Published in final edited form as:

Neuron. 1991 June ; 6(6): 993–1007.

Differential Expression of the p65 Gene Family

Beverly Wendland*, Kenneth C. Miller*, James Schilling[†], and Richard H. Scheller*

*Howard Hughes Medical Institute and Department of Molecular and Cellular Physiology, Beckman Center

[†]Department of Biological Sciences, Stanford University, Stanford, California 94305

Summary

The genome of the marine ray *Discopyge ommata* contains at least three p65-related genes. o-p65-A is 84% identical, o-p65-B is 78% identical, and o-p65-C is only 41% identical to a previously characterized rat p65. The cytoplasmic domain, particularly the two regions that are similar to the regulatory domain of protein kinase C, are most highly conserved. The three genes are expressed in different but overlapping patterns in the central nervous system. o-p65-A immunoreactivity is found predominantly in forebrain, cerebellum, and neuroendocrine cells, while o-p65-B immunoreactivity is predominantly localized to the spinal cord, brainstem, and midbrain. Many synaptic vesicle proteins are members of small gene families that are differentially expressed, resulting in several unique combinations of these molecules in specific brain regions.

Introduction

Intercellular communication in the nervous system is accomplished by the regulated secretion of neurochemical messengers from the presynaptic nerve terminal. Fast- and slow-acting messengers are stored in synaptic vesicles, and upon calcium influx, the lumen of the vesicle becomes continuous with the extracellular space to release transmitter. The vesicle membrane then recycles, regenerating the initial state. This secretory process is modulated by neuronal activity and provides many potential molecular targets for regulation during learning and memory. Crucial to understanding the events that underlie neuronal communication is a characterization of the cell biology of membrane flow mediated by the synaptic vesicle (Kelly, 1988). The proteins associated with synaptic vesicles in the nerve terminal are certain to play a critical role in this process. Several of these peripheral and integral membrane synaptic vesicle proteins have been characterized, and their functions are now being elucidated (reviewed in Trimble et al., 1991; Südhof, 1989; Reichardt and Kelly, 1983; Almers, 1990).

The first group of synaptic vesicle proteins to be studied was the synapsins, a family of peripheral phosphoproteins that may regulate the availability of small clear vesicles for docking at the active zone (Südhof et al., 1989b; Linas et al., 1985). Integral membrane synaptic vesicle proteins include synaptophysin and the related synaptoporin, which have been proposed to be hexameric channels in the vesicle membrane (Knaus et al., 1990;

Navone et al., 1986; Thomas et al., 1988; Buckley et al., 1987; Cowan et al., 1989; Leube et al., 1987; Südhof et al., 1987). Other proteins include SV2 (Buckley and Kelly, 1985; Floor and Feist, 1989), VAT (Linial et al., 1989), and the VAMPs (Trimble et al., 1988), also called synaptobrevin (Südhof et al., 1989a), which are small molecules with a carboxy-terminal membrane anchor and a cytoplasmic domain. Importantly, one or more low molecular weight GTP-binding proteins have been found to be associated with synaptic vesicles (Ngsee et al., 1990; Fischer von Mollard et al., 1990, 1991), probably through covalent attachment to a lipid. The GTP-binding proteins are similar to *ras* and the yeast proteins SEC4 and YPT1 and may be molecular switches involved in regulating vectorial membrane flow in the secretory pathway (Bourne, 1988).

Recently the primary sequence of an additional synaptic vesicle protein, p65 (Matthew et al., 1981), has been determined (Perin et al., 1990). This molecule has a 52 amino acid amino-terminal luminal domain, a hydrophobic membrane anchor, and a carboxy-terminal cytoplasmic domain of 342 amino acids. The cytoplasmic portion of p65 has two 115–116 amino acid stretches of homology to the regulatory region of the calcium-dependent protein kinase C (PKC) (Nishizuka, 1988). Experiments with recombinant p65 protein suggest that this region binds acidic phospholipids (Perin et al., 1990). The lipid-binding and red blood cell agglutination properties of p65 have led to the proposal that the molecule is involved in membrane fusion events associated with exocytosis.

Several of the synaptic vesicle proteins that have been characterized, including synapsin (Südhof et al., 1989b), VAMP (Elferink et al., 1989), synaptophysin (Knaus et al., 1990), and the GTP-binding proteins (Ngsee et al., 1991), are members of gene families. The individual members of these gene families are differentially expressed within the nervous and endocrine systems and, in some cases, other tissues. For example, VAMP-1 is expressed in a limited number of nuclei, particularly those that modulate somatomotor functions, while VAMP-2 is expressed more widely in autonomic, sensory, and integrative nuclei (Trimble et al., 1990). Differential expression of the genes encoding these molecules results in unique sets of protein forms on various vesicle classes. While the functional significance of this differential expression is not yet known, particular combinations of these molecules are likely to suit the specific requirements for membrane flow, metabolism, and synaptic modulation in different neurons.

In this report, we demonstrate that the marine ray *Discopyge ommata* differentially expresses at least three p65-related genes. The three genes have the same overall structure and are most similar in the PKC-homologous domain. Antibody studies reveal differential localization patterns for the o-p65-A and o-p65-B gene products.

Results

Three p65 Genes

Synaptic vesicles were purified from the electric organ of the marine ray *D. ommata* using differential centrifugation, sucrose gradient floatation, and column chromatography (Carlson et al., 1978). The vesicles were TCA-precipitated and the proteins fractionated by polyacrylamide gel electrophoresis (Figure 1). The proteins were then electroblotted to

polyvinylidene difluoride (PVDF) paper, and several synaptic vesicle-specific bands were sequenced. Amino-terminal sequencing of 62 kd and 74 kd bands revealed the identical sequence MLVPQAFVAP, with the additional carboxy-terminal residues MA included in the longer sequencing run with the 62 kd band. In addition to having identical amino termini, these two proteins are also antigenically related and have identical CNBr cleavage patterns. To obtain internal amino acid sequences, the two proteins were cleaved with CNBr. The cleavage fragments were refractionated on SDS-polyacrylamide gels and transferred to PVDF paper, and the amino acid sequences of two fragments were determined. The resulting amino acid sequences were XXTSDPYVXVFLLPD and XVGGLSDPYVKIDLLQN.

The second CNBr amino acid sequence was used in conjunction with the published nucleotide sequence of rat p65 to design a synthetic oligonucleotide for screening (see Experimental Procedures). The synthetic 54 nucleotide DNA fragment was used to screen a cDNA library constructed with poly(A)⁺ RNA isolated from the electromotor neurons of the marine ray *D. ommata*. The libraries were then rescreened with various DNA fragments generated by restriction enzymes and the polymerase chain reaction (PCR). During the course of screening, three classes of p65-homologous cDNAs were identified on the basis of restriction enzyme mapping and nucleotide sequencing. The classes are designated o-p65-A, o-p65-B, and o-p65-C. Overlapping sets of near full-length cDNAs from each class were isolated and the predicted proteins were defined by analysis of the DNA sequence.

The o-p65-A sequence is derived from eight cDNA clones (Figure 2A). Two smaller clones end with a poly(A) tract, whereas six of the clones with longer 3' untranslated sequence end with a natural EcoRI site (see Experimental Procedures). The 3' untranslated regions are identical up to the site of the poly(A) tract. This suggests that the two 3' ends arise from alternate polyadenylation sites. The predicted 5' untranslated region exists in two configurations: one form contains an 85 nucleotide insert (nucleotides 98–182), while the other does not. Alternative splicing of the 5' untranslated region is likely to be the source of the insert. The first in-frame methionine is predicted to define the initiation of the open reading frame, which extends for 427 amino acids. The open reading frame is flanked by in-frame stop codons in the form with the insert; however, the form lacking the insert has an open reading frame extending to the end of the defined sequence. In both configurations the same methionine is predicted to initiate translation. Some clones also lack residues 48 and 49, 122–124, and Glu-276 (Figure 2A). These minor differences may be due to polymorphisms, cloning deletions, or small, alternatively spliced sequences.

