Vamp/Synaptobrevin Isoforms 1 and 2 Are Widely and Differentially Expressed In Nonneuronal Tissues

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Abstract. VAMP/synaptobrevin is part of the synaptic vesicle docking and fusion complex and plays a central role in neuroexocytosis. Two VAMP (vesicle-associated membrane protein) isoforms are expressed in the nervous system and are differently distributed among the specialized parts of the tissue. Here, VAMP-1 and -2 are shown to be present in all rat tissues tested, including kidney, adrenal gland, liver, pancreas, thyroid, heart, and smooth muscle. The two isoforms are differ- entially expressed in various tissues and their level may depend on differentiation. VAMP-1 is restricted to exo- crine pancreas and to kidney tubular cells, whereas VAMP-2 is the predominant isoform present in Langerhans islets and in glomerular cells. Both iso- forms show a patchy vesicular intracellular distribution in confocal microscopy. The present results provide ev- idence for the importance of neuronal VAMP proteins in the physiology of all cells.

EUKARYOTIC cells possess organized secretory path- ways for the exocytosis of proteins and other mole- cules. These secretory routes consist of several dis- crete membrane compartments and of transport vesicles that shuttle between these compartments (Rothman and Orci, 1992; Pryer et al., 1992; Simons and Zerial, 1993; Bennett and Scheller, 1993; Rothman and Warren, 1994). Membrane fusion of carrier vesicles may be constitutive (i.e., vesicle budding from the endoplasmic reticulum and fusing with the Golgi), or regulated (i.e., synaptic trans- mission). Though exocytosis takes place in virtually every cell (Burgoyne and Morgan, 1993), certain tissues are highly specialized in such a function and nerve cells pos- sess the most sophisticated and tightly controlled secretory apparatus (Kelly, 1993a).

Presynaptic nerve terminals are filled with small synap- tic vesicles (SSV)[1], which contain chemical neurotransmit- ters, and with the less abundant larger dense-core vesicles loaded with neuropeptides. Most SSVs are attached to the cytoskeleton via the synapsins and constitute a reserve neurotransmitter pool (Hirokawa et al., 1989; Valtorta et al., 1992; Greengard et al., 1993; De Camilli, 1995). A small percentage of SSVs is bound to active zones of the presyn- aptic membrane, ready to discharge their content in the in- tersynaptic space. Neuroexocytosis is triggered by a local increase in calcium concentration consequent to opening of voltage-gated calcium channels by membrane depolar- ization. The SSV membrane fuses very rapidly (200–300 μsec) with the presynaptic membrane (Almers, 1994). Fused SSVs are then rapidly retrieved and recycled in a dynamin-dependent process (Heuser and Reese, 1973; Bennett and Scheller, 1993, 1994; Takei et al., 1995; Süd- hof, 1995).

The abundance of SSVs in brain and the availability of a well defined and efficient protocol of isolation has lead to the molecular characterization of many SSV protein com- ponents (Hüttner et al., 1983; Bennett and Scheller, 1993, 1994; Südhof, 1995). Apart from proteins involved in the uptake of neurotransmitters (Schuldiner et al., 1995), very little is known about the function of these proteins. Söllner et al. (1993a,b) have identified a 20-S multi-subunit com- plex likely to be responsible for vesicle docking and fusion in the cell. This complex includes the vesicular protein VAMP (vesicle-associated membrane protein; also known as synaptobrevin), the presynaptic membrane-bound pro- teins SNAP-25 (synaptosomal-associated protein of 25 kD), syntaxin and soluble factors which include NSF (N-ethyl- maleimide sensitive factor), α/β and γ SNAPs (Malhotra et al., 1988; Trimble et al., 1988; Oyler et al., 1989; Clary et al., 1990; Bennett et al., 1992). Each of these proteins has a

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1. *Abbreviations used in this paper:* VAMP, vesicle-associated membrane protein; NSF, N-ethyl-maleimide sensitive factor; SNAP, soluble NSF at- tachment protein; SNAP-25, synaptosomal-associated protein of 25 kD; SSV, small synaptic vesicles; TeNT, tetanus neurotoxin; NRK, normal rat kidney cells; PC12, rat pheochromocytoma cells; RIN, rat insulinoma cells.

homologue in yeast and it is believed that the vesicle membrane fusion apparatus is conserved from yeast to man (Pryer et al., 1992; Ferro-Novick and Jahn, 1994; Südhof, 1995).

VAMP, a major component of the SSV membrane, is clearly implicated in neuroexocytosis since it is the specific target of the zinc-endopeptidase activity of tetanus and botulinum B, D, F, and G neurotoxins, which cause a sustained inhibition of neurotransmitter release (Schiavo et al., 1992a,b; 1993a,b,c; 1994; Yamasaki et al., 1994). VAMP was first identified by screening a cDNA library of the *Torpedo* electric organ (Trimble et al., 1988) and later cloned from rat, bovine, human, *Drosophila*, yeast, squid, and *Aplysia* (Elferink et al., 1989; Südhof et al., 1989; Archer et al., 1990; Chin et al., 1993; Di Antonio et al., 1993; Protopopov et al., 1993; Hunt et al., 1994; Yamasaki et al., 1994; Sweeney et al., 1995). Four regions can be identified in the primary structure of VAMP. An amino-terminal part is very rich in prolines and is not conserved among species and isoforms both in terms of sequence and length. A central hydrophilic and charged domain is highly conserved and contains the sites of cleavage of clostridial neurotoxins. A third transmembrane region is followed by a short carboxyl terminal hydrophilic sequence of variable length in the different species. Proteolysis, antibody binding, and the knowledge that clostridial neurotoxins operate in the cytosol (Trimble et al., 1988; Südhof et al., 1989; Montecucco and Schiavo, 1993) indicate that VAMP is a type II protein with a cytosolic amino terminus and a membrane-anchoring carboxyl end (Kutay et al., 1993, 1995).

Two isoforms of VAMP were identified in the nervous and neuro-endocrine tissues with dissimilar amino-terminal proline-rich domain part (Baumert et al., 1989; Elferink et al., 1989; Archer et al., 1990; Chin et al., 1993). In situ hybridization shows that they are differently distributed among the various brain regions (Trimble et al., 1991; Chin et al., 1993). More recently, a third VAMP isoform with a shorter amino terminal, termed cellubrevin, has been characterized by McMahon et al. (1993). Cellubrevin was suggested to be a nonneuronal member of the VAMP family mainly associated to endosomes.

Using polyclonal antisera raised vs rat brain VAMPs, glucose transporter-containing vesicles, isolated from adipose tissue, were shown to contain a doublet of VAMPs (Corley-Cain et al., 1992). Moreover, in situ hybridization shows the presence of VAMP in Malpighian tubes and in the gastro-intestinal epithelia of *Drosophila melanogaster* (Chin et al., 1993). More recently, VAMPs were also detected in skeletal muscle and pancreas (Ralston et al., 1994; Braun et al., 1994; Gaisano et al., 1994; Regazzi et al., 1995). Finally, tetanus neurotoxin, which specifically cleaves VAMP, inhibits the exocytosis of lysozyme in macrophages (Pitzurra et al., 1989). Taken together these results indicate that neuronal VAMP isoforms or related proteins may be expressed outside the nervous tissue. Here, we document the distribution of neuronal VAMP-1 and -2 isotypes outside the nervous tissue with various techniques. We show that the two isoforms are differentially expressed in different tissues. These results suggest that these proteins play an active role in exocytosis in many cell types and that the two isoforms have specialized functions.

