A protein of $M_r$ 100,000 (MVP100) is highly enriched in the electromotor system of electric rays. Biochemical analysis indicates that MVP100 is contained in the cholinergic nerve terminals of Torpedo electric organ as part of a large cytosolic complex. On sucrose density gradient centrifugation MVP100 comigrates with synaptic vesicles or synaptosomes. It can be partially separated from synaptic vesicles by gel filtration or glycerol velocity gradient centrifugation. Within the complex MVP100 behaves like a hydrophobic protein and is protected against proteolytic attack. MVP100 can be immunodetected by an antibody against phosphotyrosine, and it becomes phosphorylated on incubation with $\left[\gamma^3P\right]$ATP. By screening an electric ray electric lobe cDNA library the primary structure of MVP100 was analyzed. MVP100 is highly homologous to the major vault proteins of slime mold and rat and to the human lung resistance-related protein. Compared with non-neural proteins of slime mold and rat, whereas the composition in the other, the sequence similarity in the major vault protein is more than 70% of the total complex. The major vault protein is about 100 kDa, the major vault protein, accounting for more than 70% of the total complex. The major vault protein is highly enriched in microglia (7). Mutational analyses of major vault protein in slime mold indicate an involvement of vaults in cell growth (8). Evidence has been provided that in D. discoideum and rat liver a small amount of the vault ribonucleoprotein particles localizes to the nuclear pore complex (6). Vault subpopulations have also been observed at the ruffling edges of spreading rat fibroblasts and along cytoskeletal elements, suggesting a possible role in motility (5). In the developing rat brain vaults are highly enriched in microglia (7). Analyses of the major vault protein in slime mold indicate an involvement of vaults in cell growth.

The Major Vault Protein (MVP100) Is Contained in Cholinergic Nerve Terminals of Electric Ray Electric Organ*

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 Vaults are ubiquitous, evolutionarily conserved cytoplasmic ribonucleoprotein particles of unknown function (1). The vault particle was originally identified as a small ovoid body in preparations of clathrin-coated vesicles and named for its globular structure, which is reminiscent of cathedral vaults (2). Vaults are multimeric protein complexes with a predominant member of about 100 kDa, the major vault protein, accounting for more than 70% of the total complex. The major vault protein is phylogenetically conserved between the amoeba Dictyostelium discoideum and rat, whereas the composition in the other minor protein members of vaults differs (3, 4). Recently, the lung resistance-related protein (LRP), which has a high predictive value for resistance to chemotherapy in myeloid leukemia and ovarian carcinoma, was found to be a homolog of the major vault protein (5).

One interesting feature of vaults is the presence of a single species of small RNA whose size varies among species (9). By Northern blot analysis vault RNA was found in all tissues and cells analyzed with the lowest level of expression in brain tissue (9). Vaults as described in rat and slime mold are similar in mass (12.9 MDa). Estimates for particle width and length range between 26 and 35 nm, and 35 and 60 nm, respectively, depending on the method used (6, 8).

When analyzing proteins that copurify with cholinergic synaptic vesicles from the electric ray electric organ we identified a protein of $M_r$ 100,000 (MVP100). Primary structure analysis of this protein revealed that it is an electric ray homolog of the major vault protein identified in rat and D. discoideum. Expression of MVP100 in brain is high relative to liver with the highest level in the electric lobe containing the cell bodies of the cholinergic neurons innervating the electric organ. MVP100 is localized abundantly in cholinergic nerve terminals of Torpedo in close proximity to synaptic vesicles. Comparison of the primary structure of MVP100 reveals highly conserved domains in the protein architecture. Distribution, subcellular localization, and biochemical properties of the electric ray MVP100 are compared with those of the synaptic vesicle protein SV$_2$, a transmembrane glycoprotein (10, 11) representing a putative transporter molecule (12, 13).