The o-p65-B clone is 3.5 kb and predicts an open reading frame of 439 amino acids flanked by in-frame stop codons (Figure 2B). The amino acid sequences from the 62 kd and 74 kd vesicle proteins are underlined in Figure 2B. The first methionine residue is shown to be the start of translation; however, this amino terminus does not correspond exactly to the amino-terminal sequence obtained from the 62 kd and 74 kd vesicle proteins. The amino-terminal sequence of the two vesicle proteins differs in the first 3 residues. This is not likely to be a cloning artifact, since analysis of multiple independent clones resulted in the same nucleotide sequence. We do not understand the origin of these differences. Several possibilities arise; for example, a common contaminant may be present in both 62 kd and 74

kd protein samples, or an alternative mRNA form of this message may give rise to the form of the protein that was directly sequenced. Another explanation for the amino-terminal sequence might be proteolytic processing of o-p65-B at Arg-22 by a trypsin-like activity.

o-p65-C is defined by a 2632 nucleotide sequence and predicts a 537 amino acid open reading frame flanked by in-frame stop codons (Figure 2C). The 3' untranslated region is 773 nucleotides and ends with a natural EcoRI site. Some clones contain a second fragment of 1 kb terminating with a poly(A) tract to complete the 3' untranslated region of this mRNA.

The predicted ray p65 proteins are aligned with rat p65 in Figure 3, and Table 1 summarizes the percent identity and similarity among the molecules. o-p65-A, o-p65-B, and rat p65 are closely related, with overall identities of 84% and 78%, respectively. This suggests that o-p65-A is the ray homolog of the rat protein. Table 1 further demonstrates that o-p65-B is equally divergent from o-p65-A and rat p65, suggesting that a duplication event leading to o-p65-A/B occurred prior to divergence of mammalian and ray species. A somewhat less likely explanation of the data is that the expression in different brain regions (see below) resulted in unique patterns of selection, resulting in the amino acid sequence differences observed. In contrast, o-p65-C is only 39%–41% identical to rat p65, o-p65-A, and o-p65-B (Figure 3 and Table 1).

All of the p65-homologous proteins have a 26–27 residue hydrophobic domain that is predicted to span the synaptic vesicle membrane. This transmembrane region is highly conserved between rat p65, o-p65-A, and o-p65-B, with 3 proline residues in the amino terminus and 4 cystine residues in the carboxyl terminus of the domain. While o-p65-C has a hydrophobic domain at a similar distance from the predicted initiator methionine, the precise sequence is not highly conserved (Figure 4). The major differences in the membrane-spanning region of o-p65-C are the lack of the proline and cystine residues and the presence of 5 serine residues and a single threonine residue.

The amino-terminal domains are between 52 and 74 amino acids in length and display a relatively low level of conservation between the sequences (Figure 4). Rat p65, o-p65-A, and o-p65-B all contain potential N-linked glycosylation sites, while o-p65-C does not. Perin et al. (1991a) have demonstrated that this glycosylation site is used in rat, but whether the sites are used in *D. ommata* is unknown. Immediately carboxy-terminal to the membrane-spanning domain, single lysine and cystine residues are conserved among all four of the sequences. Of the next 13 residues, 8 are lysine in rat p65, o-p65-A, and o-p65-B, while only 5 are positively charged in o-p65-C. In o-p65-C, this domain is immediately followed by a sequence of 13 amino acids, 9 of which are histidine residues, perhaps compensating for the lower number of lysine residues in this region (Figure 3; Figure 4). Moving carboxy-terminal along the sequence, o-p65-C has an insert of 93–98 amino acids relative to the other p65 genes. The insert appears between the putative transmembrane domain and the first PKC-homologous domain and has no known homology to proteins in the data bank.

The region of the p65 proteins from the PKC-A domain to the carboxyl terminus is the most highly conserved (Figure 3; Figure 4). This part of the protein includes the two domains homologous to the regulatory domain of PKC, which display a conserved core composed of the sequence SDPYVK (residues 300–305 and 435–440; Figure 3), followed by a highly basic stretch of amino acids (residues 312–315 and 449–454; Figure 3). This region of the protein has been proposed to be an acidic lipid-binding domain, and it is therefore likely that much of the sequence conservation preserves this function. The 23–25 amino acid stretch between the two PKC-homologous domains is somewhat less well conserved between the four sequences. The identity in this region between rat p65 and o-p65-A is 94%, while the identity to o-p65-B is 65%. In o-p65-C, conservation of the domain between the PKC repeats is not markedly lower than the conservation of the repeats, unlike o-p65-A and o-p65-B (Figure 4). This may suggest that the entire carboxy-terminal domain of o-p65-C has diverged as a single unit rather than maintaining the PKC repeats as discrete functional units. While the carboxy-terminal 39 amino acids have no homology to other proteins in the data bank, this region is highly conserved among the four proteins. Again, o-p65-C differs from the other sequences in that the stop codon is 21 amino acids beyond the stop of the other p65 proteins.

Differential Expression of the p65 Genes

The three *D. ommata* cDNAs were used in RNA blotting experiments to determine the pattern of expression in nervous system tissues. Because of the seasonal variability in obtaining animals, we have not been able to conduct all of the blotting experiments on all of the same tissues. Regardless of this problem, the data clearly demonstrate that the three genes are differentially expressed (Figure 5). o-p65-A is expressed relatively abundantly in the forebrain, where two mRNA species of 2.4 kb and 3.9 kb are observed. A very weak hybridization signal to mRNAs of the same size is observed in electric lobe and spinal cord; however, the level of the signal is at least an order of magnitude lower. o-p65-B shows the opposite pattern of expression. Two mRNAs of 3.7 kb and 8.3 kb are observed in electric lobe and spinal cord, with a lower signal in total brain and little or no signal in forebrain. No mRNAs are detected in non-nervous system tissues including liver, heart, muscle, and electric organ. This pattern of expression for o-p65-A and o-p65-B is consistent with the immunocytochemical data presented below. While we do not have immunological reagents that recognize the protein encoded by o-p65-C, the RNA blotting again shows that this gene is expressed in specific brain regions. A large transcript of 9.5 kb is observed in electric lobe but not in forebrain or liver.

The p65 Homologs on Electric Organ Synaptic Vesicles

To define further the differences in expression patterns of the p65 gene family, antibodies that specifically recognize o-p65-A or o-p65-B were used in a series of histochemical studies. None of the proteins on electric organ synaptic vesicles is immunolabeled with monoclonal antibody (MAb) 48, which recognizes o-p65-A and rat p65. However, these vesicles do have a prominent protein of 62 kd that is a p65 homolog by virtue of its vesicle specificity, molecular weight, and protein sequence (see above). We produced an antiserum in rat to the 62 kd protein and affinity purified it using the 62 kd band immobilized on a PVDF membrane. Western blot experiments demonstrate that monoclonal 48 reacts with o-

p65-A fusion protein and not with o-p65-B or o-p65-C. Furthermore, the affinity-purified 62 kd antiserum specifically reacts with an o-p65-B fusion protein and not with o-p65-A or o-p65-C.

To determine whether the 62 kd protein copurifies with synaptic vesicles, the antibodies were used to probe fractions from the final stage of the synaptic vesicle purification. Figure 6A shows a graph illustrating this step, in which larger membrane contaminants from the previous sucrose gradient are separated from the smaller synaptic vesicles by controlled pore glass (CPG) chromatography. The ATP and absorbance profiles shown here are typical for electric organ synaptic vesicles, which contain a high concentration of ATP along with acetylcholine (Carlson et al., 1978). The CPG fractionation of the 62 kd protein and VAMP-1, another synaptic vesicle protein (Trimble et al., 1988), is shown in the immunoblot below (Figure 6B). Surprisingly, the antibodies that had been affinity purified against the 62 kd protein also reacted strongly with a band at 74 kd and two other bands at 185 kd and 215 kd, all of which copurify with the vesicles.

One possible explanation for the 185 kd and 215 kd bands that were immunolabeled by the anti-62 kd antibodies is that they represent undissociated complexes. When we solubilized the vesicles in SDS buffer prepared with fresh dithiothreitol, the 185 kd and 215 kd bands were no longer detectable, while the mobilities of the 62 kd and 74 kd bands remained unchanged. An example of this is shown in Figure 7B, lane 1. In separate experiments, using both total midbrain protein and purified synaptic vesicles, we have confirmed that the high molecular weight complex is present in nonreducing gel conditions and not detectable under reducing conditions (data not shown). This suggests that the 185 kd and 215 kd bands do not represent contiguous polypeptide chains, but rather complexes that are covalently linked by one or more disulfide bonds.

The o-p65-A and o-65-B Proteins Are Differentially Localized in the Ray CNS

The relative distributions of o-p65-A and o-p65-B were examined by immunoblotting various regions of the ray CNS (Figures 7A and 7B). o-p65-A immunoreactivity was detected with MAb 48 (Matthew et al., 1981), while o-p65-B immunoreactivity was detected with antibodies affinity purified against the 62 kd protein of electric organ vesicles.

