate fraction. However, Triton X-100 (0.5%) completely solubilized p35, suggesting that p35 is an integral membrane protein. Solubilization of the membrane preparation with Triton X-114 before phase partitioning (*20*) resulted in the recovery of a large fraction of p35 in the detergent phase (Fig. 4B), consistent with it being an integral membrane protein. To determine if p35 is associated with synaptic vesicles or another membrane compartment, we performed anti-p65 immunoprecipitations (Fig. 4C) of crude synaptic vesicle fractions that were either solubilized with CHAPS (2%) or treated with 0.5 M NaCl. Three synaptic vesicle proteins (p65, SV2, and synaptophysin) and p35 were coimmunoprecipitated from the vesicle preparation solubilized with CHAPS. In contrast, only p65, SV2, and synaptophysin were immunoprecipitated from the salt-washed intact vesicle preparation. This result, in conjunction with the inability of 0.5 M NaCl to extract p35 from membranes (Fig. 4A), indicates that p35 is not a synaptic vesicle protein but rather is an integral component of another membrane compartment. The absence of p35 in anti-p65 immunoprecipitates of salt-washed membranes suggests that an interaction between synaptic vesicles and p35-containing membranes is disrupted under these conditions. Indeed, the in vitro interaction between bacterially expressed p65 and p35 is disrupted in 0.5 M NaCl (*13*).

The direct interaction between the proteins p65 and p35 (Fig. 3), their localization to different membrane compartments (Fig. 4C), and their membrane topology (*5*) (Fig. 2) raise the possibility that p35 participates in the docking of synaptic vesicles with the presynaptic plasma membrane. To test this possibility, we determined the localization of p35 within dissociated hippocampal neurons by indirect immunofluorescence microscopy (*21*). To evaluate the localization of p35, we compared its distribution with that of the synaptic vesicle protein synaptophysin. In these neurons, both synaptophysin and p35 were concentrated in numerous varicosities (Fig. 5). However, the distribution of p35 differed from that of synaptophysin. Whereas synaptophysin immunoreactivity uniformly filled the varicosities, p35 immunoreactivity was often restricted to a ring or a crescent-shaped pattern. In several cases the p35 staining was concentrated at the

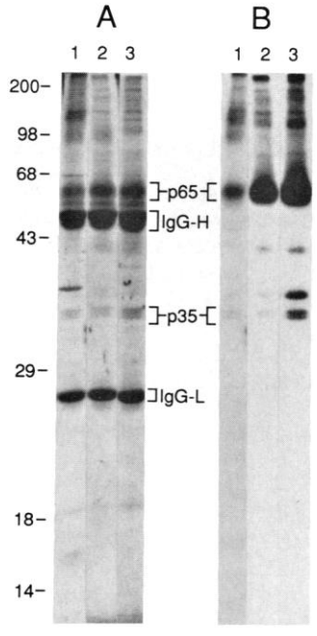

Fig. 1. Coimmunoprecipitation of p65, p35, and a protein kinase. Anti-p65 immunoprecipitations were analyzed for total protein composition (**A**) and in vitro protein phosphorylation activity (**B**). In (A) a synaptic vesicle–enriched fraction [LP2 (*12*), 0.5 mg of protein] was solubilized with detergent [lane 1, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS); lane 2, Triton X-100; lane 3, octylglucoside], and proteins were immunoprecipitated with anti-p65 (*4*) bound to protein A–Sepharose beads as described (*10*). For detection of in vitro phosphorylation (B), the immune complexes were resuspended in reaction buffer (90 μl) [50 mM tris (pH 7.5), 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.4 mM EGTA, CHAPS (1%)] and incubated at 30°C for 1 min. The phosphorylation reaction was initiated by addition of 10 μl of γ-^{32}P–labeled adenosine triphosphate (0.2 mM, 50 μCi/ml). After incubation for 5 min at 30°C, the reaction was terminated with ice-cold HKA buffer (400 μl) [10 mM Hepes-KOH (pH 7.5), 140 mM potassium acetate, 1 mM $MgCl_2$, 0.1 mM EGTA] containing CHAPS (1%), 20 mM EDTA, and 1 mM EGTA. The immune complexes were recovered by brief centrifugation in a microfuge and eluted with SDS-PAGE sample buffer. After SDS-PAGE, we determined the total protein patterns by Coomassie blue staining, and we detected the pattern of protein phosphorylation by autoradiography. The positions of molecular size standards, p65, p35, and the immunoglobulin G heavy (IgG-H) and light (IgG-L) chains are indicated.