**EXPERIMENTAL PROCEDURES**

Subcellular Fractionation—All purification steps were performed at 4 °C. Synaptic vesicles from frozen electric organ of Torpedo marmorata (120–140 g, wet weight) were extracted in 0.4 M NaCl (3.5 mM EGTA, 10 mM HEPES/NaOH, pH 7.4) and purified further using a discontinuous sucrose gradient as described previously (14). In brief, synaptic vesicles extracted in saline buffer were separated on a simple sucrose step gradient consisting of 22 ml of 0.4 M sucrose, 0.2 M NaCl, and 10 ml of 0.2 M sucrose, 0.3 M NaCl (both adjusted to pH 7.4 with 10 mM HEPES/NaOH and centrifuged at 80,000 $\times$ g$_{av}$ for 2 h). Synaptic vesicles were collected as the band at the 0.2/0.4 M sucrose interface (fraction F$_s$). Fraction F$_s$ was diluted 2-fold with isotonic glycyne solution (0.8 M glycine, 3.5 mM EGTA, 10 mM HEPES/NaOH, pH 7.4) and centrifuged at 188,000 $\times$ g$_{av}$ for 1 h. The resulting pellet was resuspended in 2 ml of isotonic glycyne solution yielding fraction F$_p$. Resuspended fractions were separated on a Sephacryl-1000 column (1.6 × 150 cm) equilibrated...
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1.1.1.1

with 0.8 mM glucose, 3.5 mM NaCl, 10 mM HEPES/NaOH, pH 7.4, at a flow rate of 20 ml/h. In some experiments fractions from the column effluent were centrifuged for 1 hr at 188,000 × g, and the resulting pellet and supernatant fractions were analyzed.

1.1.1.1

Purification of MVP100 and Amino Acid Sequence Analysis—The synaptic vesicle fraction F3 was subjected to glycerol gradient velocity centrifugation as described previously (17). In brief, synaptic vesicles were resuspended in gradient buffer consisting of 150 mM NaCl, 0.1 mM MgCl2, 1 mM EGTA, 10 mM HEPES, pH 7.4. 300 μl of fraction were loaded per gradient (4.4 ml) with a linear glycerol concentration ranging from 5 to 30% (in gradient buffer) on top of a cushion (0.4 ml) containing 30% glycerol and in addition 50% sucrose. Gradients were centrifuged for 1 hr at 150,000 × g, and the 300 μl were collected as described previously (20). In brief, precocooned Torpedo X-114 was used as a 22% (w/v) stock solution. Synthetic vesicles (fraction F3) were solubilized using a Branson sonifier (2 × 4 s, small pestle) in 10 ml Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% Triton X-114 at 0°C (200 µM in 200-µl suspension). The procedure results in two phases, an aqueous supernatant and a detergent-rich phase. The aqueous supernatant still contains residual Torpedo X-114. It was centrifuged for 45 min at 188,000 × g, and pelleted and supernatant fraction were analyzed. The detergent-rich phase and the remaining top of the sucrose cushion were washed twice with Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The detergent-rich phase was then collected and subjected to analysis.

1.1.1.1

Intracellular Treatment of Intact Synaptic Vesicles—Synaptic vesicles derived from 20 g of tissue obtained after discontinuous sucrose gradient centrifugation and high speed centrifugation were resuspended in isotonic saline solution (1 ml of 0.4 M NaCl, containing 10 mM HEPES/NaOH, pH 7.4). They were incubated (4°C for 30 min with gentle rotation) at acidic or basic pH (pH 3.0 or 11.5) or in media containing 0.4 M KCl, 2 M KCl, or 1% Triton X-100 in addition to sodium chloride. Samples were then loaded onto a sucrose cushion (0.25 M sucrose, 0.28 M NaCl, and 10 mM HEPES/NaOH, pH 7.4) and centrifuged for 45 min at 150,000 × g. Resulting supernatant and pellet fractions were analyzed by Western blotting.

1.1.1.1

Analytical Procedures—Protein content was determined according to Bradford (21) or Schaffner and Weissmann (22) using bovine serum albumin as a standard. The activity of acetylcholinesterase was measured according to Ellman et al. (23), and analysis of ATP was performed using the luciferin-luciferase bioluminescence method of Stanley and Williams (24) as described previously (25).

1.1.1.1

Immunocytochemistry—Immunolabeling with colloidal gold on tissue sections was performed using the preembedding protocol described in detail by Janetzko et al. (26). Briefly, this includes fixation of tissue blocks with paraformaldehyde (2.6%), glutaraldehyde (0.05%), and picric acid (0.04%) in 0.3 M cacodylate buffer, pH 7.4, and subsequent washing with 0.1 M sodium phosphate buffer, pH 7.4, containing sucrose (3.4%) and NaCl (0.7%). Vibratome sections were incubated with antibodies. After application of antibody-coated colloidal gold particles, sections were postfixed with 2% glutaraldehyde and with 1% OsO4 in, respectively, washing solution and postfixation solution. After several washes, secondary gold-conjugated-antibodies were applied for 4 h. Nitrocellulose strips were fixed with 2% glutaraldehyde and postfixed with 1% OsO4 in the washing solution used for tissue blocks. All steps were carried out at 4°C. Subsequently strips were embedded in Epon and processed further as for tissue sections.