In general, we found a marked segregation of the two p65s in the nervous system. The o-p65-A immunolabeled band was found to be expressed mainly in cerebellum and forebrain and at a much lower level in midbrain (Figure 7A). No o-p65-A immunolabeling was detected in electric organ synaptic vesicles, spinal cord, or brainstem by this analysis. The o-p65-B immunolabeled band was most highly expressed in electric organ synaptic vesicles, spinal cord, brainstem, midbrain, and to a much lesser extent in the cerebellum (Figure 7B). No o-p65-B immunolabeling was detected in forebrain, which also shows that the anti-62 kd antibodies do not cross-react with the o-p65-A form. o-p65-B was also not detected in muscle or liver, confirming that this form of p65 is also neural specific. The 74 kd form of o-p65-B was also detected in all regions of the CNS where the 62 kd form was observed, although at a much lower level. o-p65-A was detected as a single band of 62 kd.

o-p65-A and o-65-B Are localized to Different Nerve Terminal Populations in the CNS

The fact that o-p65-B copurifies with synaptic vesicles predicts that its primary site of localization in neurons will be nerve terminals. Because the neuromuscular junction is a large and easily identifiable set of nerve terminals, we first looked here for o-p65-B by immunofluorescence staining of electric ray muscle fibers. Figure 8A shows that o-p65-B immunoreactivity is indeed found at these nerve terminals, which were identified by labeling with α -bungarotoxin (Figure 8B). Nerve terminals in the CNS are much smaller and harder to identify, especially in the frozen sections required for most antibody staining. In thin sections, however, nerve terminals in the CNS can be resolved as punctate spots, sometimes outlining the somata of larger neurons (De Camilli et al., 1983). Figure 8C shows an example of o-p65-B immunoreactivity in the brainstem, in which terminals can be seen outlining the soma of a large neuron. This staining pattern could be entirely blocked by preincubation of the antibody with the 62 kd protein of electric organ synaptic vesicles (data not shown). The pattern seen in Figure 8C is similar to the light-level staining seen with other synaptic vesicle proteins (Buckley and Kelly, 1985; Navone et al., 1986; Baumert et al., 1989, 1990).

To examine the localization of o-p65-A and o-65-B in the nervous system at a higher resolution, frozen sections from different regions were probed for either o-p65-A or o-p65-B immunoreactivity using indirect immunofluorescence staining. A monoclonal antibody recognizing SV2, a widely distributed synaptic vesicle protein (Buckley and Kelly, 1985), was used for comparison.

In the spinal cord (Figures 9A–9C), o-p65-A immunoreactivity, which was not detected in the immunoblot, was seen in sparsely distributed nerve terminals. In contrast, o-p65-B immunoreactive nerve terminals had a distribution and density very similar to SV2 immunoreactive terminals. In the cerebellum, on the other hand (Figures 9D–9F), o-p65-A immunoreactivity was indistinguishable from that of SV2. Here, o-p65-A and SV2 immunoreactivities occur in the form of small spots in the molecular layer and large, irregular clumps in the granule cell layer, which is the expected distribution for nerve terminals in this region. o-p65-B was not detected at all in the molecular layer, but there was some faint immunoreactivity in the glomeruli of the granule cell layer. In the midbrain (Figures 9G–9I), o-p65-A immunoreactive nerve terminals were again sparse, although they were present at a higher density than in the spinal cord. The distribution of o-p65-B immunoreactive terminals was similar to that of SV2, but a slightly lower density of terminals seemed to be stained. In the forebrain (Figures 9J–9L), as in the cerebellum, o-p65-A immunoreactive terminals were indistinguishable in density and distribution from SV2 immunoreactive terminals. o-p65-B immunoreactive terminals, in contrast, were very sparsely distributed, with some fields only containing a few detectable spots of immunoreactivity.

Localization of o-p65-A and o-65-B in Neuroendocrine Cells

Most of the widely distributed synaptic vesicle proteins have been shown to be expressed in neuroendocrine cells (reviewed in Matteoli et al., 1989). We compared o-p65-A and o-65-B

immunoreactivity in frozen sections of anterior pituitary and in pancreatic islet cells, again using SV2 for comparison.

In the anterior pituitary (Figures 10A–10C), strong o-p65-A immunoreactivity was seen, especially in the neuroendocrine cells lining the capillary passages of the pituitary. This distribution was identical to that of SV2. o-p65-B immunoreactivity, on the other hand, was not found inside any cells in the pituitary, although some weak reactivity was observed outlining many of the cells. In the pancreas (Figures 10D–10F), all three antibodies stained similar structures, presumably the insulin-secreting islet cells. The o-p65-B staining did seem less intense, however. Weak, irregular o-p65-B immunoreactivity outlining non-islet pancreatic cells was also observed, similar to what was seen in the pituitary. The cause of this apparent cross-reactivity is not known.

Localization of p65-A to a Subset of Nerve Terminals in Rat

Although the p65 recognized by MAb 48 (p65-A) was previously shown to be widely distributed in the rat nervous system (Matthew et al., 1981), other synaptic vesicle protein probes were not available for comparison at that time. We have reexamined the rat spinal cord for p65-A immunoreactivity using MAb 48 and compared this pattern with SV2 immunoreactivity. Immunofluorescence staining of frozen sections was again used for the comparison (Figures 11A and 11B). As in the ray spinal cord, p65-A immunoreactive nerve terminals were sparsely distributed in the spinal cord compared with the abundant SV2 immunoreactive terminals, which could be seen tightly packed around large neurons. Anti-62 kd antibodies did not cross-react with rat tissues and thus could not be used in this comparison.

Discussion

The p65 Gene Family

There are at least three p65-related genes in the genome of the marine ray *D. ommata*. In other systems, two general functions for multiple genes have been noted. The first is as a means of regulating gene expression in a complex set of cells or tissues. Alternatively, amino acid differences between members of the p65 family may have been selected to fit the functional requirements of specific neurons and secretory cells in which they are expressed. It is possible that the p65 protein(s) is involved in targeting recycled membrane to a synaptic endosome-like structure or may be involved in other aspects of membrane flow at the nerve terminal (Kelly, 1988). Because of the homology to the PKC regulatory domain and in vitro lipid-binding activity, it has been proposed that p65 mediates membrane fusion or that the molecule may be involved in docking synaptic vesicles at the active zone (Perin et al., 1990). This docking could occur by the p65 protein binding plasma membrane lipids via the PKC-homologous domains. However, more specificity is required to direct vesicles to the region of the active zone membrane where fusion occurs. Perhaps specific nerve terminals require particular p65 proteins to interact with different molecular machinery present at the active zone.

The high degree of homology to rat p65, in conjunction with the immunological studies presented here, makes it quite certain that o-p65-A and o-p65-B are integral membrane components of the synaptic vesicle. The strong conservation in the lipid-binding domain makes it likely that they bind phosphoserine lipids. Perin et al. (1991a) have proposed that the region between the membrane span and the first PKC repeat serves as a dimerization domain via interactions of an amphipathic helix. While all but one of the proposed hydrophobic and charged regions are conserved in o-p65-A, many substitutions in the homologous domain make it unlikely that this region can cause dimerization in o-p65-B or o-p65-C.

The overall domain structure and sequence identity in the PKC-homologous region clearly put the predicted o-p65-C protein in the p65 family. Since the PKC region is the most highly conserved part of the protein, and since an mRNA is expressed, it is not likely that o-p65-C is a pseudogene. The o-p65-C sequence is more distantly related to rat p65, o-p65-A, and o-p65-B than a recently reported *Drosophila* p65 homolog (Perin et al., 1991b). This suggests that there were at least two p65 genes in the common ancestor of *Drosophila* and vertebrates and that a duplication event occurred over 100 million years ago. Neither the fly homolog of p65 nor o-p65-C has predicted N-linked glycosylation sites amino-terminal to the predicted transmembrane domain.

The o-p65-B Protein in Electric Organ Synaptic Vesicles

Cholinergic synaptic vesicles purified from electric organ contain two p65 proteins that are closely related. Although the 62 kd form is more prevalent in other parts of the CNS, the 74 kd form can still be detected in our immunoblots, showing that it is not confined to electric organ vesicles. We do not know, however, if these two forms are actually found together on the same vesicles or whether they exist on separate subpopulations that are not resolved during our isolation procedure. Electric organ vesicles have been shown to exist in two populations, an active pool and a reserve pool, which can be partially resolved by their different densities (Zimmerman and Denston, 1977).