Materials and Methods

Probes Used

Rat VAMP-1 and -2 cDNA clones corresponding to full-length mRNA were described previously (Elferink et al., 1989).

Northern Blotting and RNase Protection Assay

Total RNA was extracted from adult rat tissues using the procedure described by Chomczynski and Sacchi (1987). RNA concentration was determined by O.D. measurement and ethidium bromide staining of formaldehyde agarose gel electrophoresis.

For Northern blot, 30 μg from each tissue was size fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon filter by capillary elution. (Sambrook et al., 1989) Blotted RNA was fixed following manufacturer's instructions and hybridized overnight at 52°C with 5 × 10^6 full-length VAMP-1 and -2 probes, labeled with ^{32}P-dATP with Klenow DNA polymerase. The final washings were performed in 0.5 × SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6), 0.1% SDS at 65°C. Filters were exposed to x-ray film for 15 d.

For RNase protection assay antisense cRNA probes corresponded to the 3′UTR of each mRNA: VAMP-1 probe was 330 nt in length and was transcribed from VAMP-1 cDNA after digestion at the XbaI site; VAMP-2 probe was 415 nt in length and was transcribed from VAMP-2 cDNA after digestion at the BamHI site. Nucleotide sequence analysis confirmed the existence of high divergence at this site between the two mRNAs. RNase protection assay was performed using RPA II kit (Ambion, Austin, TX). 5 μg of total RNA from adult rat brain and spinal cord, 50 μg from kidney, adrenal gland, heart atrium, ventricle, and liver were hybridized overnight at 45°C in 80% formamide, 100 mM Na citrate, 300 mM Na acetate, pH 6.4, 1 mM EDTA with 10^5 cpm of gel purified ^{32}P-labeled VAMP-1 or VAMP-2 antisense probe. 1 μg of total RNA from each sample was hybridized with 6 × 10^4 cpm of mouse β actin cRNA probe as a control. After digestion with RNases A and T1, samples were separated by electrophoresis with 5% denaturing polyacrylamide gel. Molecular weight of protected fragments was determined using 100 bp DNA ladder (Pharmacia LKB Biotechnology, Piscataway, NJ) labeled with ^{32}P-dCTP with Klenow DNA polymerase. Autoradiography was developed after 4 d at −80°C.

In Situ Hybridization

Adult male Wistar rats (200–250 gr) were anesthetized with sodium pentobarbital (50 mg/ml) and perfused *trans*-cardiacally with isotonic saline buffer, followed by 4% paraformaldehyde in PBS. The organs of interest were removed, postfixed for 3 h in the perfusion buffer, and cryoprotected by immersion in a series of cold, graded sucrose solution in isotonic saline buffer to a final concentration of 18%. The organs were frozen with dry ice and cut into 8-μm-thick sections with microtome and mounted onto chrome potassium/gelatin slides. Prehybridization treatments and hybridization conditions were essentially as described by Gorza et al. (1993). Antisense and sense [^{35}S-RNA] transcripts corresponding to the full-length VAMP-1 and VAMP-2 mRNAs were synthesized using T$_3$ or T$_7$ RNA polymerase (Boehringer-Mannheim) to a specific radioactivity of 1.2 × 10^8 cpm/μg after linearization of VAMP-1 and VAMP-2 cDNA clones. Radiolabeled sense and antisense cRNA were digested by mild alkali hydrolysis to 100 nt fragments. Before hybridization, sections were digested with Proteinase K (Boehringer Mannheim Corp., Indianapolis, IN) 20 μg/ml in 10 mM TrisHCl, 5 mM EDTA, pH 7.8 at room temperature for 7.5 min. Hybridization was carried on using 10^5 cpm of ^{35}S-labeled probe in 50% formamide, 20 mM Tris-HCl, 0.3 M NaCl, 5 mM EDTA, 10 mM NaH$_2$PO$_4$, 10% dextran sulfate, 1× Denhardt's solution, 10 mM DTT and 0.1 mg/ml of denatured yeast tRNA and incubating overnight at 52°C. The highest stringency wash was performed in 50% formamide 2× SSC, 10 mM DTT for 20 min at 65°C and was followed by digestion with 20 μg/ml RNase A in 10 mM TrisHCl, 0.4 M NaCl, 5 mM EDTA pH 7.5 for 30 min at 37°C. After air drying, sections were placed in film cartridges and exposed to Kodak x-Omat film for 1–3 d. Slides prepared for autoradiography were coated with NTB-2 emulsion (diluted 1:2 with water), exposed at 4°C and developed with Kodak D-19 developer. In each experiment, pairs of slides hybridized with sense and antisense cRNAs were developed after 2 and 7 d of exposure. Some slides were counterstained with hema-

toxylin. A Zeiss Axioplan microscope equipped with dark-field and phase contrast optics was used for examination.

Antibody Production

Synthetic NH_2-terminal 1-33 peptides of rat VAMP-1 and VAMP-2 were synthesized using an automated solid-phase peptide synthesizer with Fast-Moc chemistry (Applied Biosystems, Foster City, CA). The two peptides were purified by HPLC using a reverse phase C_8 Ultra-Sphere preparative column (Beckman Intrs., Fullerton, CA), conjugated to keyhole limpet hemocyanin (Pierce, Rockford, IL) and mixed with Freund's adjuvant and 3 mg of heat-killed *Mycobacterium tuberculosis* (Difco, Detroit, MI). New Zealand white rabbits were immunized subcutaneously followed by intramuscular injections 3 wk apart (Vaitukaitis, 1981). Serum was affinity purified by binding to, and eluting from, the peptide bound to an AH-Sepharose 4B matrix (Pharmacia). These affinity purified antibodies recognize the bands of the correct size corresponding to VAMP-1 and VAMP-2, respectively, both in recombinant full-length VAMP-1 and VAMP-2 GSH fusion protein (Fig. 5), and in purified rat brain small synaptic vesicles (not shown) run in high resolution SDS-PAGE gels (Hüttner et al., 1983; Schiavo et al., 1992b).

Tissue Sample Preparation and Western Blot

Organs were isolated from adult rats and homogenated in homogenation buffer containing 50 mM Tris-HCl, 2% SDS, 0.5 mM EDTA, 0.4 mM benzamidine, 0.1 mM N-p-tosyl-L-lysinechloromethyl ketone (TLCK), 0.1 mM N-tosyl-L-phenylalanin chloromethyl ketone (TPCK) 2 μg/ml pepstatin A and 0.2 mM PMSF, (Sigma Chem.Co., St. Louis, MO) pH 7.0, and centrifuged for 5 min at 15,000 rpm. Supernatants were precipitated with trichloracetic acid in order to eliminate DNA, centrifuged for 10 s and pellets were resuspended in homogenation buffer without protease inhibitors. Protein concentration was determined with the method of Lowry (Markwell et al., 1981) using bovine serum albumin (BSA) as a standard. Tissue protein samples (175 μg/lane) were loaded onto a 13–18% SDS-polyacrylamide linear gradient gel (Laemmli, 1970) transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) for 3 h at 400 mA (Towbin et al., 1979). Stripes were probed with affinity purified antibody anti–VAMP-1 and anti–VAMP-2 (1:200 dilution, 16 h at 4°C) and stained with goat anti–rabbit IgG conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) (1:10,000 dilution, 2 h at 20°C).