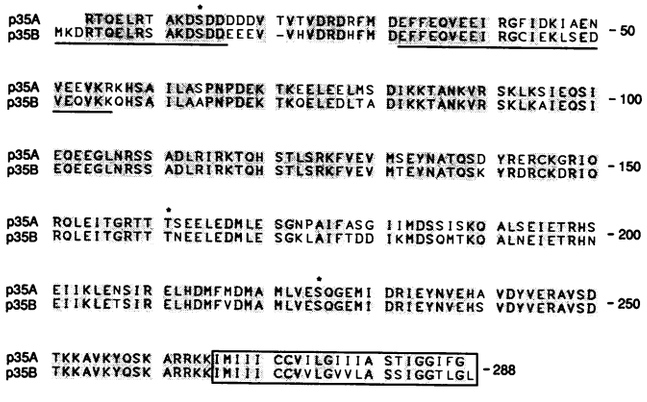

Fig. 2. Comparison of p35A and p35B protein sequences. The protein sequences predicted from the two classes of p35 cDNA clones (*15*) are aligned. Identical amino acids are shaded, the sequences determined by direct microsequencing of the p35 protein (*14*) are underlined, and the hydrophobic COOH-terminal sequences are boxed. The positions of three potential casein kinase II phosphorylation sites (*32*) conserved between the two proteins are indicated (•).

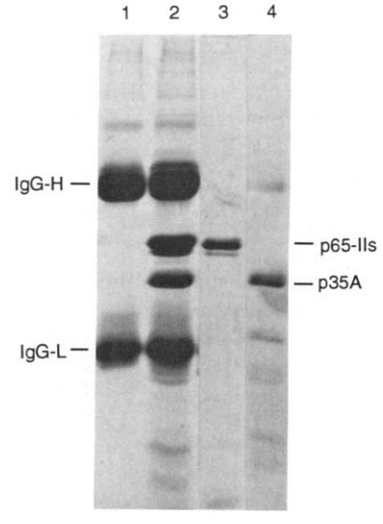

Fig. 3. In vitro interaction of p65 and p35. Purified bacterially expressed p35A (15 μg/ml) was incubated in the presence (lane 2) or absence (lane 1) of purified bacterially expressed p65-IIs (15 μg/ml) in HKA buffer containing CHAPS (1%), bovine serum albumin (0.1%), and gelatin (0.1%). After incubation at 4°C for 12 hours, the samples were subjected to immunoprecipitation with anti-p65 as described (*10*) and analyzed with SDS-PAGE and Coomassie blue staining. The positions of the heavy (IgG-H) and light (IgG-L) chains of the IgG, purified p65-IIs (lane 3), and purified p35A (lane 4) are indicated.

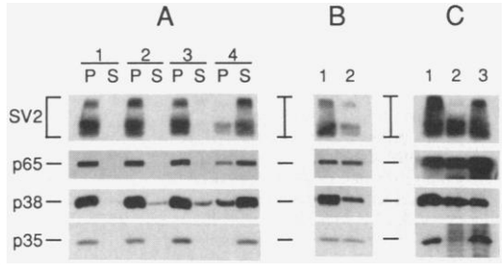

Fig. 4. Biochemical characterization of p35. A synaptic vesicle–enriched fraction (LP2) was subjected to fractionation under different conditions, and the behavior of SV2, p65, synaptophysin (p38), and p35 was monitored with protein immunoblotting. (**A**) We separated LP2 (30 μg) into soluble (S) and particulate (P) fractions by centrifugation at 100,000*g* for 1 hour after extraction under the following conditions: HKA buffer (lane 1); HKA buffer containing 0.5 M NaCl (lane 2); 0.2 M Na_2CO_3 (pH 11.0) (lane 3); and HKA buffer containing Triton X-100 (0.5%) (lane 4). (**B**) LP2 (30 μg) was solubilized in Triton X-114 (2%) and separated into detergent (lane 1) and aqueous (lane 2) phases as described (*20*). (**C**) LP2 (300 μg) was immunoprecipitated with anti-p65 after treatment with 0.5 M NaCl (to reduce background binding of membranes to the protein A–Sepharose beads) (lane 2), or solubilization in CHAPS (2%) (lane 3) as described (*10*). LP2 (30 μg) was loaded in lane 1.