1.1.1.1

RESULTS

Copurification of Synaptic Vesicles and MVP100—We initially identified MVP100 as a protein copurifying with cholinergic synaptic vesicles. Antibodies raised against the protein were used to investigate further the vesicular association of the protein in a number of different purification protocols. The routinely applied isolation procedure for obtaining purified cholinergic synaptic vesicles from the Torpedo electric organ in-
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The particulate nature of MVP100 is supported further by a series of centrifugation experiments. Three major column-derived fractions (see, Fig. 2A, bottom) were subjected to high speed centrifugation (1 h at 188,000 × g): a synaptic vesicle fraction almost devoid of MVP100, an intermediate fraction containing MVP100 and the synaptic vesicle membrane and volume markers, and the "p100 fraction" where the majority of MVP100 immunoreactivity elutes. Pellet and supernatant fractions were analyzed for MVP100 immunoreactivity by Western blotting (Fig. 2B). In all cases MVP100 is solely recovered in membrane marker largely elutes with the void volume (100-120 ml). Synaptic vesicles as identified by the markers ATP and SV2 are retarded and elute as a broad and symmetrical peak. In contrast, MVP100 elutes significantly later, indicative of a difference in size between the synaptic vesicle compartment and MVP100-containing particles.

MVP100 Separates from Synaptic Vesicles on Column Chromatography—The sedimentation behavior of MVP100 on density gradient centrifugation is indicative of a large complex with density similar to that of the synaptic vesicle compartment. Dissociation of this MVP100-containing complex from synaptic vesicles becomes apparent when synaptic vesicle harvested from the 0.2/0.4 m sucrose interface are pelleted, resuspended, and subjected to column chromatography on a Sephacryl-1000 column. Separation of the synaptic vesicle fraction on the sizing column results in a bimodal distribution of protein (Fig. 2A). Activity of acetylcholinesterase as a plasma

cludes homogenization, pore filtration, and differential centrifugation. During all these steps MVP100 as identified by a monospecific polyclonal antibody (α-p100d) copurifies with synaptic vesicles. The resulting micromolar fraction enriched in synaptic vesicles is then fractionated further by discontinuous sucrose density gradient centrifugation, and the fractions obtained are analyzed for various markers (Fig. 1). Protein contents reveal a peak at the top and the bottom of the sucrose gradient. ATP as a luminal constituent of cholinergic synaptic vesicles is contained in fractions 10–16 corresponding to 0.3–0.4 m sucrose with a maximum in fractions 14 and 15. Western blot analysis using an antibody against the synaptic vesicle integral SV2 demonstrates that the peak of immunoreactivity overlaps with the peak of ATP-containing fractions. The bulk of MVP100 comigrates with the other synaptic vesicle proteins and ATP. However, some of the MVP100 immunoreactivity is shifted to less dense fractions. A monoclonal antibody raised against the p97 subunit of 15S Mg2+-ATPase was applied to a Sephacryl-1000 sizing column. Panel A, synaptic vesicles identified by their ATP contents separate from the membrane fragments in the void volume peak enriched in acetylcholinesterase (AChE) (upper graph). Elution of MVP100 as analyzed by immunoblotting only partially overlaps with the synaptic vesicle specific protein SV2. It is shifted to later effluent fractions (middle graph). The distribution of lanes in the immunoblots corresponds to the elution profile of the column. Effluent fractions containing synaptic vesicles (VP), intermediate fractions (IM) representing coelution of MVP100 and the synaptic vesicle specific protein SV2, and the MVP100 fraction (p100 frac.) that is largely devoid of vesicle markers were subjected to high speed centrifugation.