Cell Cultures

The PC12 subclone used here was kindly provided by Dr. T. R. Rogers (University of Maryland School of Medicine). PC12 were cultured at 37°C on plastic dishes (100 mm diam) coated with collagen as described by Walton et al. (1988) and grown in DMEM (Flow, U. K, supplemented with 44 mM sodium bicarbonate), 7.5% horse serum, 7.5% FCS, pH 7.4 in a humid atmosphere of 5% CO_2 in air. NRK and RIN were cultured at 37°C on plastic dishes (100 mm diameter) in DMEM and RPMI, respectively, supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Weekly subcultivations were performed at initial cell density of $0.5-1 \times 10^6$ cells per 100-mm diameter dish. Immunofluorescence experiments were performed on cells seeded on coverslips ($10-50 \times 10^4$ cells/well in 12 well plates) coated with poly-lysine.

Immunofluorescence Microscopy

Cells. All procedures were carried out at room temperature. After three washings in PBS, cells were fixed in 4% paraformaldehyde in PBS for 20 min, rinsed once with PBS and twice for 10 min in PBS containing 0.38% glycine and 0.27% NH_4Cl. After three PBS washes, cells were permeabilized, in PBS containing 0.5% BSA, 0.1% saponin for 30 min (blocking solution). Cells were incubated with affinity purified antibody anti–VAMP-1 or VAMP-2 diluted 1:200 in blocking solution for 1 h. After five washes with blocking solution, they were incubated with fluorescein-conjugated pig anti–rabbit IgG secondary antibody (DAKO) diluted 1:100 in blocking solution for 30 min. For double staining, the primary step was with mouse anti-synaptophysin (Boehringer-Mannheim), in combination with rabbit anti–VAMP-1 or anti–VAMP-2. This was followed by FITC-conjugated pig anti–rabbit IgG in combination with Texas red–coupled horse anti–mouse immunoglobulins (Vector Labs, Burlingame, CA). Cells were washed five times with blocking solution and mounted in 90% glycerol-PBS containing 3% N-propylgallate. Specimens were observed using a Nikon RCM 8000 confocal microscope.

Cryosections. Prefixed cryosections of rat pancreas, kidney, adrenal gland and liver were incubated at room temperature for 3 h with affinity purified anti–VAMP-1 or -2 diluted 1:200 in PBS containing 3% BSA. After three washings in PBS, they were incubated with rhodamine-conjugated pig anti–rabbit IgG secondary antibodies (DAKO) diluted 1:200 in PBS-3% BSA. Slides were washed three times in PBS, mounted in Mowiol (Sigma), and observed under a light microscope.

Results

Expression in Different Tissues of VAMP-1 and -2 mRNAs

Previous studies on the distribution of VAMPs among species and tissues have suggested that VAMP isoforms are involved in cellular functions specific for nervous and neuro-endocrine tissues. The identification in nonneuronal cells of cellubrevin, an isoform with a distinct primary structure, is also in keeping with a tissue restricted distribution of VAMPs. However, a scattered set of data indicated the presence of VAMPs in other tissues.

Preliminary Northern blotting experiments with mRNA extracted from different tissues and hybridized with ^{32}P-labeled cDNA probes showed that the rat brain VAMP isoforms mRNAs were expressed in all nonneuronal tissues tested (data not shown). To exclude cross-reactivity due to high homology between coding regions of VAMP-1 and VAMP-2 mRNAs and to increase signals, RNase protection assay was performed using antisense cRNAs corresponding to the 3' untranslated region as a probe. Fig. 1 reports the result of such an assay performed on rat brain, spinal cord, kidney, adrenal gland, heart atrium, and ventricle and liver. VAMP-1 and -2 mRNAs protected fragments have the same size in all tissues tested and in brain
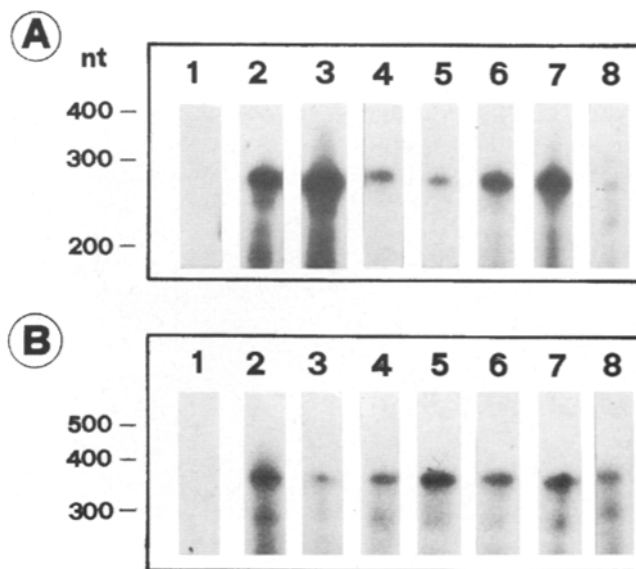
Figure 1. RNase protection assay of VAMP-1 (*A*) and VAMP-2 (*B*) mRNAs in rat tissues. 50 μg of yeast tRNA was used as negative control (lane *1*); lanes *2* and *3* were loaded with 5 μg of total RNA from brain (*2*) and spinal cord (*3*); lanes *4–8* were loaded with 50 μg of kidney (*4*), adrenal gland (*5*), heart atrium (*6*), heart ventricle (*7*), and liver (*8*). Hybridization results in a protected fragment of about 280 nt for VAMP-1 (*A*) and a fragment of about 350 nt for VAMP-2 (*B*).

VAMP-1

A

B

C

VAMP-2

D

E

F
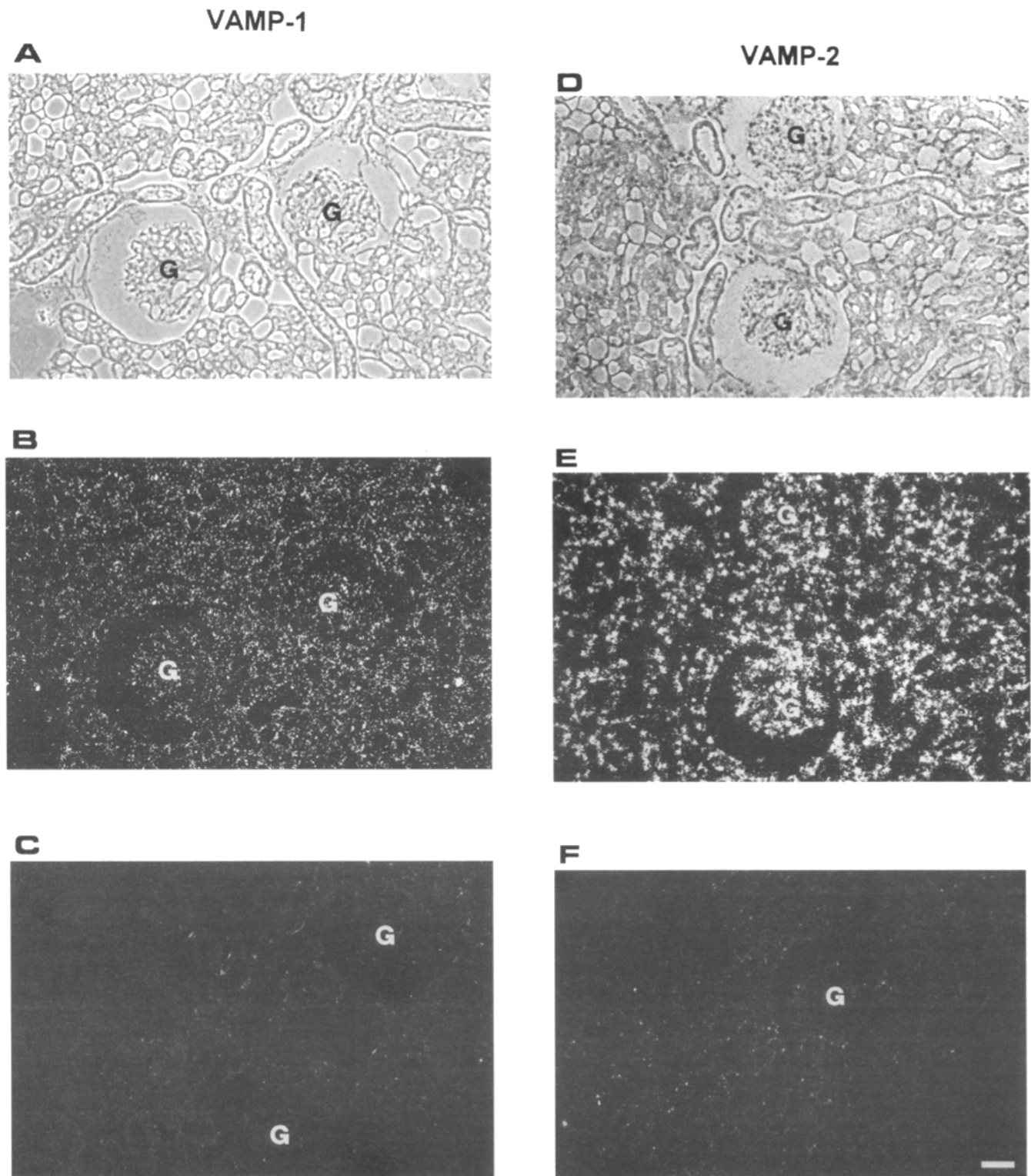
Figure 2. In situ hybridization of kidney with antisense and sense VAMP-1 and -2 cRNA probes: phase contrast (*A* and *D*) and dark-field microphotographs (*B, C, E,* and *F*) of in situ hybridization with antisense [^{35}S-cRNA] probes of VAMP-1 (*A* and *B*) and VAMP-2 (*D* and *E*) and with sense [^{35}S-cRNA] probes of VAMP-1 (*C*) and VAMP-2 (*F*) of kidney. *G*, glomerula. Bar, 50 μm.