Although we have not determined what the exact difference is between the 62 kd and 74 kd polypeptides, the fact that they are closely related structurally and antigenically suggests either that they may be the products of differentially spliced transcripts or that one form is a posttranslational modification of the other. It is interesting to note that previous studies have suggested that more than one form of synapsin may be expressed in a particular nerve terminal population (Südhof et al., 1989b).

Quaternary Structure of p65

The results suggest that the electric organ p65 proteins are tightly associated either with other p65 polypeptides or with another synaptic vesicle protein(s). The molecular weight of the observed complex is consistent with the formation of a trimer assemblage of the 62 kd protein. The possibility that p65 associates into trimers is intriguing, because this is a property that it would share with the influenza virus fusion protein, hemagglutinin (reviewed in Wiley and Skehel, 1987), although the two proteins share no significant homology at the amino acid level (unpublished data). p65-A has also recently been observed to migrate as a

high molecular weight band on an SDS-polyacrylamide gel, although in this case it was proposed to be migrating as a homodimer (Perin et al., 1991a).

We cannot determine at this stage whether the 185 kd and 215 kd complexes are stabilized by disulfide linkages or noncovalent forces in native p65 on vesicles. Previous studies with synaptophysin, which forms dimers, trimers, and tetramers under nonreducing conditions, suggested that synaptophysin monomers are noncovalently associated in the native state, but that rearrangement of intramolecular disulfide bonds under certain storage conditions can lead to the covalent linkage of adjacent monomers (Johnston and Südhof, 1990).

Differential Expression and Localization of o-p65-A and o-65-B

The Northern blot, immunoblot, and immunofluorescence results are summarized in Table 2. To some extent, one can correlate the distribution of o-p65-A and o-65-B immunoreactivities with broad functional divisions in the CNS. o-p65-A immunoreactivity is primarily found in regions involved in higher level integrative behavior (cerebellum and forebrain), whereas o-p65-B immunoreactivity is primarily found in areas involved in reflexes and low level integration of primary sensory information (spinal cord, brainstem, and midbrain). There was no obvious correlation between the form expressed in anyone region and neurotransmitter type or sensory modality. We have not determined what properties of o-p65-A and o-65-B make one form more suited than another to a particular region of the brain.

The only sites where the two forms are apparently expressed together are in the cerebellar granule layer and the islet cells of the pancreas. However, in both cases, the o-p65-B reactivity was weak compared with that of o-p65-A. This could either mean that the two forms are coexpressed at these locations, with o-p65-B being expressed at a lower level, or that there is yet another different p65 that is recognized by MAb 48 and yet is only weakly cross-reactive to the anti-62 kd antibodies.

By Northern blot analysis, the mRNA encoding o-p65-C has a pattern of expression similar to the o-p65-B protein in the CNS; however, two observations demonstrate that o-p65-C is not the form we are detecting in purified electric organ synaptic vesicles and other brain regions. The protein sequence obtained for the 62 kd protein does not match the predicted molecular weight or sequence of o-p65-C, and a recombinant trp E-o-p65-C fusion protein does not cross-react with either the anti-62 kd antibodies or MAb 48. The localization of the o-p65-C protein, therefore, awaits the production of specific antibody probes.

Comparison with Other Multifunctional Synaptic Vesicle Proteins

It is important to determine whether the expression pattern described here for the p65 gene family bears any resemblance to the expression patterns of other synaptic vesicle protein gene families. Of the three other synaptic vesicle proteins known to be encoded by a gene family, only VAMPs 1 and 2 have been systematically compared in a rostral/caudal analysis in the CNS (Trimble et al., 1990). Although this study compared the distribution of VAMP-1 and VAMP-2 mRNAs in rat, there is some correlation with the immunolocalization of the different forms of p65 in ray. VAMP-2, like o-p65-A, is the

dominant form expressed in forebrain areas and pituitary, while VAMP-1, like o-p65-B, is the dominant form in brainstem and spinal cord.

Our results lead us to expect that this general pattern of expression will be similar in other vertebrates, including mammals. Although another form of p65 has not yet been demonstrated in mammals, our immunofluorescence study in rat spinal cord shows that p65-A immunoreactivity is only found in a subset of nerve terminals in this region, thus suggesting that a p65-B-like protein may be expressed in the remaining terminals.

The recent findings that synaptic vesicle proteins exist in multiple forms that are differentially expressed suggests that the precise properties of synaptic transmission may vary in different regions of the brain. It now becomes important to determine the extent of this variation and to understand the roles of synaptic vesicle proteins, including the p65 proteins characterized here, in governing synaptic transmission.

Experimental Procedures

Synaptic Vesicle Purification

Cholinergic synaptic vesicles from the electric organ of *D. ommata* were isolated as previously described (Linial et al., 1989; Carlson et al., 1978).

Protein Isolation

Purified vesicles were precipitated in 10% TCA and washed with acetone. Laemmli gels (8%–13% gradient) (Laemmli, 1970) were run and blotted to Millipore Immobilon PVDF paper. Proteins were visualized with Coomassie blue. A small region of the blot was excised and immunoblotted with the antiserum directed against the 62 kd protein. Iodinated secondary antibody, followed by autoradiography, indicated the location of immunoreactive protein. Bands of interest were excised from the remainder of the blot. Amino-terminal protein sequence and internal sequence derived from CNBr fragments were obtained. The predicted amino acid sequences in o-p65-B that match the vesicle protein sequence are indicated in Figure 2B. The histidine residue at position 333 was incorrectly assigned; due to technical difficulty, we were unable to resolve histidine residues at the time.

CNBr Cleavage and Protein Sequencing

Protein bands from PVDF paper were cut out, transferred to a microfuge tube, and covered with 70% formic acid. CNBr, which cleaves at methionine residues (Gross, 1967), was added, and the reaction was allowed to proceed for 16–24 hr. The CNBr solution was then removed from the slice to a separate tube. To elute fragments still bound to the paper, a solution of 70% n-propanol, 1% trifluoroacetic acid (TFA) in water was then added to the slice and incubated for 30 min at room temperature. This solution was then removed and added to the CNBr solution, and the entire contents were dried in a SpeedVac. The dried fragments were redissolved in SDS-PAGE sample buffer and fractionated on an 18% SDS-polyacrylamide gel. The resolved fragments were then electroblotted onto Immobilon-P and stained with Coomassie blue. Bands were excised and loaded directly onto the protein sequencer (Applied Biosystems, Foster City, CA).

Library Screening and cDNA Clones

The 54 nucleotide fragment

GTTCTGCAGCAGGTCAATCTTCACGTAGGGATCAGATAAGCCACCCACATCCAT was synthesized according to the minus strand sequence of the rat p65 cDNA clone from nucleotides 1429–1482. Two base changes were made to accommodate amino acid differences in the *D. ommata* vesicle protein: position 40 was changed from C to G, and position 46 was changed from A to C. Plaques (300,000) from a *D. ommata* electric lobe λ GT10 cDNA library were screened using the 54-mer end-labeled with ^{32}P . Filters were hybridized according to Maniatis et al. (1982) at 37°C and washed in 6× SSC at 55°C. Nine clones were analyzed by subcloning into KS– (Stratagene) and dideoxy sequencing (Sanger et al., 1977). Eight of these clones corresponded to o-p65-A but can be divided into three classes (Figure 2A). Two clones were 3.3 kb fragments with a natural RI site at the 3′ end, one with a 5′ end at nucleotide 1, the other at nucleotide 8. The second class is a 2.1 kb fragment with a poly(A) tail and the 5′ end at nucleotide 8. The third class is composed of 3.2 kb fragments with a natural RI site at the 3′ end and an 85 nucleotide deletion from nucleotides 92 to 176, with 5′ ends at nucleotides 6 and 8. Three partial clones beginning 3′ to the deletion were also sequenced. One has a poly(A) tail and 5′ end at nucleotide 1121. Two other clones with 3′ natural RI sites began at nucleotides 581 and 1166 (Figure 2A). Two of these clones were sequenced on both strands using oligonucleotide primers synthesized as needed. All of these clones had 6 mismatches with the oligonucleotide probe. The final 2.4 kb clone from this initial screen corresponded to o-p65-C. This clone had 13 mismatches with the oligonucleotide probe.