site of contact between varicosities or between a varicosity and a cell body. The labeling of varicosities and putative synaptic sites by anti-p35 was strongly reduced by preincubation of the antibody with bacterially expressed p35A (*13*). The pattern of p35 staining is consistent with localization not only to the plasma membrane of synaptic vesicle–containing varicosities but to a restricted domain of the plasma membrane corresponding to the site of neurotransmitter release.

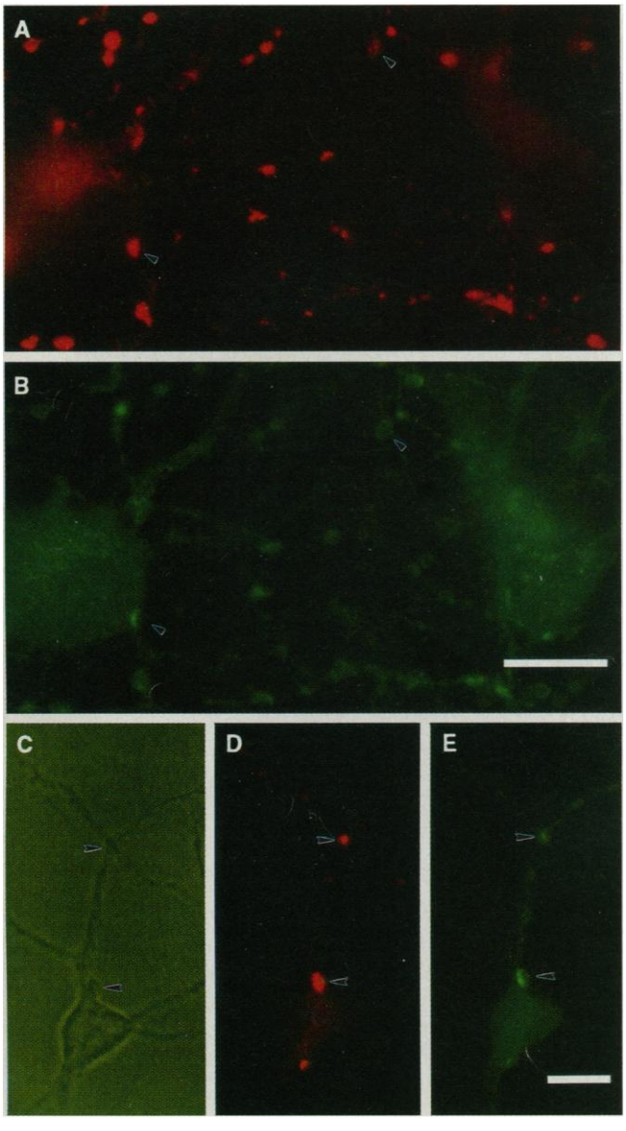

Fig. 5. Localization of p35 by immunofluorescence microscopy. Indirect immunofluorescence microscopy on dissociated hippocampal neurons was performed as described (*21*). Double staining for synaptophysin (**A**) and p35 (**B**). Phase contrast image (**C**) and double staining for synaptophysin (**D**) and p35 (**E**). Arrowheads indicate varicosities and potential synaptic sites where p35 appears localized to the plasma membrane. Scale bars = 14.5 μm.

If p35 functions in the docking of synaptic vesicles with the presynaptic plasma membrane, it might interact with other components of the presynaptic active zone. One such component is the N-type voltage-gated calcium channel. This channel, which is selectively blocked by ω-conotoxin, is responsible for the influx of Ca^{2+} that triggers synaptic vesicle exocytosis in a variety of preparations (*22*). We immunoprecipitated proteins from digitonin-solubilized rat brain synaptosomes with anti-p35 and tested for the coprecipitation of ^{125}I-labeled ω-conotoxin prebound to its receptor (*23*) (Table 1). Anti-p35 immunoprecipitated 22% of the ω-conotoxin receptor. A variety of control antibodies did not immunoprecipitate the ω-conotoxin receptor (<5%). Anti-p35 did not precipitate the plasma membrane-localized Na^+/K^+ adenosine triphosphatase (ATPase) as determined by protein immunoblotting (*24*), further demonstrating the specificity of the anti-p35 immunoprecipitations. The apparent abundance of p35 (Fig. 1A) and the lack of a 35-kD subunit in the isolated N-type calcium channel (*25*) suggest that p35 is not a stoichiometric component of the N-type calcium channel. A 35-kD antigen that interacts with the ω-conotoxin receptor has recently been described (*26*). It will be of interest to determine the relationship between this antigen and p35.