and spinal cord, indicating that the same neuronal mRNA species are expressed outside the nervous tissue. This conclusion is also based on the fact that there are no introns in the rat VAMP genes (Elferink et al., 1989).

A relative comparison of the level of the signal given by the various nonneuronal tissues with respect to that of brain indicates that, in general, VAMP-2 mRNA signal is higher than that of VAMP-1, except for heart. The marked

increased presence of isoform 1 and decreased presence of isoform 2 in the spinal cord with respect to brain is noteworthy. The level of expression of the two VAMP isoforms found here appears to correlate with the known secretory activity of kidney, liver, and adrenal glands. The large amount of VAMPs in the heart was unexpected and its significance remains to be explained.

To obtain information on the distribution of the two VAMP mRNA isoforms within the different parts of a tissue, an in situ hybridization study with [^{35}S]RNA sense and antisense probes spanning the entire coding region of the two rat brain VAMP isoforms was performed. Figs. 2, 3, and 4 show the distribution of VAMP-1 and -2 mRNAs in kidney, in pancreas and in thyroid, liver, adrenal gland and aorta, respectively. Both antisense probes show strong hybridization signals with neuronal cells (not shown) whereas no signal is observed with the sense probes (Fig. 2, C and F). In the kidney, isoform 1 shows a uniform distribution (Fig. 2, A and B), but hybridization signals are much weaker than those observed in the brain. Conversely, strong hybridization signals are observed for VAMP-2 mRNA in both tubular and glomerular cells (Fig. 2, D and E). In pancreas there is a clear differential distribution of the two isoforms: VAMP-1 mRNA predominates in the exocrine acinar cells (Fig. 3, A and B), whereas VAMP-2 mRNA is enriched in the endocrine Langerhans islets (Fig. 3, C and D). It is noteworthy that the arterial wall contains VAMP-2, but appears to be devoid of VAMP-1 mRNA.