MVP100 Separates from Synaptic Vesicles on Column Chromatography—The sedimentation behavior of MVP100 on density gradient centrifugation is indicative of a large complex with density similar to that of the synaptic vesicle compartment. Dissociation of this MVP100-containing complex from synaptic vesicles becomes apparent when synaptic vesicles harvested from the 0.2/0.4 m sucrose interface are pelleted, resuspended, and subjected to column chromatography on a Sephacryl-1000 column. Separation of the synaptic vesicle fraction on the sizing column results in a bimodal distribution of protein (Fig. 2A). Activity of acetylcholinesterase as a plasma

FIG. 1. Codetermination of the bulk of MVP100 with synaptic vesicles on a discontinuous sucrose gradient. The synaptic vesicle volume marker ATP is exclusively retained at the 0.2/0.4 m sucrose interface, whereas protein contents are highest in the top and bottom fractions of the gradient. The distribution of MVP100, of the vesicle specific protein SV2, and of the 97-kDa (p97) subunits of the ubiquitous cytosolic Mg2+-ATPase was analyzed by Western blotting with the respective antibodies (bottom panel). Fractions of 1 ml were collected from the gradient, and every second fraction was analyzed by immunoblotting. Material corresponding to 100 μl was loaded per lane. Thus, the distribution of lanes corresponds exactly to the sucrose gradient in the upper graph.

FIG. 2. Separation of MVP100 from the synaptic vesicle peak on column chromatography. Synaptic vesicles obtained after discontinuous sucrose density centrifugation were applied to a Sephacryl-1000 sizing column. Panel A, synaptic vesicles identified by their ATP contents separate from the membrane fragments in the void volume peak enriched in acetylcholinesterase (AChE) (upper graph). Elution of MVP100 as analyzed by immunoblotting only partially overlaps with the synaptic vesicle specific protein SV2. It is shifted to later effluent fractions (middle graph). The distribution of lanes in the immunoblots corresponds to the elution profile of the column. Effluent fractions containing synaptic vesicles (VP), intermediate fractions (IM) representing coelution of MVP100 and the synaptic vesicle specific protein SV2, and the MVP100 fraction (p100 frac.) that is largely devoid of vesicle markers were subjected to high speed centrifugation. Panel B, the resulting pellet (P) and supernatant (S) fractions were analyzed for MVP100 immunoreactivity. Material corresponding to 0.3 ml was applied per lane. Fractions denoted VP, IM, and p100 frac. correspond to pooled effluent fractions as indicated in the graph. Material corresponding to one-fifth of each fraction was loaded per lane.
the pellet fraction. Thus, independent of its position in the column effluent, the sedimentation behavior of MVP100 is identical to that of the parent fraction prior to loading onto the column, indicating that MVP100 is still part of a large complex.

Purification of MVP100 by Glycerol Velocity Gradient Centrifugation—Upon glycerol velocity gradient centrifugation MVP100 immunoreactivity fractionates throughout gradient fractions 5–15 (Fig. 3A). Part of the MVP100 immunoreactivity clearly separates from immunoreactivity of the synaptic vesicle marker SV2, which is restricted to the densest fractions. MVP100-containing fractions in the middle portion of the gradient were devoid of any synaptic vesicle marker analyzed. As judged by protein staining MVP100 was by far the most abundant protein in these fractions (Fig. 3C, lane 1). Other polypeptides were only visible when gels were heavily overloaded. These fractions were used for producing antibodies against native MVP100 (α-p100n; see Fig. 3A, n) and for direct microsequencing of the protein. In immunoblots all three antibodies reveal the same sedimentation profile for MVP100 (Fig. 3A).

When prepared by the same method as MVP100 from electric organ the sedimentation behavior of MVP100 from Torpedo liver differs. A Western blot using an antibody against a synthetic peptide of MVP100 (α-p100p, see “Experimental Procedures”) is shown as an example (Fig. 3B). The bulk of the liver MVP100 protein sediments at less dense fractions, and little immunoreactivity is found in fractions where synaptic vesicles would be sedimenting (see Fig. 3A). The apparent molecular mass of MVP100 from liver is identical to that from electric organ (Fig. 3C, lane 2).

Purification of MVP100, Cloning Strategy, and Amino Acid Analysis—By direct microsequencing of MVP100 obtained after glycerol gradient centrifugation several stretches of amino acid residues were obtained. An oligonucleotide was designed from one of these sequences for screening a cDNA library derived from mRNA of the electric lobe of the related electric ray D. australis. By screening 160,000 recombinants two independent positive clones were obtained and used to isolate a full-length clone (accession number X87771). The full-length clone of 2717 nucleotides has a short 5’-untranslated region without an in-frame stop codon, an open reading frame encoding 852 amino acids followed by a short 3’-untranslated flanking region containing a polyadenylation signal and the poly(A) tail. The predicted protein has a calculated molecular mass of 95.8 kDa, similar to its migration behavior on SDS-polyacrylamide gel electrophoresis. The calculated pI of MVP100 is 5.5.

All stretches of amino acid residues obtained by direct sequencing were found to be contained in the linear structure of MVP100, indicating that the MVP100 band is devoid of contaminating proteins that might be masked by MVP100.