A 24 nucleotide fragment (nucleotides 382–405; Figure 2C) corresponding to the 5′ end of the original o-p65-C clone was end-labeled and used to screen 300,000 plaques of the electric lobe cDNA library. Filters were hybridized at 37°C and washed in 6× SSC at 50°C. The positive clones were rescreened using a hexamer-primed 2.4 kb insert from the original clone. Three additional clones were isolated. The full-length clone had two RI fragments of 2.7 kb and 1.0 kb with a poly(A) tail on the 1.0 kb fragment and natural RI sites at the 5′ end of the 1.0 kb and 3′ end of the 2.7 kb fragments. The other clones began at nucleotides 242 and 1000 (Figure 2C). Both strands of the full-length clone were sequenced by ExoIII-SI deletions as outlined by Stratagene.

To isolate o-p65-B, a PCR-generated fragment amplified from o-p65-A (nucleotides 561–1255) within the coding region was hexamer primed and used to screen 4.5×10^6 plaques of the λ GT10 electric lobe library by hybridizing at 50°C and washing in 2× SSC at 50°C. There were an average of 230 positives per 30,000 plaques, of which about 25% remained positive after subsequent 60°C washing. Six of 27 clones analyzed by cross-hybridization with o-p65-A at 60°C and washing in 0.1× SSC at 70°C had low cross-reactivity. Three of these clones were subcloned into KS– and sequenced by dideoxy sequencing. The 3.5 kb clone was sequenced on both strands with ExoIII-SI deletion, and the overlapping clones included nucleotides 30–1233 and 125–676. O-p65-B had 11 mismatches with the original 54 nucleotide oligo, but these mismatches were evenly distributed across the length, possibly resulting in greater destabilization.

Northern Blot Analysis

Two or 3 μg of poly(A)⁺ RNA (Figures 5A and 5B, respectively) or 25 μg of total RNA (Figure 5C) from various tissues of *D. ommata* was separated on 1% agarose formamide gels and blotted to Nytran. Blots were treated according to Nytran directions from Schleicher and Schuell and probed with a hexamer-primed insert from o-p65-A (nucleotides 8–2160), o-p65-B (nucleotides 30–1233), or o-p65C (nucleotides 382–1886). The presence of intact RNA in the lanes was confirmed by probing with human β -actin and methylene blue staining of the Nytran (data not shown). In the experiment shown in Figure 5B, the RNA was somewhat degraded in the muscle lane; but when repeated with intact RNA, there was still no signal with o-p65-B.

Antibody Production

The protein from the pooled vesicle peak fractions was precipitated with 10% TCA, resuspended in SDS-PAGE sample buffer, and fractionated on an 8%–13% SDS-PAGE gradient gel (Laemmli, 1970). After staining with Coomassie blue, the gel fragment was excised and homogenized in PBS and adjuvant (RIBI Immunochemical, Hamilton, MT). A male rat (Sprague-Dawley) was given a primary subcutaneous injection of 5 μg of protein followed by two boosts of 5 μg at 3 week intervals.

Affinity Purification of Anti-62 kd Antibodies

All 62 kd immunoblots and immunofluorescent staining were done with affinity-purified antibodies. The antibodies were purified essentially as described by Snyder et al. (1987), with the exception that the 62 kd band on PVDF paper was used as the solid support.

Immunoblot Analysis

Western blotting was done as described by Towbin et al. (1979). After transfer, the blots were incubated in blocking solution for 15 min. Primary antibody incubations were done for 16 hr at 4°C. ¹²⁵I-labeled goat anti-rat or anti-mouse antibodies (ICN Biomedicals, Costa Mesa, CA) were used to visualize binding of the primary antibody.

Immunofluorescence Staining

The immunofluorescence techniques used were modified from De Camilli et al. (1983). Electric rays were anesthetized and perfused with 4% formaldehyde. Tissues were then removed and immersed in the same fixative for 3 hr at 0°C. At this time, excess fixative was removed by several changes of PBS. Tissue specimens were then infiltrated for 16 hr with 18% (w/v) sucrose in PBS, embedded in Tissue-Tek (Miles, Elkhart, IN), and frozen in isopentane chilled with liquid nitrogen. Frozen sections (5–10 μm) were cut on a cryostat at –25°C, mounted on subbed glass coverslips that had been coated with poly-L-lysine (>300,000 MW; Sigma), and briefly air dried. For immunostaining, the sections were washed for 10 min with 0.1 M glycine buffered at pH 7.4 with Tris base. Permeabilization and blocking of the sections were done by incubating in 0.3% Triton X-100, 1% BSA, 5% normal goat serum in TBST (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween-20, 0.2% BSA) for 15 min. Incubations of primary antibodies (diluted in blocking solution) were done for 16 hr at 4°C followed by washes with five changes of TBST. Fluorescein isothiocyanate-

or rhodamine-conjugated secondary antibodies (Sigma) were then diluted 1:50 and incubated with the sections for 1 hr at room temperature. After washing in TBST, CitiFluor was used to mount coverslips, and the slides were viewed under fluorescence optics.

Acknowledgments

The first two authors (K. C. M. and B. W.) contributed equally to this work. We thank Regis Kelly and Louis Reichardt (University of California, San Francisco, CA) for supplying the SV2 and monoclonal 48 antibodies, respectively, and Bill Trimble (University of Toronto, Toronto, Ontario) for supplying the VAMP antibodies. We also thank Dr. Lisa Elferink for help with the RNA blotting experiments and Dr. Johnny Ngsee for helpful advice and discussion. This work is supported by the Howard Hughes Medical Institute and the National Institute of Mental Health.

References

- Almers W. Exocytosis. *Annu. Rev. Physiol.* 1990; 52:607–624. [PubMed: 2184769]
- Baumert M, Maycox PR, Navone F, De Camilli P, Jahn R. Synaptobrevin: an integral membrane protein of 18000 daltons present in small synaptic vesicles of rat brain. *EMBO J.* 1989; 8:379–384. [PubMed: 2498078]
- Baumert M, Takei K, Hartinger J, Burger PM, Fischer von Mollard G, Maycox PR, De Camilli P, Jahn R. P29: a novel tyrosine-phosphorylated membrane protein present in small clear vesicles of neurons and endocrine cells. *J. Cell Biol.* 1990; 110:1285–1294. [PubMed: 2182650]
- Bourne HR. Do GTPases direct membrane traffic in secretion? *Cell.* 1988; 53:669–671. [PubMed: 2836065]
- Buckley K, Kelly RB. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol.* 1985; 700:1284–1294. [PubMed: 2579958]
- Buckley KM, Floor E, Kelly RB. Cloning and sequence analysis of cDNA encoding p38, a major synaptic vesicle protein. *J. Cell Biol.* 1987; 705:2447–2456. [PubMed: 3121632]
- Carlson SS, Wagner JA, Kelly RB. Purification of synaptic vesicles from elasmobranch electric organ and the use of biophysical criteria to demonstrate purity. *Biochemistry.* 1978; 77:1188–1199. [PubMed: 418798]
- Cowan D, Linial M, Scheller RH. Torpedo synaptophysin: evolution of a synaptic vesicle protein. *Brain Res.* 1989; 509:1–7.
- De Camilli P, Cameron R, Creengard P. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. 1. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J. Cell Biol.* 1983; 96:1337–1354. [PubMed: 6404910]
- Elferink LA, Trimble WS, Scheller RH. Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J. Biol. Chem.* 1989; 264:11061–11064. [PubMed: 2472388]
- Fischer von Mollard C, Mignery GA, Baumert M, Perin MS, Hanson TJ, Burger PM, Jahn R, Südhof TC. rab3 is a small GTP binding protein exclusively localized to synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 1990; 87:1988–1992. [PubMed: 2155429]
- Fischer von Mollard G, Südhof TC, Jahn R. A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. *Nature.* 1991; 349:79–81. [PubMed: 1845915]
- Floor E, Feist BE. Most synaptic vesicles isolated from rat brain carry three membrane proteins, SV2, synaptophysin and p65. *J. Neurochem.* 1989; 52:1433–1437. [PubMed: 2496198]
- Gross E. The cyanogen bromide reaction. *Meth. Enzymol.* 1967; 11:238–255.
- Hoar, WS.; Randall, DJ., editors. *The Endocrine System. Vol. II.* New York: Academic Press; 1969. Fish Physiology.
- Johnston PA, Südhof TC. The multisubunit structure of synaptophysin. *J. Biol. Chem.* 1990; 265:8869–8873. [PubMed: 2111326]
- Kelly RB. The cell biology of the nerve terminal. *Neuron.* 1988; 1:431–438. [PubMed: 2908445]