Fig. 4 shows the distribution of the mRNAs of VAMP-1 and -2 in thyroid, liver, adrenal gland, and aorta. The two isoform mRNAs are present at the same level in thyroid and are localized mainly in the follicular cells (panels A and E). In liver, the signal for VAMP-1 is lower than that of VAMP-2 in agreement with the RNase protection assay (panels B and F). VAMP-2 appears to be expressed at different levels in different liver cell types. Hematoxylin staining of VAMP-2 prehybridized slides identifies hepatocytes as VAMP-2 mRNA expressing liver cells, whereas Kupffer cells display only background levels of hybridization (not shown). VAMP-1 mRNA is enriched in the medulla of the adrenal gland. In contrast, VAMP-2 mRNA is higher in the cortical than in the medullar portion (Fig. 4, C and G). Fig. 4 also shows that VAMP-2 mRNA is clearly present in the smooth muscle cells of aorta, in a larger amount with respect to VAMP-1 mRNA (panels D and H).
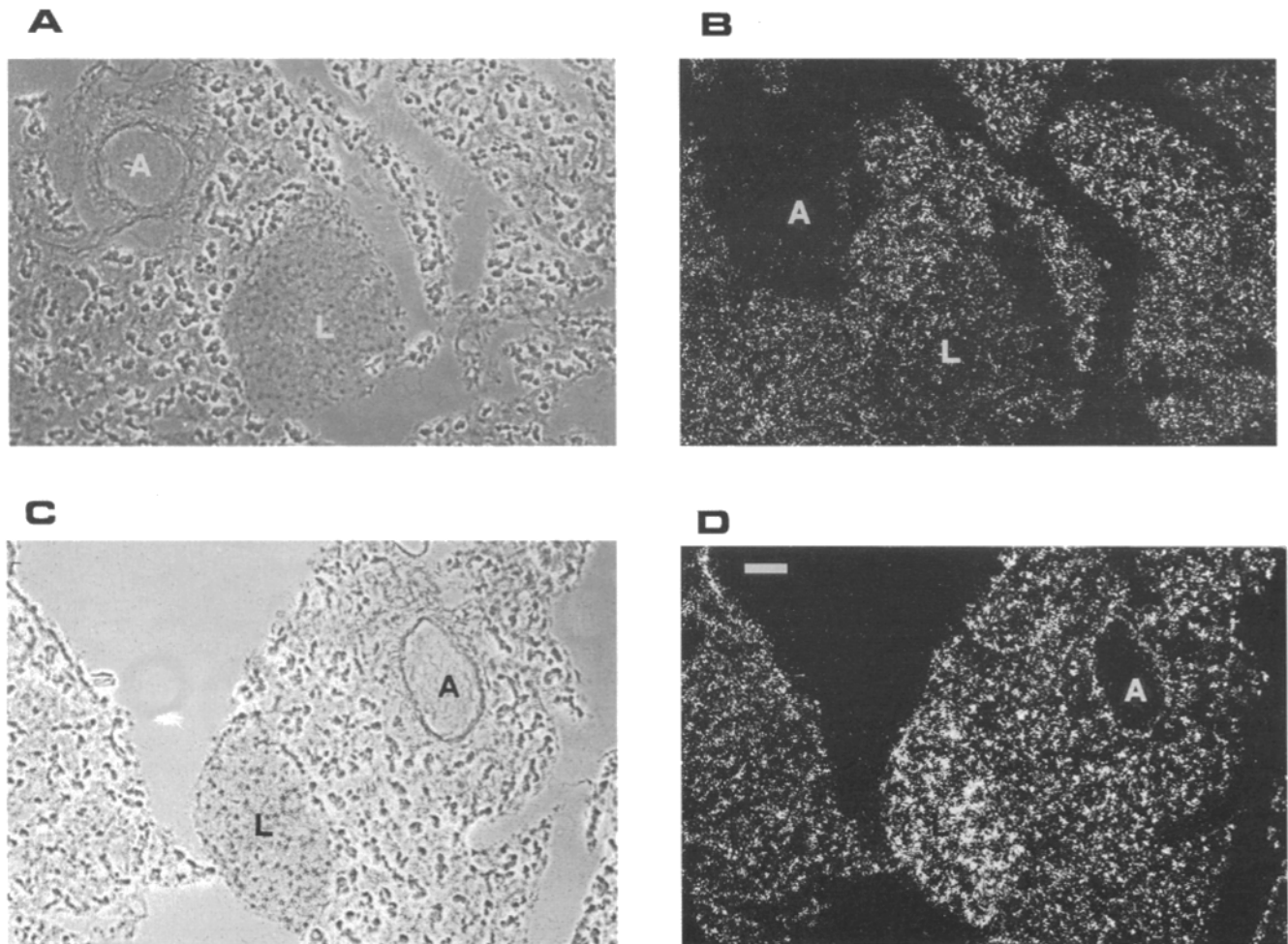
Figure 3. In situ hybridization of pancreas with antisense VAMP-1 and -2 cRNA probes. Phase contrast (A and C) and dark-field (B and D) microphotographs of in situ hybridization with antisense [^{35}S-cRNA] probes of VAMP-1 (A and B) and VAMP-2 (C and D) of pancreas. A, artery; L, islet of Langerhans. Bar, 50 μm.
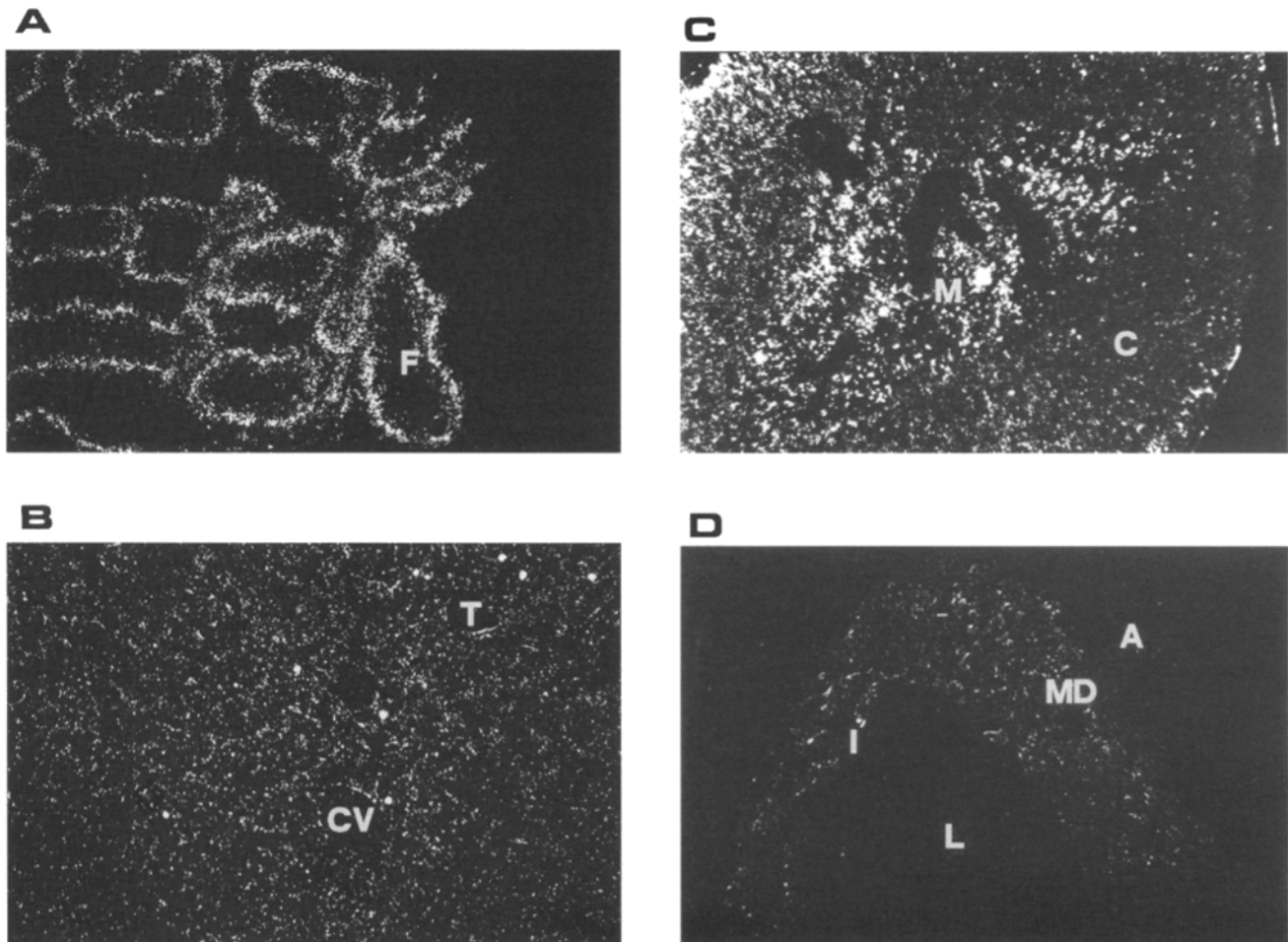
VAMP-1

Figure 4. In situ hybridization of thyroid, liver adrenal gland and aorta with antisense VAMP-1 and -2 cRNA probes. Dark-field microphotographs of in situ hybridization with antisense [^{35}S-cRNA] probes of VAMP-1 (*A–D*) and VAMP-2 (*E–H*) of thyroid (*A* and *E*), liver (*B* and *F*) adrenal gland (*C* and *G*) and aorta (*D* and *H*). *F*, folliculum; *CV*, central vein; *T*, Glisson trias; *M*, medulla; *C*, cortex; *L*, lumen; *I*, intima; *MD*, media; *A*, adventitia. Bars: (*A, B, D, E, F*, and *H*) 50 μm; (*C* and *G*) 150 μm.

VAMP Proteins Are Present in Different Amounts in Nonneuronal Tissues

The presence and tissue distribution of the two "neuronal" VAMP isoforms was assayed with antibodies raised in rabbits against the 1-33 amino-terminal part of VAMP. This part of the VAMP molecule is not conserved among species and isoforms. After affinity purification on antigen-bound columns, these antibodies showed no cross-reaction among recombinant full-length rat brain VAMP-1 and VAMP-2 produced as fusion protein with glutathione *S*-methyltransferase (Fig. 5, *A* and *B*). Alternatively, isoform specificity was probed in Western blots on small synaptic vesicles, isolated from rat brain cortex (Hüttner et al., 1983), where the two VAMP isoforms were differentiated on the basis of their slightly different electrophoretic mobility (not shown). These anti–VAMP-1 and VAMP-2 antibodies do not recognize cellubrevin because they do not stain protein bands of electrophoretic mobility higher than those of VAMP-1 and VAMP-2 in any of the tissues tested.