MVP100 Is the Electric Ray Homolog of Evolutionarily Conserved Major Vault Proteins—A computer-based search in nucleotide and protein data bases revealed a high homology of the electric ray protein with the major vault protein of human, rat, and slime mold (Fig. 4). The electric ray protein shows 69.1% amino acid identity (in an 851-amino acid overlap) with the major vault proteins of human (27) and 68.7% (847 amino acids) identity with that of rat origin (28). 55.8% (840 amino acids) and 52.1% (849 amino acids) amino acid identity is found with the two isoforms MvPA (4) and MvPB (8) of the slime mold D. discoideum, respectively. The identity between the mammalian proteins is 89.9% (868 amino acids) and between the amoeba isoforms is 60.4% (839 amino acids). Thus MVP100 is an electric ray homolog of the major vault protein.

Highly Conserved Domains in the Protein Structure of MVP100—Manual alignment of the predicted amino acid sequences of MVP100 from electric ray, human, rat spleen and the two isoforms MvPA and MvPB from D. discoideum revealed four domains that are phylogenetically highly conserved in the primary structure of the proteins (Fig. 4). Alignment of the sequences indicates that 10 amino acids from the amino terminus of the rat protein (the first of two putative translation starts) can be omitted from the sequence published previously.

Whereas the amino termini (domain A) are conserved, the carboxyl termini show a long hypervariable region interrupted by one small conserved domain. Secondary structure analyses predict a very long (about 150 amino acids) α-helical domain near the carboxyl terminus. This indicates the presence of an elongated tail in all proteins. Within this region the proteins reveal homologies to tail regions of other elongated proteins, such as the myosin heavy chain (18.1% over 386 amino acids). There is a 60% prediction for the formation of a long coiled coil structure in this α-helical domain. This might be of relevance for the formation of the vault complex.

MVP100 contains numerous putative phosphorylation sites for a variety of protein kinases. In this context it is interesting to note that several potential phosphorylation motifs for protein kinase C (four) and casein kinase II (three) and one for tyrosine-protein kinase are evolutionarily conserved in all major vault proteins (Fig. 4). Only in MvPB from Dictostelium is the phosphorylation motif for tyrosine protein kinase missing.

Biophysical Properties of MVP100—Subcellular fractionation of MVP100 suggested that it forms or is part of a large stable cytosolic complex. To investigate further the biophysical properties of complexed MVP100 we studied its solubility and protease resistance. These were compared with those of a membrane integral synaptic vesicle protein occurring in the same subcellular fraction (SV2, Fig. 5). The fraction (Fp) containing both MVP100 and synaptic vesicles was subjected to temperature-induced phase separation using Triton X-114 yielding detergent-rich and aqueous phases. MVP100 immunoreactivity segregates completely into the detergent phase indicative of hydrophobic interactions of MVP100 in vaults (Fig. 5A). The hydrophobic protein SV2 with its 12 putative transmembrane
domains behaves rather similarly, but a small amount of SV$_2$ immunoreactivity is retained within the aqueous phase containing residual amounts of detergent.

Further evidence that MVP100 is part of a stable complex is derived from another set of experiments. Synaptic vesicles (fraction F$_3$) subjected to various chemical treatments were loaded onto a sucrose cushion (0.25 M sucrose containing 0.28 M NaCl, adjusted to pH 7.4 with 10 mM HEPES/NaOH) and centrifuged at 150,000 g$_{av}$ for 45 min. After previous incubation of intact synaptic vesicles with 0.4 M KCl or 2 M KCl, or at acidic pH (3.0) MVP100 is recovered in the pellet fraction to the same extent as SV$_2$. Only after incubation at basic pH (11.5) substantially more MVP100 than SV$_2$ is recovered in the supernatant fraction. In contrast to SV$_2$, MVP100 is only partially soluble in 1% Triton X-100.

MVP100 contained in the complex is resistant to limited proteolysis. A fraction (F$_3$) containing MVP100 and synaptic vesicles was incubated in the absence or presence of trypsin (1 unit) for 5 min or for 30 min at 4°C. After trichloroacetic acid precipitation samples were analyzed by Western blotting using MVP100 and SV$_2$ antibodies (Fig. 5B). Even after 30 min of incubation the majority of MVP100 is still intact with only a faint degradation product around 40 kDa, whereas SV$_2$ becomes rapidly degraded (5 min). Limited proteolysis in the presence of the detergent Triton X-100 (0.1%) yields identical results. Denatured MVP100 on SDS gels or electroeluted from gel slices after SDS-polyacrylamide gel electrophoresis could, however, be degraded by several proteases tested. Moreover, MVP100 disintegrates when stored for a longer period of time at 2°C, giving rise to several polypeptides that are immunodetected by the monospecific antibodies.