- Knaus P, Marqueze-Pouey B, Scherer H, Betz H. Synaptoporin, a novel putative channel protein of synaptic vesicles. *Neuron*. 1990; 5:453–462. [PubMed: 2206533]
- Laemmli U. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
- Leube RE, Kaiser P, Seiter A, Zimbelmann R, Franke WW, Rehm H, Knaus P, Prior P, Betz H, Reinke H, Beyreuther K, Wiedenmann B. Synaptophysin: molecular organization and mRNA expression as determined from cloned cDNA. *EMBO J*. 1987; 6:3261–3268. [PubMed: 3123215]
- Linial M, Miller K, Scheller RH. VAT-I: an abundant membrane protein from Torpedo cholinergic synaptic vesicles. *Neuron*. 1989; 2:1265–1273. [PubMed: 2483112]
- Linás R, McGuinness TL, Leonard CS, Sugimori M, Greengard P. Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. USA*. 1985; 82:3035–3039. [PubMed: 2859595]
- Maniatis, T.; Fritsch, EF.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory; 1982.
- Matteoli M, Navone R, Haimann C, Cameron PL, Solimena M, De Camilli P. Secretory organelles of neurons and their relationship to organelles of other cells. *Cell Biol. Int. Rep*. 1989; 73:981–992. [PubMed: 2699836]
- Matthew WD, Tsavalier L, Reichardt LF. Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J. Cell Biol*. 1981; 91:257–269. [PubMed: 7298720]
- Navone F, Jahn R, Di Gioia G, Stukenbrok H, Greengard P, De Camilli P. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol*. 1986; 70:2511–2527. [PubMed: 3097029]
- Ngsee JK, Miller K, Wendland B, Scheller RH. Multiple GTP-binding proteins from cholinergic synaptic vesicles. *J. Neurosci*. 1990; 70:317–322. [PubMed: 2105379]
- Ngsee JK, Elferink LA, Scheller RH. A family of ras-like CTP-binding proteins expressed in electromotor neurons. *J. Biol. Chem*. 1991; 266:2675–2680. [PubMed: 1899244]
- Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*. 1988; 334:661–665. [PubMed: 3045562]
- Perin MS, Fried VA, Mignery CA, Jahn R, Stidhot TC. Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature*. 1990; 345:260–263. [PubMed: 2333096]
- Perin MS, Brose N, Jahn R, Südhof TC. Domain structure of synaptotagmin (p65). *J. Biol. Chem*. 1991a; 266:623–629. [PubMed: 1985919]
- Perin MS, Johnston PA, Ozcelik T, Jahn R, Franke U, Südhof TC. Structural and functional conservation of synaptotagmin (p65) in *Drosophila* and humans. *J. Biol. Chem*. 1991b; 266:615–622. [PubMed: 1840599]
- Reichardt LF, Kelly RB. A molecular description of nerve terminal function. *Annu. Rev. Biochem*. 1983; 52:871–926. [PubMed: 6137190]
- Sanger F, Nicklen F, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 1977; 74:5463–5467. [PubMed: 271968]
- Snyder M, Elledge S, Sweetser D, Young RA, Davis RW. Lambda gt 11: gene isolation with antibody probes and other applications. *Meth. Enzymol*. 1987; 754:107–128. [PubMed: 2448583]
- Südhof TC. Synaptic vesicles. *Curr. Opin Cell Biol*. 1989; 1:655–659.
- Südhof TC, Lottspeich F, Greengard P, Mehl E, Jahn R. A synaptic vesicle protein with a novel cytoplasmic domain and four transmembrane regions. *Science*. 1987; 238:1142–1144. [PubMed: 3120313]
- Südhof TC, Baumert M, Perin MS, Jahn R. A synaptic vesicle membrane protein is conserved from mammals to *Drosophila*. *Neuron*. 1989a; 2:1475–1481. [PubMed: 2560644]
- Südhof TC, Czernik AJ, Kao H-T, Takei K, Johnston PA, Horiuchi A, Kanazir SD, Wagner MA, Perin MS, De Camilli P, Greengard P. Synapsins: mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins. *Science*. 1989b; 245:1474–1480. [PubMed: 2506642]

- Thomas L, Hartung K, Langosch D, Rehm H, Bamberg E, Franke WW, Betz H. Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science*. 1988; 242:1050–1053. [PubMed: 2461586]
- Thomas TB. Islet tissue in the pancreas of Elasmobranchii. *Anat. Rec.* 1940; 76:1–18.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 1979; 76:4350–4354. [PubMed: 388439]
- Trimble WS, Cowan DM, Scheller RH. VAMP-1: a synaptic vesicle-associated integral membrane protein. *Proc. Natl. Acad. Sci. USA*. 1988; 85:4538–4542. [PubMed: 3380805]
- Trimble WS, Gray TS, Elferink LA, Wilson MC, Scheller RH. Distinct patterns of expression of two VAMP genes within the rat brain. *J. Neurosci.* 1990; 70:1380–1387. [PubMed: 2329380]
- Trimble WS, Linial M, Scheller RH. Cellular and molecular biology of the presynaptic nerve terminal. *Annu. Rev. Neurosci.* 1991; 74:93–122. [PubMed: 1851609]
- Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 1987; 56:365–394. [PubMed: 3304138]
- Zimmerman H, Denston CR. Separation of synaptic vesicles of different functional states from the cholinergic synapses of the Torpedo electric organ. *Neuroscience*. 1977; 2:715–730. [PubMed: 593552]

Figure 1. Discopyge Ommata Synaptic Vesicle Proteins

Cholinergic synaptic vesicles from the electric organ of *D. ommata* were purified and fractionated by SDS-PAGE. The gel was stained with Coomassie blue, revealing two vesicle-specific bands at 62 kD and 74 kD (arrows). These bands were sequenced directly or cleaved with CNBr, and some of the resulting fragments were sequenced.

A

[illegible]

B

[illegible]