It has recently been reported that two different antibody preparations, one a monoclonal antibody generated against chick synaptic membranes (*27*) and the other derived from the sera of patients with Lambert-Eaton myasthenic syndrome (*28*), recognize a common 58-kD antigen and

Table 1. Immunoprecipitation of ω-conotoxin receptor. The percentage of ^{125}I-labeled ω-contoxin immunoprecipitated by various antibody preparations was determined as described (*23*). Values presented are the mean ± SD from *n* experiments.

Antibody	^{125}I-labeled ω-conotoxin immunoprecipitated (%)
Anti-p35 (15 μl) (*19*)	21.9 ± 3.9 (*n* = 7)
Anti-p35/blocked (15 μl) (*18, 19*)*	1.5 ± 2.1 (*n* = 2)
Preimmune serum (15 μl) (*19*)	4.6 ± 4.7 (*n* = 4)
Anti-mouse IgG (25 μg)	4.5 ± 0.5 (*n* = 3)
Anti-Na^+/K^+ ATPase α-subunit serum (10 μl) (*33*)	0 ± 0 (*n* = 2)

*Preincubated with p35A (5 μg).

immunoprecipitate the ω-conotoxin receptor. Expression cloning of the cDNA encoding this antigen demonstrated that it is p65 (*28*). Precipitation of the ω-conotoxin receptor by antibodies to p65 could result from either a direct or a p35-mediated interaction. The latter possibility is supported by our observation (*24*) that anti-p65 coprecipitates both p35 and ω-conotoxin receptor after Triton X-100 or CHAPS solubilization but does not coprecipitate either p35 or ω-conotoxin receptor after digitonin solubilization.

The molecular properties of p35 that we have described suggest that it may be involved in synaptic vesicle docking or fusion. Because of this, we propose the name syntaxin, from the Greek συνταζισ meaning "putting together in order." Our working model is that syntaxin, by virtue of its interactions with p65 and the N-type calcium channel, brings into close proximity the two membranes involved in the fusion reaction and the source of a factor that regulates membrane fusion. This arrangement would ensure that exocytosis occurs both at restricted sites and with an extremely rapid time course. The interaction between p65 and syntaxin could serve as an intermembrane scaffold on which the molecular machinery that catalyzes the fusion reaction is assembled. This fusion machinery is likely to include additional components of both the synaptic vesicle (*1*) and plasma membranes (*6*, *8*), as well as soluble factors (*29*).

The processes of synaptic vesicle docking and fusion are potential targets for the physiological modulation of synaptic transmission. A common mechanism for the regulation of protein function is through phosphorylation. It is of interest that casein kinase II, an enzyme that is activated during long-term potentiation in hippocampal slices (*30*), coisolates with and phosphorylates both p65 and syntaxin (*13*). Long-term potentiation, a form of synaptic plasticity thought to mimic the modulation of synaptic efficacy involved in the processes of learning and memory, is likely to involve both pre- and postsynaptic mechanisms (*31*). The phosphorylation of p65 and syntaxin by casein kinase II could represent a presynaptic mechanism for modulating synaptic efficacy through the regulation of synaptic vesicle docking or fusion.