Fig. 5 shows that these antibodies recognize rat brain VAMP isoforms in all the tissues tested. From their elec-

trophoretic mobility, it appears that the VAMP proteins of the various tissues have very similar sizes, close or identical to that of neuronal VAMPs. Slight differences in electrophoretic mobility between the brain VAMPs and the other tissue VAMP bands is most likely due to the higher and different lipid content of brain, which includes gangliosides and cerebrosides. This result is in full agreement with the recent cloning and sequencing of the rat pancreas VAMP-2 gene (Regazzi et al., 1995), which was found to be identical to the neuronal one (Elferink et al., 1989). The amount of VAMP-1 and -2 isotypes in the various tissues, as judged from the intensity of immunostaining, correlates with the level of expression of their respective mRNAs, reported in Fig. 1.

Immunofluorescence Analysis of the Distribution of VAMP Proteins in Different Tissues

The distribution of VAMP-1 and -2 proteins within each tissue was studied by immunofluorescence of cryosections with isoform specific antibodies. Fig. 6 *A* shows that VAMP-1 is present in the exocrine cells of pancreas and is

VAMP-2

E
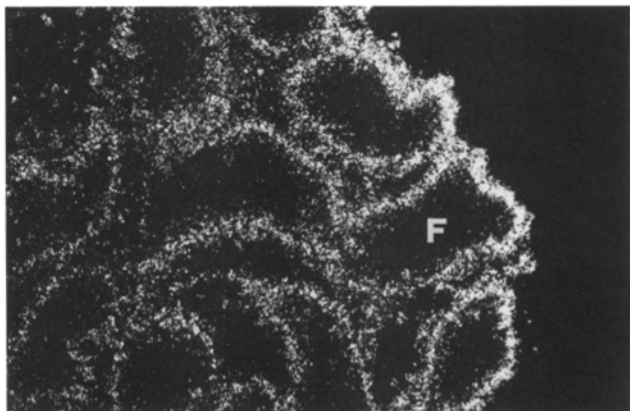
G

F

H

below detection in the endocrine portion. On the other hand, VAMP-2 is present both in endocrine and exocrine pancreas, but is predominant in the Langerhans islets of adult rat pancreas (Fig. 6 *B*). This latter result correlates with the presence of VAMP on zymogen granules of exocrine pancreas (Braun et al., 1994; Gaisano et al., 1994). Moreover, it is fully consistent with the fact that only VAMP-2 and cellubrevin, but not VAMP-1 genes were id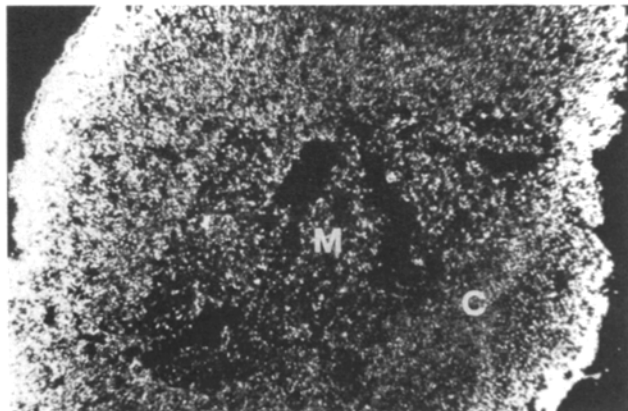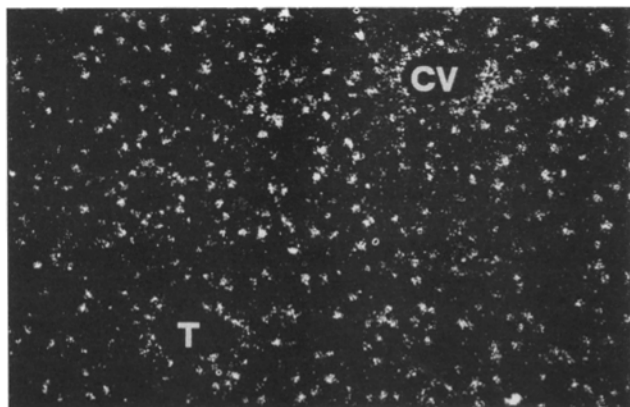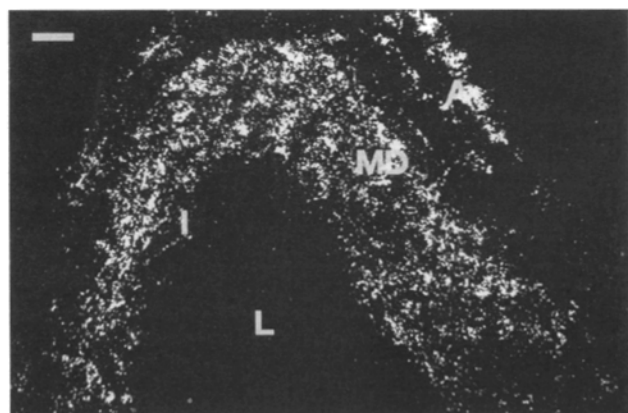entified in a pancreatic β-cell derived c-DNA library (Regazzi et al., 1995). The differential distribution of the two neuronal VAMP isoforms is much more evident than that assumed from the in situ hybridization experiments of Fig. 3. In kidney, VAMP-1 stains the tubular cells and is absent from glomeruli, while VAMP-2 is present in both tubular and glomerular cells (Fig. 6, *C* and *D*). Panel *F* of Fig. 6 shows that VAMP-2 is concentrated in the medullar part of the adrenal gland, whereas VAMP-1 stains more the cortical than the medullar portion. When preimmune antisera were used there was no fluorescent staining. This is also the case when VAMP-1 or VAMP-2 amino-terminal peptides were used as competition to the purified antibodies. In pancreas, kidney, and adrenal gland there appears to be no close correlation between the level of VAMP-1 and -2 mRNA and protein expression. This is not unprecedented since it has been detected before for different mRNA species (Gorza et al., 1993; Moschella et

al., 1995; Kelly et al., 1995). Such discrepancies may be accounted for by a different stability of VAMP-1 and -2 mRNA and/or proteins in different cell types. Moreover partial cross-hybridization of the full-length cRNA probes and different posttranslational regulation of the two VAMP isoform mRNAs may also be involved.

Intracellular Distribution of VAMP-1 and -2

The intracellular distribution of neuronal VAMP proteins was studied by confocal immunofluorescence microscopy on rat insulinoma cells (RIN), rat normal kidney cells (NRK), and rat pheochromocytoma cells (PC12). Cells were fixed, permeabilized, and stained with affinity purified rabbit antibodies anti–VAMP-1 or anti–VAMP-2. Fig. 7 shows that in RIN cells VAMP-2 staining has a spotty distribution in the entire cytosol (*b*), whereas VAMP-1 is absent (*a*) in agreement with the data obtained in pancreas. At variance, in NRK cells both VAMP isoforms are present and they show a vesicular-type staining (*c* and *d*).

Fig. 8 shows that in undifferentiated PC12 cells, VAMP-1 gives a fluorescent signal much lower than that of VAMP-2, whose amount differs in individual cells of the same culture (*b*), though it shows in each cell a spotty distribution within the cytosol. Neuronal differentiation of these cells is accompanied by a large increase in the amount of

Figure 5. Western blot of recombinant GST–VAMP-1 and GST–VAMP-2 fusion proteins and tissue homogenates with affinity purified anti–VAMP-1 and anti–VAMP-2 polyclonal antibodies. Isoform specific polyclonal antibodies were raised in rabbits and purified by affinity chromatography. The specificity of these two antibodies were tested by immunoblot of 5 μg of recombinant GST–VAMP-1 and -2 fusion proteins. Different tissue homogenates were treated as detailed in Materials and Methods, electrophoresed, blotted, and revealed with rabbit affinity purified anti–VAMP-1 (*A*) and anti–VAMP-2 (*B*) (brain, *1*; kidney, *2*; adrenal gland, *3*; liver, *4*; heart atrium, *5*; heart ventricle, *6*; pancreas, *7*; thyroid, *8*). The high amount of proteins loaded on the gel leads to a distortion of the VAMP bands and may slightly affect also electrophoretic mobility.