C

```

1      GGCTCCTGACCCGACCCCAACAGCTCTCCAGGATCGCAATCTCTGACTCAGGGCGTCAATGACCCCTCAACCGACTCCTCCTGCTGAAGCTCGCAGATCAGCCACTGCAGATCAAGGGC
141    TTGAGATTCCACAGAGGAGGTCCACATTTTAGTCAGGATTCGGTGGAGGCCAGAGCCGCCCAACCCAGGAGCCTCTGACTTCACCTCCAGCGGTGCCGCCGTGGCAAGGCGATGCT

1      M S G D G E D E L C R N A L A L V N E L C F S V R G N H N N E K C I E F S
241    GGGACAAGATGTCAAGTGATGGGAGGACGAGTTGTGAGGAATGCGCTGGCGCTGGTCAACGAGCTGTGCTTCAGTGTCCGTGGGAACCAACAACGAGAAGTGCATCGAGTTCTCCT

368    Y L L R D R D R T R H I E T D I S V S L L S V I V T F C G I V L L L G V S L F V S
361    ACCTCCTGGTGATCGAGACCGACGCCACATCGAGACAGATATCTCGGTGAGTCTGCTCTCTGTGATCGTCACTTTCTCGGCATCGTGCTTCTTGGCGTCTCCCTCTTTGTCTCCT

488    W K L C W I P W R D K G L N P Q R R D S Q H H P H Q H L H H H H S H F T D L T V
481    GGAAGCTGTGCTGGATCCCATGGCGGACAAGGTCTGAACCCACAGCGCGGGACTCCCAACCCATCCGACCGAGCCTCCACACCGACCACTCAGCTTACCGAGCTCACCGTCC

608    E R V D C G P E M P E R S Y L D L E S Y P E S G I K L S Q T S P D I P V D T S S
601    AGAGAGTGGATCGGGGCCGAGATGCCGAACGTTCTTACCTGGACCTGGAGTCTATCCCGAGTCCGGCATTAGCTGAGCCAGACCTCCCGGATATCCGGTGGACACGAGCTCGG

728    G S K E N N I P N A H S Q Q Q V S A P P P A T R F N S L P R P I P Q Q L S S P E
721    GGAGCAAGGAGAACCAATCCCAATCGGCACTCCGACAGGTCTGTGCGCTCCGCAACCGAGCTGACAGCAAGGTTCAACTCACTCCGAGGCCGATCCGACGAGCTGAGCGCCGAAT

848    F G T Q A D E K V E Q V T S I G Q I K P E L Y K Q R S I D T E A K K H Q K V N C
841    TTGGAACCTCAGGCCGAGAGAAGTGGAAAGGTGACAGGATCGGCGAGATCAAACCCGAGCTGACAGCAAGCGGTGATCGACACAGGCGGATCCGACAGGAGCTGAGCGCCGAAT

968    G R I N F M L R Y T Y T T E Q L V V K I L K A L D L P A K D A N G F S D P Y V K
961    GCCGATTAACTTCATGCTACGCTACACCTACACCGAGCAGCTGGTGGTCAAGATCCTGAAAGCCCTTGACCTGCCGCCAAAGATGCCAATGGATTCTCTGATCCTTACGTGAAGA

1088   I Y L L P D R K K K F Q T K V H R K T L N P I F N E T F Q F N V P F N E L Q N R
1081   TTTACCTGCTGCCCGACCGCAAGAAGTTTTCAGACCAAGGTCCATCGCAAGCCTTGAAACCAATCTTCAACGAAACCTTTCAGTTCATGTGCCCTTTAAAGAGCTCCAGATCGCA

1208   K L H F S V Y D F D R F S R H D L I G Q V V L D N L L E F S D F S E D T T I W R
1201   AGCTGCATTTAGTGTTTACGACTTTGACCGCTTCTCTCGCATGACCTCATTGGCCAGGTGGTGTGTCACAACTGCTGGAGTTCTCCGACTTCTCCGAGGACGACCATCTGCGGGG

1328   D I L E A T S E K A D L G E I N F S L C Y L P T A G R L T I T I I K A T N L K A
1321   ACATCCTGGAAGCCACCTCGGAAAGGACAGATCTGGGCGAAATCAACTCTCGCTGTGCTACCTGCGGACAGCTGGAACACTTACCATCACTATTATCAAGGCCACTAAGCTCAAGCGA

1448   M D L T G F S D P Y V K A S L I C D E R R I K K R K T S I K K N T L N P V Y N E
1441   TGGACCTGACTGGATTTTCAGACCCCTACGTGAAGGCTCGCTGATCTGTGACGAGCGAGCGGTGAAGAAGCGGAAGACCTCAATAAAGAGAAGACGCTTAACCCGGTGTACAATGAGG

1568   A L V F D I P N E N M E H V N V I I A V M D Y D C I G H N E V I G M C R V G N A
1561   CGCTGGTCTTCGACATCCCAATGAGAACATGAGGACGCTCAACGTCATCATCGCGGTCTGAGTACGATTGCATCGGACACAAAGAGGTGATCGGGATGTGCGGTGTGGGAATGCCA

1688   T D G P G R E H W N E M L A N P R K P I E Q W H Q L I E E K V M N S Y M T K S F
1681   CTGACGGGCGGGTGGGAGCACTGGAACGAAATGCTGGCAATCCACGCAACCCATTGAGCAATGGCACAGCTGATTGAGGAAAAGGTGATGAACAGCTACATGACAAAGAGTTTCG

1808   A A G T G A T K P V T I V V E S P H S V *
1801   CCGCTGGTACAGGTGCTACCAAGCGGTCAACATTGTGGTGGAGAGTCTCACTCAGTCTGAGAAGCCCACTCCACATGGTAAAAATAAACCAAGAACACCTTGGTGTAGAAAGTAG

1921   GATAGAGGATGAAGTAAGACCACTTGGGCTTCAGGAGAACATAAAGACACATTTACCTCATATCTTTCATGGTCTCAGAGCAATCATTTTACAGCAATGACTTACCTTTGATGCAAG
1921   CAGCTGGTAATTTGTGCACGGCAAGCTCACAAACAGCAATACTTCCATAACCGTGATGTCAACCGCAAGAGGACAGGGGAAGCAGCCCCACGACAAACACAGCTCCTGGAAGTTCC
2041   TCCCGAGCTGCACATCATTCGGGCTGGTCTTCAGCGTCTCCGAGTCTCTCTGGAACCTCTCTGACGACAACTGTGGGAGCAATCCTCACACATGCAGTTCCAGAAAGGTGGCT
2041   CACCTTGTCAAGAACAATAAAGATGAGCAATGAACACTGGTCTCACCCAGATGGCAGGAAATGGTTCAATTAAGAAAGAAATTAGCAATGATCCGTGGACTGAGAGGAACTATTGGCTC
2161   AGGTGCTGGGAGCACTCGTTATTGAAGAGCTTGAAGAAGACAGGTGACCAATAAGTTGCTGCTGGGTTGTGGTGTGGGTAAGAGTGATGGTGTGGTTCCCTAATTTCAATGCAGGAG
2161   GAAATGTACGATTGGACATCCTGACATCGTTGCAGCTCTTGGGTCAATGTGACAGCTATGACTGGAGAAGCAATGACCGCAAGGGGAATGGGCTGGAGGAGGGGAATTC

```

Figure 2. Nucleotide Sequences of Three p65 Related cDNAs

The nucleotide and predicted amino acid sequences for representatives of three p65 cDNAs are shown. (A), o-p65-A; (B), o-p65-B; and (C), o-p65-C. The region corresponding to the amino acid sequence determined from the CNBr cleavage fragments and amino terminus is underlined in o-p65-B. In-frame upstream stop codons are also underlined.


```

1
r-p65      MVSASHPEALAAPVTT  VATLVP  HNATEPASPGEGK
o-p65-A    MKLTEAYHDAALPATPLPTAVA  NATEAAGSEEGK
o-p65-B    MQAEMNOSAAEVTMTKFTFTMKTRETHPQAFVAPMAT  ATMMPIDTGDNSTEAGVPGEK
o-p65-C    MSGDGEDELCRNALAL  VNELC  FSVRGNHNNEK

61
r-p65      EDAFSKL  KQKFMNELHKIPLPPWALIAIAIVAVILVVTCFCVCKKCLFKKKKNKKK
o-p65-A    QDGFSKVKV  KEKFMNELNKIPLPPWALVAIAIVAILGLTCCFCICKKCLLKKKNKKK
o-p65-B    NDVFEKL  KEKFMNELQKIPLPPWALIAIAIVSGLLLTCCLCICKKCCCKKKKNKK
o-p65-C    CIEFSYLLRDRDRTRHIETDISVSLLSVI  VTFCGIVLLGVSLFVSWKLCWIPWRDKGL

120
r-p65      GKEKGGKNAINMKDVKDLGKTMKDQALK  DDDAETGLTDGEEK
o-p65-A    GKEKGGKNAMTMKDVKEGKSGKEQALKDEDAETGLT  TDGK
o-p65-B    EKGKGGKNDINMKDVKGSG  GNQDDDAETGLTEGEDK
o-p65-C    NPQRDSQHHPHQHLLHHHSHFTDLTVERVDCGFEMPERSYLDLESYPESGIKLSQTSPP

180
r-p65
o-p65-A
o-p65-B
o-p65-C    IPVDTSSGSKENNIPNAHSQQQVSAPPPATRFNSLPRIPQQLSSPEFGTQADEKVEQVT

240
r-p65      EEPKEEEKL  GKLYSLDYDFQNNQLLVGIIQAAELPALDM
o-p65-A    EEEKEDEKL  GKLFSLDYDFQNNQLLVGIIQAAELPALDV
o-p65-B    EEEAKEEEKL  GKIQFSLDYDFQANQLTVGIIQAAELPALDM
o-p65-C    SIGQIKPELYKQRSIDTEAKKHQKVNCGRINFMLRYTYTTEQLVVKILKALDLPARDA

298-----PKC-A-----
r-p65      GGTSDPYVVKVFLLPEKKKKFETKVHRKTLNPFNEQFTFKVPYSELGGKTLVMVAVY
o-p65-A    GGTSDPYVVKVFLPDKKKKYETKVHRKTLNPFNESFIFKIPYSELGGKTLVMVAVY
o-p65-B    GGTSDPYVVKVFLPDKKKKYETKVQKTLNPFNESFVFKVPYQELGGKTLMMVAVY
o-p65-C    NGFSDPYVVKIYLLPDRKKKFQTKVHRKTLNPIFNETFQFMVPPNELQNRKLFHSVY

355----->-----<-----
r-p65      DFDRFSKHDIGEF  KVPMTVDVFGHVTEEWRDLQSAEKEEQEKLGDICFSLRYVP
o-p65-A    DFDRFSKHDVIGEA  KVPMTVDVFGHVTEEWRDLQGAEEKEEQEKLGDICFSLRYVP
o-p65-B    DFDRFSKHDCIGQV  TVLMTKVLDLGGQLEEWRDLESAAEKEEPEKLGDICTSLRYVP
o-p65-C    DFDRFSRHDLIGQVVLNLLFESDFSEDTTIWRDILEATSEKAD  LGEINFSLCYLP

412-----PKC-B-----
r-p65      TAGKLTVVVILEAKNLKKMDVGGLSDPYVKIHLMQNGKRLKKKKTTIKKNTLNPYY
o-p65-A    TAGKLTVVVILEAKNLKKMDVGGLSDPYVKIHLMQNGKRLKKKKTTIKKNTLNPYY
o-p65-B    TAGKLTVCILEAKNLKKMDVGGLSDPYVKIHLLQNGKRLKKKKTTVKKNTLNPYY
o-p65-C    TAGRLTITIIKATNLKAMDLTGFSDPYVKASLICDERRLKKRKTSIKKNTLNPVY

467----->-----
r-p65      NESFSFEVPFEQIQKVQVVVTVLDYDKIGKNDAIDKVFVGYNSTGAELRHWSDMLAN
o-p65-A    NESFSFEVPFEQIQKVQVVVTVLDYDKIGKNDAIGKVFVGYNSTAAELRHWSDMLAN
o-p65-B    NESFSFEIPFEQIQKVQVCVTVLDYDKIGKNDAIGKIFVGSNASGTELRHWSDMLAN
o-p65-C    NEALVFDIPNENMEHVNVIIAVMDYDCIGHNEVIGMCRVGNATDGPGREHWNEMLAN

524
r-p65      PRRPIAQWHTLQVEEEVDAMLAVKK
o-p65-A    PRRPIAQWHTLQPEEEVDATLGMKK
o-p65-B    PRRPIAQWHTSLKPEEEVDVALGLKK
o-p65-C    PRKPIEQWEQL  IEEKVMNSYMTKSFAAGTGATKPVTIVVESPHSV