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14. Proteins interacting with p65 were isolated, were resolved by SDS-PAGE, were electrophoretically transferred to polyvinylidene fluoride membrane, and were detected with Coomassie blue staining. Amino acid sequences were obtained from intact p35 [MKDRTQELR(T or S)AKDXKK] and from a 14-kD fragment of p35 generated with CNBr digestion (XEFFEQVEEIRGFIDKIAENVEXVKR). The tenth cycle of sequencing of the intact protein yielded both Thr and Ser, suggesting that more than one related sequence was present in the 35-kD region. An oligonucleotide (5′-GCIATCT-TGTCIATGAAICCICGIATCTCCTCIACCACCT-CAAAGAACTC-3′) corresponding to amino acids 2 through 18 of the CNBr fragment was synthesized according to codon usage frequencies with inosine incorporated at degenerate positions. The oligonucleotide was used to screen 500,000 plaques of a rat brain cDNA library constructed in the Lambda Zap II vector (Stratagene, La Jolla, CA). Twelve potential positives were selected, seven of which remained positive on secondary screening. 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; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
15. The four p35A clones totaled 2.1 kb in length and included 856 nucleotides of coding sequence (encoding 285 amino acids beginning at the fourth amino acid from the NH_2-terminus of the protein as determined by microsequencing) and ~1.2 kb of 3′ untranslated sequence. The three p35B clones totaled 3.0 kb in length and included 211 nucleotides of 5′ untranslated sequence, 864 nucleotides of coding sequence (encoding 288 amino acids beginning at the NH_2-terminus of the protein as determined by microsequencing) and ~1.9 kb of 3′ untranslated sequence. An in-frame stop codon was present in the 5′ untranslated region, indicating that p35 is not derived from a larger precursor. The two cDNA clones were 77% identical over the entire coding region and completely divergent in the 3′ untranslated region. The p35A and p35B cDNA sequences have been submitted to the GenBank data bank as accession numbers M95734 and M95735, respectively.
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18. Fusion proteins encoding glutathione-*S*-transferase (GST) in frame with either p35A or the cytoplasmic domain of p65 were prepared by insertion of the appropriate cDNA into the vector pGEX-KG [K. L. Guan and J. E. Dixon, *Anal. Biochem.* **192**, 262 (1991)] and transfection into the AB1899 strain of *Escherichia coli*. After induction of expression with 100 μM isopropyl-β-D-thiogalactopyranoside for 5 hours, the bacteria were suspended in phosphate-buffered saline (PBS) containing Tween 20 (0.05%), 2 mM EDTA, and β-mercaptoethanol (0.1%) and lysed by two passages through a French Press. The fusion protein was recovered by binding of the GST domain to glutathione agarose beads. The recombinant portion of the fusion protein was eluted from the beads with 50 mM tris (pH 8.0), 150 mM NaCl, 2.5 mM $CaCl_2$, and β-mercaptoethanol (0.1%) containing thrombin (3 μg/ml).
19. A rabbit polyclonal antibody to bacterially expressed p35A (prepared by Berkeley Antibody Company, Richmond, CA) was generated by primary injection of GST-p35A fusion protein (200 μg) and boost injections of p35A (200 μg). Both proteins were purified by SDS-PAGE before injection. The gels were stained with 4M sodium acetate [R. C. Higgins and M. E. Dahmus, *Anal. Biochem.* **93**, 257 (1979)], and the appropriate region was excised, equilibrated with PBS, and homogenized with Freund's adjuvant.
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21. Dissociated neuronal cultures from hippocampal regions CA1 and CA3 of 2-day-old rats were grown for 6 days on polyornithine-coated glass cover slips in modified Eagle's medium supplemented with fetal calf serum (10%). The cultures were fixed with paraformaldehyde (4%) in 125 mM sodium phosphate (pH 7.0) for 20 min, quenched with 10 mM NH_4Cl, 10 mM glycine in PBS for 30 min, and permeabilized and blocked with saponin (0.3%) and normal goat serum (3%) in PBS (PBS-SG) for at least 30 min. Samples were incubated with primary antibodies [1:10 dilution of affinity-purified anti-p35A and 1:10 dilution of monoclonal antibody to synaptophysin (SY-38) in PBS-SG] for 4 hours in a humid chamber, were washed three times with PBS containing saponin (0.3%), and were incubated with secondary antibodies (1:50 dilution of fluorescein-conjugated goat antibody to rabbit immunoglobulin G and 1:50 dilution of rhodamine-conjugated goat antibody to mouse IgG in PBS-SG) for 1 hour. The cover slips were washed three times with PBS containing saponin (0.3%), mounted on glass slides, and observed with a Zeiss Axiophot fluorescence microscope (Thornwood, NY).
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TECHNICAL COMMENTS

Carbon and the Antarctic Marine Food Web

M. Huntley *et al.* present (*1*) a model of an Antarctic marine food web and suggest that top predators in the Southern Ocean are responsible for a large efflux of carbon into the atmosphere, up to 20 to 25% of primary production. These values are probably an order of magnitude too large because Huntley *et al.* have used an oversimplified food web and invalid assumptions and parameter values.