VAMP-1 and in the appearance of intense staining of both VAMP-1 and -2 in synaptic terminals (panels *c* and *d* of Fig. 8), identified by the presence of synaptophysin, a protein specific of synaptic vesicles (panels *e* and *f* of Fig. 8).

Discussion

Docking and fusion of vesicles with target membranes within the cell is mediated by a set of proteins conserved from yeast to mammals (Rothman and Orci, 1992; Bennett and Scheller, 1994; Rothman and Warren, 1994; Ferro-Novick and Jahn, 1994). On the basis of their topological localization, they can be divided into three groups: proteins located on the vesicle, proteins of the target membrane, and cytosolic proteins. These proteins form a multi-subunit complex of 20 S that assembles sequentially with a preliminary involvement of VAMP, syntaxin, and SNAP-25 (Söllner et al., 1993a,b; Rothman and Warren, 1994). Its synaptic vesicle location suggested that VAMP might play a major role in vesicle recognition of the target membrane via interactions with the presynaptic membrane proteins SNAP-25 and syntaxin. The identification of several syntaxin isoforms and of the nonneuronal VAMP isoform cellubrevin lend support to this view. On this basis, it was anticipated that vesicular trafficking among different intracellular compartments would be mediated by organelle and/or tissue specific isoforms (Bennett et al., 1993; Kelly, 1993b; Söllner et al., 1993b; Warren, 1993; Rothman and Warren, 1994).

So far three VAMP isoforms have been characterized in mammals: two neuronal isoforms (VAMP-1 and -2) and one nonneuronal isoform (cellubrevin). Previous studies of distribution of VAMP-1 and -2 within the rat central nervous system with RNA probes showed that VAMP-1 expression is mainly restricted to cells involved in somatomotor functions and to cells of the spinal cord, whereas VAMP-2 is more widely expressed and present in nuclei associated with autonomic, sensory, and integrative roles (Elferink et al., 1989; Trimble et al., 1991). Another study employed an anti-VAMP monoclonal antibody and found VAMP in the nerve and neuro-endocrine cells, but not in other tissues (Baumert et al., 1989). This result is possibly due to a nonconservation of the epitope recognized by the monoclonal antibody used. Other studies have found that neuronal VAMP isoforms are expressed in the rat adipose tissue (Corley-Cain et al., 1992), in the gut and Malpighian tubules of *D. melanogaster* (Chin et al., 1993) and in the exocrine pancreas (Braun et al., 1994).

Here, the presence and distribution of both neuronal VAMP isoforms among various rat tissues are clearly documented with different experimental approaches. These proteins are highly expressed in tissues with a high secretory activity such as thyroid and pancreas, and, in lower proportion, in all tissues tested including liver and smooth muscle cells. It was suggested that VAMPs are absent in chromaffin cells (Stecher et al., 1989; Höhne-Zehl et al., 1993), but the results reported here demonstrate that they are indeed expressed in these cells.

Figure 6. Light immunofluorescence microscopy of cryosections of rat pancreas, kidney, and adrenal gland with isoform specific VAMP antibodies: fluorescence images of cryosections of adult rat pancreas (*A* and *B*), kidney (*C* and *D*) and adrenal gland (*E* and *F*) stained with affinity purified anti–VAMP-1 (*A, C,* and *E*) or anti–VAMP-2 (*B, D,* and *F*). *L*, islet of Langerhans; *G*, glomerula; *M*, medulla; *C,* cortex. Bar, 50 μm.
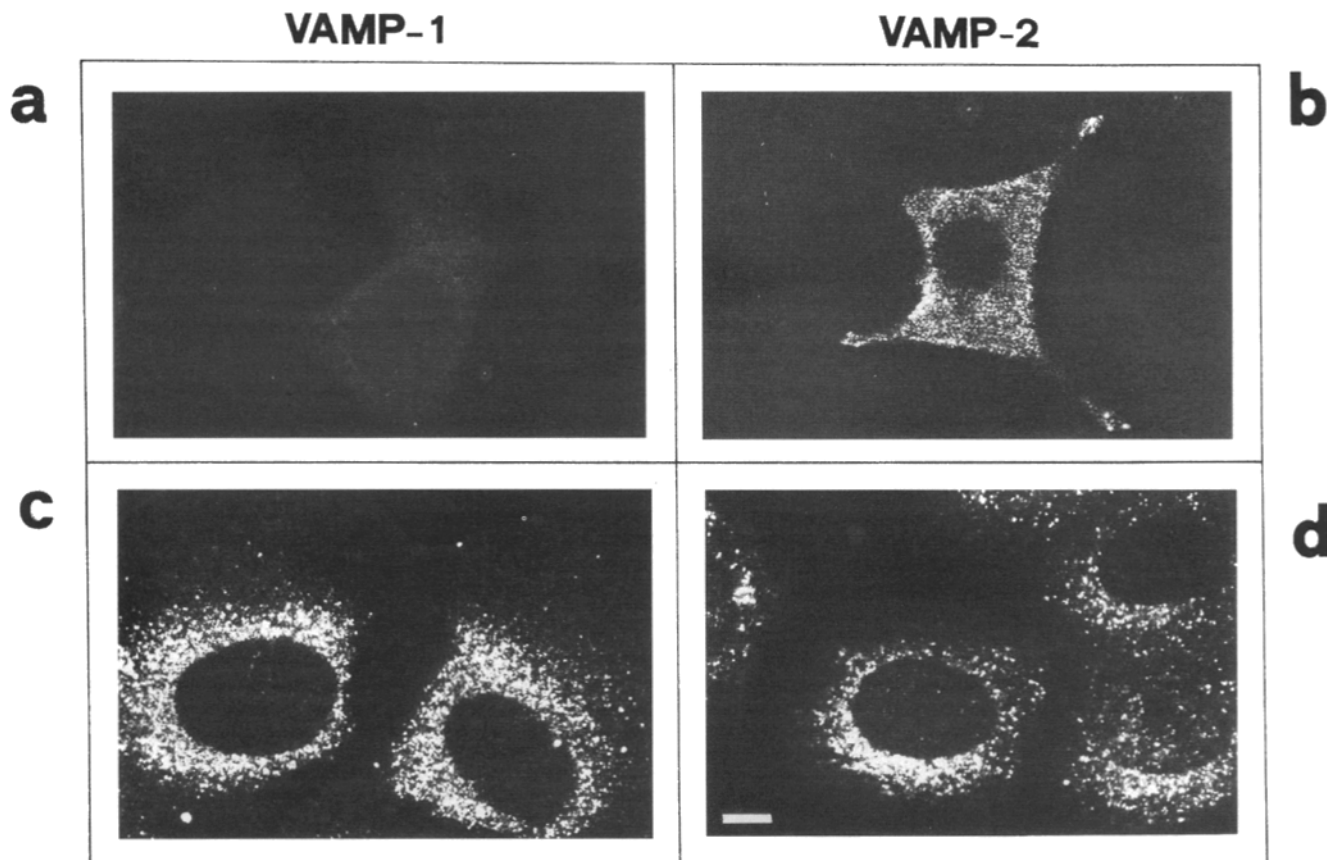
VAMP-1   VAMP-2

Figure 7. Confocal immunofluorescence microscopy of RIN (*a* and *b*) and NRK (*c* and *d*) cell lines: stained with anti–VAMP-1 (*a* and *c*) and with anti–VAMP-2 (*b* and *d*). Bar, 5 μm.