MVP100 Becomes Phosphorylated in Vitro and in Vivo—When fractions of intact synaptic vesicles obtained after sucrose density centrifugation (fraction F$_3$) were incubated with radiolabeled ATP in the presence of divalent cations but without the addition of exogenous protein kinases MVP100 becomes phosphorylated. The autoradiographs reveal that MVP100 is the major phosphoprotein in this fraction (Fig. 6, lane 1). Other precipitation samples were analyzed by Western blotting using MVP100 and SV$_2$ antibodies (Fig. 5B). Even after 30 min of incubation the majority of MVP100 is still intact with only a faint degradation product around 40 kDa, whereas SV$_2$ becomes rapidly degraded (5 min). Limited proteolysis in the presence of the detergent Triton X-100 (0.1%) yields identical results. Denatured MVP100 on SDS gels or electroeluted from gel slices after SDS-polyacrylamide gel electrophoresis could, however, be degraded by several proteases tested. Moreover, MVP100 disintegrates when stored for a longer period of time at -20°C, giving rise to several polypeptides that are immunodetected by the monospecific antibodies.

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Fig. 5. Behavior of MVP100 upon detergent and protease treatment compared with that of SV2. Panel A, identification of proteins by immunoblotting on temperature-induced phase separation of synaptic vesicle proteins. Lane 1, parent fraction prior to phase partitioning. Lane 2, detergent phase after phase separation using Triton X-124. MVP100 immunoreactivity segregates completely into the detergent phase. Lane 3, aqueous phase subjected to high speed centrifugation. The resulting aqueous supernatant fraction is devoid of MVP100 and SV2. Lane 4, corresponding pellet fraction. It apparently contains residual amounts of detergent and reveals some immunoreactivity of SV2. Proteins were precipitated with 10% trichloroacetic acid (final concentration), and one-fourth of each fraction was applied per lane. Panel B, resistance of MVP100 to limited proteolysis. Intact synaptic vesicles (100 μg) were incubated in the absence (lane 1) or presence of trypsin (1 unit) for 5 min (lane 2) or 30 min (lane 3) at 4°C. After trichloroacetic acid precipitation samples were analyzed by Western blotting using MVP100 and SV2 antibodies. Protein corresponding to one-fifth of each sample was applied per lane. Note that even after 30 min of incubation the majority of MVP100 is still intact with only a faint degradation product around 40 kDa appearing (arrows). SV2 immunoreactivity is virtually destroyed.

Fig. 6. Phosphorylation of MVP100. Lane 1, intact synaptic vesicles obtained after discontinuous sucrose gradient centrifugation (fraction F3) were incubated with [γ-32P]ATP in the presence of Mg2+ and Ca2+ as described under "Experimental Procedures." MVP100 is the protein with the highest incorporation of radiolabeled phosphate. Lane 2, immunodetection of MVP100 with α-p100d antibody was performed on the same nitrocellulose. Lane 3, phosphorylation of MVP100 is also observed when the p100 fraction obtained after permeation chromatography (see Fig. 2) is subjected to the same protocol. Lane 4, immunodetection of MVP100 with α-p100d antibody on the same nitrocellulose. Lanes 5 and 6, Immunodetection after application of an anti-phosphotyrosine antibody of MVP100 in the synaptic vesicle fraction F3 (lane 5) and in the MVP100-containing fraction isolated by glycerol velocity gradient centrifugation (lane 6).

less phosphorylated proteins migrate at apparent molecular masses of 70, 55, and 38 kDa. Similarly, MVP100 becomes phosphorylated in the p100 fraction (see Fig. 2) obtained after chromatography (Fig. 6, lane 3). As revealed by silver staining MVP100 is one of the most prominent proteins in the p100 fraction (not shown). In this fraction an additional phosphorylated protein band of 110 kDa makes its appearance. MVP100 is only weakly phosphorylated in the presence of 1% Triton X-100. Instead, a rather broadly migrating band around 90 kDa becomes strongly labeled. MVP100 does not become phosphorylated after it has been electroeluted from gel slices.