In the model (*1*), phytoplankton respiration is not accounted for, and all calculations refer to net rather than total primary production, which elevates the estimated contribution of all heterotrophs to the carbon flux. Net primary production, or photosynthetically fixed carbon (*P*), is apportioned between macrozooplankton (P_Z) and the microbial loop (P_L). Mean values of 0.875 (sensitivity range 0.08 to 0.95) and 0.125 (0.2 to 0.05) were assigned to P_Z and P_L, respectively. That is, in all situations, it is assumed that the majority (at least 80%) of net primary production is directly channeled to macrozooplankton, with at most 20% entering the microbial loop. It is further assumed (*1*) that all fecal material produced by the microbial loop is ingested by macrozooplankton, bringing the total proportion of net primary production reaching macrozooplankton to 95%. In general, however, much of the carbon fixed during primary production in marine pelagic systems is believed to enter the microbial food web (*2*), and studies in productive coastal regions indicate that macrozooplankton probably consume no more than 20% of primary production (*3*). The relative abundances of protozooplankton and the structure of Antarctic marine food webs are sufficiently similar to those of lower latitude regions (*4*) that the possibility of a grazing pathway dominated by microbes should be incorporated in the model.

The value of P_L in the model apparently was chosen because bacterial production has been measured as 7 to 14% of phytoplankton production near the ice edge in the austral spring (*5*) and because bacteria do not comprise a large fraction of total microbial biomass (*6*). However, this assumes that the only production that enters the microbial loop is that of bacterioplankton. Heterotrophic flagellates, other protozooplankton, and small microzooplankton feed directly on phytoplankton cells of the appropriate size (*4*). Because most chlorophyll in the Antarctic is contained in cells smaller than 20 μm and because krill generally eat particles greater than 30 μm (*7*), a considerable factor may be heterotrophic organisms within the microbial loop grazing on phytoplankton (*4*, *7*, *8*). Carbon flux *through* the microbial food web may be low, but it does not follow that carbon *entering* the microbial food web is also reduced, as assumed in the model.

Values of growth efficiencies and carbon assimilation efficiencies in the model appear to be inappropriately large. This is a result of treating aggregations of organisms as trophic levels, when it would be more appropriate to consider them as food webs. For example, the microbial loop is given a mean growth efficiency (K_L) of 0.35 and comprises bacterioplankton, heterotrophic flagellates, ciliates, and many other protozoa and small zooplankton. Carbon entering this compartment may undergo four or five trophic transfers before being packaged into a form that can be eaten by macrozooplankton. In such a scenario, growth efficiency of the compartment would be expected to be approximately 0.5 to 1.5% (*9*), rather than the 35% assumed by Huntley *et al.*

In all instances, the model overestimates the amount of carbon being transferred to macrozooplankton, which leads to invalid estimates of carbon fluxes in the food web. If revised estimates of parameters are used in the model ($P_Z = 0.20$, $K_L = 0.01$), a different result is obtained, with birds respiring approximately 5% of *net* carbon fixed and the microbial component approximately 60%. Thus, marine birds and mammals probably play a minor role as producers of atmospheric CO_2, especially when compared with microbial plankton.

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9. If it is assumed that growth efficiencies (K) for each trophic transfer within the microbial compartment are 35%, after n trophic transfers, the resulting growth efficiency of the microbial loop will be 0.35^n. Substituting $n = 4$ and 5 gives estimates of K_L of 0.015 and 0.005, respectively.

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Response: C. L. Moloney suggests that we overestimated the potential of Southern Ocean birds and mammals to respire CO_2 to the atmosphere (*1*). On the basis of estimates for the Benguela Current, she has found (*2*) that zooplankton probably use only 20% of the primary production. This would require that 80% of primary production enter the microbial loop. We have no disagreement with these results, which are characteristic of a subtropical environment. However, studies cited in our *Science* report (*3*, *4*) and results from our ongoing Research on Coastal Antarctic Ecosystem Rates (RACER) program (*5*) indicate that less than 10% of primary production enters the microbial loop of South-