```

Figure 3. Alignment of Rat and Ray p65 Amino Acid Sequences

The amino acid sequences of the rat and three ray p65 proteins are aligned to maximize the extent of homology. Amino acid positions that are conserved in all four sequences are bold. Potential N-linked glycosylation sites are underlined. The hydrophobic domain and the two PKC-homologous regions are indicated above the sequence.

Figure 4. Schematic Representation of p65 Proteins

The four p65 sequences are depicted, and the homology to the rat sequence is indicated for the various domains. The two PKC-homologous regions are indicated (dark stippling), as is the hydrophobic domain (zig zags). Glycosylation sites are depicted (solid circles). The **K** in rat p65, o-p65-A, and o-p65-B indicates a lysine-rich region, and the **H** in o-p65-C indicates a histidine-rich region. An insert and a region of low homology in o-p65-C relative to the other sequences is shaded (light stippling). An amino-terminal extension of o-p65-B and a carboxy-terminal extension of o-p65-C are depicted.

Figure 5. Differential Expression of the p65 Gene Family

Northern blots of RNA from *D. ommata* tissues are shown.

(A) Poly(A)⁺ RNA probed with o-p65-A. Lane 1, spinal cord; lane 2, electric lobe; lane 3, forebrain.

(B) Poly(A)⁺ RNA probed with o-p65-B. Lane 1, heart; lane 2, liver; lane 3, muscle; lane 4, total brain; lane 5, spinal cord; lane 6, electric organ; lane 7, electric lobe; and lane 8, forebrain.

(C) Total RNA probed with o-p65-C. Lane 1, electric lobe; lane 2, liver; and lane 3, forebrain. Blots were stained with methylene blue (A-C) and probed for actin message (B) to confirm presence of intact RNA.

Figure 6. Immunoblot Analysis of the p65 Immunoreactive Species Copurifying with Electric Organ Vesicles

(A) Graph illustrating the final stage of vesicle isolation: CPG chromatography. Open squares indicate absorbance at 310 nm, and solid diamonds indicate ATP.

(B) Immunoblot in which an equal volume of every fifth fraction from 75–145 of the same prep has been fractionated by SDS-PAGE, blotted to nitrocellulose, and probed with the anti-62 kd antibodies or antibodies to the synaptic vesicle protein, VAMP (Trimble et al., 1988). Molecular weight markers in kilodaltons and the location of VAMP are indicated on

the left. The positions of the reduced and unreduced forms of the immunolabeled bands are indicated on the right.

Figure 7. Immunoblot Analysis of o-p65-A and o-p65-B in the Ray CNS

Total protein (40 μ g) from various regions of the CNS was fractionated on a 10% SDS-PAGE gel and probed with MAb 48 to detect o-p65-A (A) or with anti-62 kd antibodies to detect o-p65-B (B). Lane 1, vesicles (4.5 μ g); lane 2, organ; lane 3, spinal cord; lane 4, brainstem; lane 5, cerebellum; lane 6, midbrain; lane 7, forebrain. Lanes 8 and 9 in (B) are muscle and liver, respectively.

Figure 8. o-p65-B Immunoreactivity in Nerve Terminals

(A) Indirect immunofluorescence localization of o-p65-B immunoreactivity in a 15 μm cross section of ray muscle fibers. The secondary antibody is fluorescein isothiocyanate-labeled. (B) Same field stained with rhodamine-labeled α -bungarotoxin to reveal neuromuscular junctions. (C) o-p65-B immunoreactivity in the ray brainstem. Immunofluorescence staining of 6 μm frozen section. Note the brightly stained nerve terminals outlining the soma of the large neuron in the center (asterisk). Bars: 40 μm (A and B); 100 μm (C).

Figure 9. Immunofluorescence Staining of o-p65-A and o-p65-B in the Ray CNS: Comparison with SV2

Shown are 7 μ m frozen sections of spinal cord (A–C), cerebellum (D–F), midbrain (G–I), and forebrain (J–L). (A, D, G, and J) SV2. (B, E, H, and K) o-p65-A. (C, F, I, and L) o-p65-B. The molecular (M) and granule cell (G) layers of the cerebellum are indicated. Bars: 40 μ m (A–D; F–I); 20 μ m (E); 100 μ m (J–L). The section in (L) was exposed longer than comparable sections, but the low amount of punctate staining demonstrates the paucity of o-p65-B immunoreactive terminals in the forebrain.

Figure 10. Immunofluorescence Staining of o-p65-A and o-65-B in Neuroendocrine Cells

Shown are 7 μm frozen sections of anterior pituitary (A–C) and pancreas (B–F). (A and D) SV2. (B and E) o-p65-A. (C and F) o-p65-B. The staining around the dark spaces in (A) and (B) represents neuroendocrine cells lining the capillary passages. Bar, 40 μm . The distribution and arrangement of the SV2- and p65-positive cells in the ray pituitary and pancreas are consistent with previous studies of the elasmobranch endocrine system (Thomas, 1940; Hoar and Randall, 1969).

Figure 11. Immunofluorescence Staining of p65-A in Rat Shown are 7 μ m frozen sections of rat spinal cord (ventral horn)

(A) p65-A and (B) SV2. Two neurons surrounded by SV2 immuno-reactive terminals are indicated with asterisks. Bar, 100 μ m. The several large fluorescent spots in (A) are background and not consistently observed.

Table 1

p65 Homologies

	r-p65	o-p65-A	o-p65-B	o-p65-C
r-p65	100	84 (4)	78 (4)	41 (11)
o-p65-A	94	100	76 (4)	40 (12)
o-p65-B	87	85	100	39 (9)
o-p65-C	64	65	58	100

The numbers in the upper right portion of the table are the percent identity, with the number of gaps introduced into the sequence in parentheses. The numbers in the lower left portion of the table are the percent similarity, as determined by the BESTFIT program. r-p65 is from the rat p65 sequence of Perin et al. (1990).

Table 2

Summary of the Distribution of Three Synaptic Vesicle Proteins in the Ray Nervous System

	SV2	p65-A	p65-B
Central nervous system			
Electric organ	++++	–	++++
Neuromuscular junction	++++	–	++++
Spinal cord	++++	+	+++
Brain stem	++++	+	+++
Midbrain	++++	+	+++
Cerebellum			
Granular layer	++++	++++	+
Molecular layer	++++	++++	–
Forebrain	++++	++++	+
Neuroendocrine cells			
Pituitary	++++	++++	–
Islet cells of pancreas	++++	++++	++++

This table provides a summary of the immunolocalization results presented in this paper. Pluses (+) indicate the relative amount of these proteins found in each region, as determined by immunoblot analysis or immunofluorescence staining. A minus (–) indicates no detectable immunoreactivity.