The primary structure of MVP100 (Fig. 4) had revealed several putative phosphorylation sites for protein kinases including tyrosine-protein kinase. To test whether MVP100 is phosphorylated in vivo we analyzed fractions of freshly isolated synaptic vesicles and glycerol velocity gradient-purified MVP100 (without previous in vitro phosphorylation) for immunoreactivity with a monoclonal anti-phosphotyrosine antibody (Fig. 6, lane 5). The results obtained closely correspond to those obtained by in vitro phosphorylation using radiolabeled ATP (Fig. 6, lanes 1 and 3). MVP100 appears as the major phosphotyrosine-containing protein in the synaptic vesicle fraction, and isolated MVP100 also contains phosphotyrosine immunoreactivity (Fig. 6, lane 6).

MVP100 Is Highly Expressed in Neuronal Tissue—Using each of the three antibodies MVP100 can be detected by Western blotting in the entire electromotor system of Torpedo (Fig. 7). This includes total tissue homogenates of the electric organ (Fig. 7, lane 1), the electric nerve (Fig. 7, lane 2), and electric lobe (Fig. 7, lane 3). Strong immunoreactivity is obtained in the synaptic vesicle fraction F3 (Fig. 7, lane 4). Since 13 times less protein than for total tissue homogenates was loaded per lane (see legend to Fig. 7) MVP100 appears to be considerably enriched in the synaptic vesicle fraction. The immunodetection signal of isolated MVP100 is shown for comparison (Fig. 7, lane 5). A weak MVP100 immunosignal was also obtained from total tissue homogenates of other brain areas, such as forebrain, cerebellum, and midbrain. MVP100 immunoreactivity could barely or not be detected in total tissue homogenates of non-neural tissues such as liver, pancreas, spleen, skeletal muscle, heart, and stomach. All three antibodies strongly recognized purified MVP100 from Torpedo liver (see Fig. 3). No cross-reactivity was observed with total tissue homogenates derived from porcine and bovine brain tissues.

MVP100 Is Localized in Cholinergic Nerve Terminals—To define further the subcellular localization of MVP100, synaptosomes were isolated from the electric organ by glycerol gradient centrifugation. 100% of the synaptosomal lactate dehydrogenase was found in the occluded form, indicating that synaptosomes were intact and sealed. On the gradient MVP100 is colocalized with occluded lactate dehydrogenase, acetylcholinesterase (a synaptosomal plasma membrane marker) (29), and the synaptic vesicle proteins analyzed (SV2, o-rab3). This suggests that MVP100 is contained inside the nerve endings. Furthermore, we performed immunolabeling using the colloidal gold technique and antibodies to MVP100 and SV2. Nerve terminals were densely labeled for MVP100, and the colloidal gold particles were found in close apposition to synaptic vesicles (Fig. 8A). Clusters of synaptic vesicles docked to the presynaptic membrane also carried gold particles. Labeling was absent from the plasma membrane or from mitochondria.
distribution is surprisingly similar to that of the membrane integral synaptic vesicle protein SV$_2$ (Fig. 8B). The SV$_2$ antibody binds to an epitope facing the cytoplasmic surface of the vesicle. These results suggest that MVP100 either is physically linked to synaptic vesicles or that the dense packing of vaults and synaptic vesicles causes nonspecific colocalization. We therefore performed in addition an ultrastructural analysis of subcellular fractions derived after column chromatography as described in Fig. 2. Synaptic vesicles derived from the synaptic vesicle peak revealed only occasional labeling with the MVP antibody, but they were densely labeled for SV$_2$ (Fig. 9). The p100 fraction contained few vesicular profiles but instead numerous electron-dense particles that presumably represent glycogen derived from electromotor nerve terminals (30). MVP100 immunoreactivity is occasionally observed in close proximity to vesicle profiles and is mainly associated with undefined structures (Fig. 9C). The small electron dense particles are not labeled. Many of the few vesicular structures contained in the fraction are labeled with the SV$_2$ antibody (Fig. 9D).

DISCUSSION

MVP100 Is Contained in a Large Complex—MVP100 forms a large cytosolic complex. This is supported by the sedimentation properties of the MVP100-containing particles, the detergent solubility of MVP100, and its resistance to proteolysis as long as the particle is intact. The long $\alpha$-helical domain of MVP100 near the carboxyl terminus could be directly involved in the formation of the polymeric protein particle. The subcellular fractionation procedures indicate that this complex has about the same density as the synaptic vesicle compartment.