There are clear differences in the distribution of the two neuronal VAMP isoforms among different areas of various tissues. The most striking case is that of pancreas, with VAMP-1 protein present in the exocrine portions and VAMP-2 being predominantly expressed in the endocrine parts. Though unexplained at the present time, these differences may reflect different functionalities of the exocytotic complexes which include the different VAMP isoforms, in terms of the kinetics of the process, calcium dependency, and molecules to be secreted. In this respect, it is noteworthy that only VAMP-2 appears to be present in the 20-S particle isolated from bovine brain in a system that secretes small molecules very rapidly (Söllner et al., 1993a; Horikawa et al., 1993). We have recently found that only VAMP-2, but not VAMP-1, forms a complex with synaptophysin and that this interaction is mediated by the amino-terminal domain (Washbourne et al., 1995).

From the present results, it appears that the VAMP isoforms detected in the various tissues are identical to the neuronal VAMP isoforms: (*a*) gene-specific probes at high stringency were used; (*b*) equal size of mRNA protected fragment were detected in the RNase digestion assay; (*c*) tissues were immunostained with affinity purified antibodies raised vs the only VAMP portion shown to vary among isoforms and species. This indicates that the amino-terminal part of the tissue VAMPs has to be closely similar to those of the neuronal isoforms; and (*d*) the electrophoretic

mobility of the VAMPs is identical in nonneuronal tissue and the difference with brain is to be attributed to a lipid shift effect. The first cloning of a VAMP from a nonneuronal tissue (insulinoma cells) has shown an identity to brain VAMP-2, extended to the noncoding flanking region of VAMP-2 (Regazzi et al., 1995).

The limited repertoire of VAMP proteins, the apparent presence of only two isoforms in some secretory cells and the ubiquity of VAMP proteins with closely similar, if not identical, amino-terminal portions which is the VAMP region expected to be associated with a specific recognition role, raise the possibility that VAMPs are not the only proteins responsible for targeting of vesicle to the plasma membrane. The apparent docking of SSV in synapses intoxicated with tetanus neurotoxin which cleaves specifically VAMP (Mellanby et al., 1988; Neale, 1989; Hunt et al., 1994) can be attributed to vesicle recognition of active zones of the presynaptic membrane mediated by other proteins. Proteins that may be involved in docking small synaptic vesicles are synaptotagmin (Söllner et al., 1993b) and the cysteine string proteins (Zinsmaier et al., 1994; Mastrogiacomo et al., 1994), shown to bind to the Ca^{2+} channels of the presynaptic membrane. Moreover, though small synaptic vesicles are one of the most characterized organelles, other vesicle proteins may be involved in target membrane recognition. Available evidence indicates that VAMP forms a hetero-trimeric complex with syntaxin and
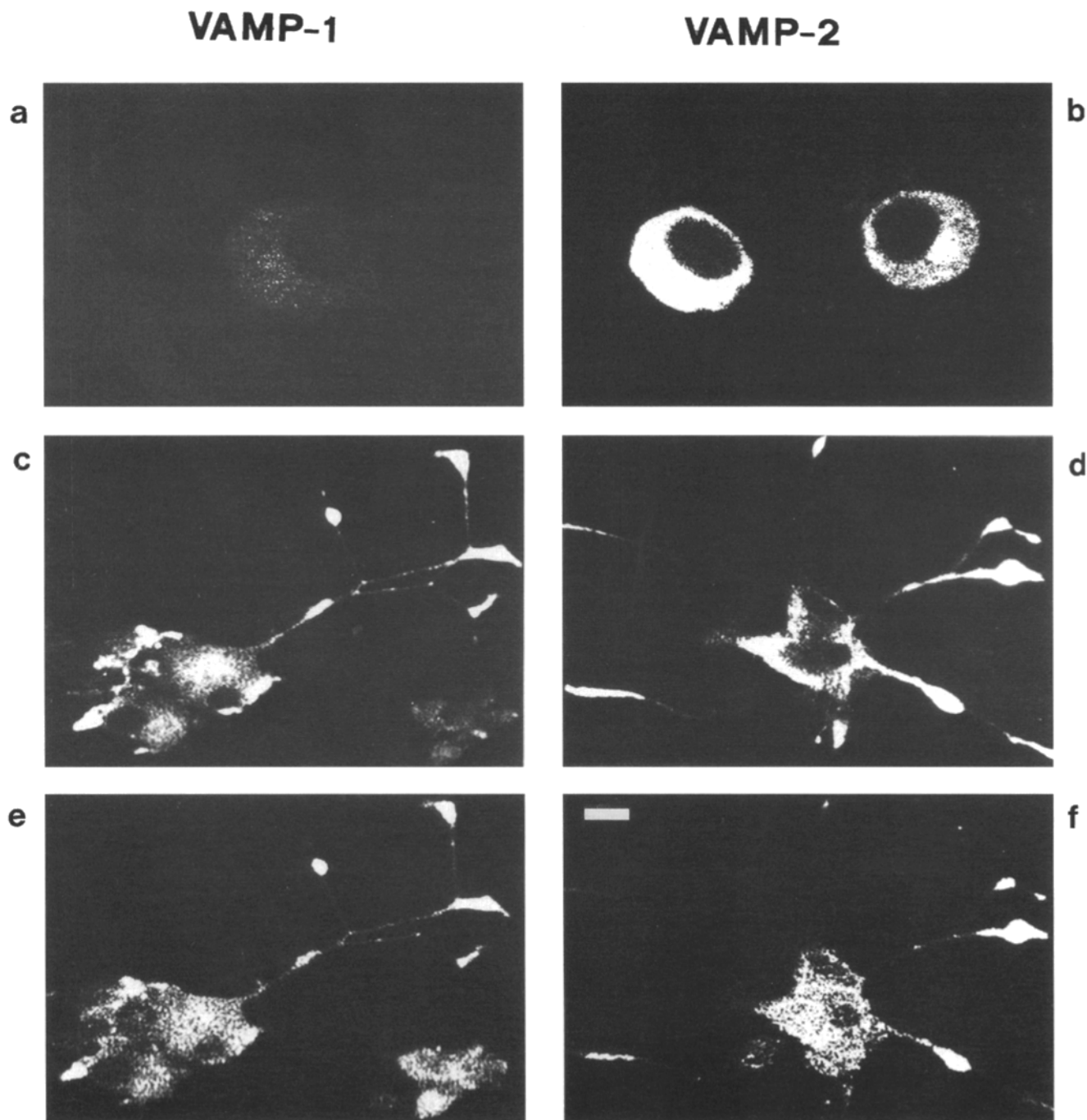
VAMP-1

VAMP-2

Figure 8. Confocal immunofluorescence microscopy of PC12 cells undifferentiated (*a* and *b*) or differentiated with NGF (*c, d, e,* and *f*) stained with anti–VAMP-1 (*a*), with anti–VAMP-2 (*b*), or double stained with anti–VAMP-1 and synaptophysin (*c* and *e,* respectively) or anti–VAMP-2 and synaptophysin (*d* and *f,* respectively). Bars: (*a* and *b*)5 μm; (*c, d, e,* and *f*) 10 μm.

SNAP-25 that constitutes the core-nucleus of the neuroexocytosis complex (Hayashi et al., 1994; Chapman et al., 1994).

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