On sucrose density gradient centrifugation MVP100 mainly cosediments with cholinergic synaptic vesicles. Vaults isolated from rat liver cofractionate with coated vesicles (2). However, according to size filtration and velocity gradient centrifugation the protein particle is significantly smaller than cholinergic synaptic vesicles of the electric ray electric organ. Values derived for the size of vaults vary depending on the technique employed. The dimensions of rat vaults have been estimated to be 35 x 60 nm (1, 8), which is about half the size of Torpedo synaptic vesicles (about 90 nm) (25).

MVP100 is by far the predominant vault component in Torpedo electric organ and liver. In preparations of purified vaults other polypeptides could be visualized only when gels where heavily overloaded. Vaults of other species have been reported to consist of several polypeptides with MVP as the most prominent member. The estimates range from 55 to 96 copies of MVP in one complex. A comparison of the polypeptide composition reported for vaults isolated from rat, bullfrog, rabbit, and D. discoideum reveals that the content of additional polypeptides varies greatly (3). Whereas other eukaryotic organisms display only a single major vault species that is the product of a single copy gene, three different major vault proteins with similar molecular masses have been identified in the slime mold (8).

Our attempts to identify a small RNA (vault RNA) (9, 31) in vaults isolated from Torpedo liver or electric organ have not been successful. Neither RNase treatment nor extensive digestion of proteins by proteinase K gave any indication of the presence of RNA. In the rat, brain tissue contained the lowest amount of transcript of the vault RNA (9). In the electric ray the expression of the MVP100 protein is higher in brain tissue than in any of the non-neural tissues investigated. We found the highest levels in the electric lobe that contains as a sole neural cell type the cholinergic electromotor neurons projecting into the electric organ.

Subcellular Localization of MVP100—Our results suggest that the cholinergic electromotor neurons are rich in MVP100-containing protein particles. The high contents in the nerve terminals suggest that the particles are transported via the axon and are enriched in the nerve terminals. At present it is not clear whether the apparent colocalization of vaults with synaptic vesicles as revealed by immunocytochemistry is due to a physical link or simply due to the dense packing inside the nerve terminals. It is noteworthy that on glycerol velocity gradient centrifugation of the synaptic vesicle fraction, MVP100-containing particles apparently separate into two populations. One sediments together with synaptic vesicles to denser glycerol fractions, and the other sediments at lighter fractions. Vaults isolated from different cellular sources by subcellular fractionation generally reveal a cytoplasmic localization. Immunofluorescence reveals a punctate cytoplasmic pattern, with some cells containing thousands of vault specific loci (5). The observation that vaults can localize with actin fibers in fibroblasts (5) is of interest since synaptic vesicles inside the nerve terminal are linked to the actin cytoskeleton (32). This could explain the colocalization of the two organelar structures in the nerve terminal and their partial association on subcellular fractionation.

Functional Considerations—At present the function of vaults is not known. MVP100 has a few conserved myristoylation sites that are not used, and none of the potential glycosylation sites is conserved between species. However, all major vault proteins contain numerous consensus motifs for phosphorylation by several protein kinases. Many of them are evolutionarily conserved from slime mold to human. Our experiments show that
MVP100 becomes highly phosphorylated in vitro. As revealed by Western blotting, freshly isolated MVP100 carries phosphotyrosine residues. This suggests that MVP100 is an in vivo substrate at least of tyrosine kinase. Our data imply that phosphorylation is of relevance for controlling vault function in vivo. On the other hand, vault proteins were not found to be phosphorylated when the rat hepatoma cell line H4 was metabolically labeled in the presence of $^{32}$P$_i$. By immunofluorescence evidence for a partial association of vaults with the nuclear pore complex has been derived. Disruption of two of the three major vault proteins in Dictyostelium reveals a mild growth defect under conditions of nutritional stress. Very recently a new avenue regarding the function of the major vault protein has been opened. LRP was found to be a homolog of the major vault proteins of rat and slime mold. Our study reveals that LRP is highly homologous also to the Torpedo MVP100. LRP overexpression was found to correlate with a poor response to chemotherapy in acute myeloid leukemia and ovarian carcinoma. It has been suggested that LRP, whose gene is closely located to the genes coding for the multidrug resistance-associated protein and protein kinase C-β, may mediate drug resistance. These observations suggest that major vault protein-containing particles play a central role in cell homeostasis. The high content of the MVP100-containing protein particles inside nerve terminals implies new and yet unrecognized functional properties of this highly specialized compartment of the nerve cell.

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