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Characterization of a GTP-binding protein implicated in both memory storage and interorganelle vesicle transport

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ABSTRACT **The phosphorylation state of cp20, a low molecular weight GTP-binding protein that is a high-affinity substrate for protein kinase C, was previously shown to change after associative conditioning of molluscs and mammals and to induce many of the biophysical and structural modifications that accompany memory retention. Here, cp20 was purified from squid optic lobes and biochemically characterized. A monoclonal antibody prepared against squid cp20 reacted with *Hermissenda* cp20 and a 20-kDa protein in rabbit hippocampus, while a polyclonal antibody also cross-reacted with Sar1p and ADP-ribosylation factor (ARF). A partial peptide sequence of squid cp20 was 50% identical (23/46 amino acids) with Sar1p, a yeast GTP-binding protein involved in vesicle transport, indicating that cp20 is probably a new member of the ARF family. This classification is consistent with our recent demonstration that cp20 affects retrograde movement of intraaxonal organelles or particles and suggests a possible role for particle traffic between intraneuronal organelles in memory acquisition.**

Associative conditioning in *Hermissenda*, involving paired light-rotation events, results in a 2- to 3-fold increase in the phosphorylation state of cp20, a low-abundance (<0.1% of total protein) 20-kDa eye phosphoprotein (1, 2), determined by several criteria to be a 20-kDa GTP-binding protein (2, 3). cp20 is also a high-affinity substrate for protein kinase C (4), which has also been implicated in memory storage (5). cp20 is a potent inhibitor in *Hermissenda* of the potassium currents I_A and $I_{K+Ca^{2+}}$, which decrease following conditioning. Purified cp20 also may inhibit axonal transport (6) and has structural effects that resemble changes that occur after associative learning (7). Thus, although levels of a few other proteins have been found to change in other types of learning (8–11), cp20 is, to our knowledge, the first protein to be causally implicated in cellular changes with molluscan or mammalian associative learning. Until now, only low nanogram amounts of cp20 have been available for study. cp20 has now been purified in microgram quantities from squid optic ganglia, characterized, and partially sequenced. A monoclonal antibody (mAb) against squid cp20 reacted with *Hermissenda* cp20 and also a 20-kDa protein in rabbit hippocampus. Polyclonal antibodies against cp20 also cross-reacted with ADP-ribosylation factor (ARF) and Sar1p. The partial peptide sequence of cp20 from squid optic lobe was 50% identical (23/46 amino acids) with Sar1p, a yeast GTP-binding protein that is a member of the ARF family.

METHODS

Animal Tissue. Optic lobes from fresh squid (*Loligo pealei*; Calamari, Inc., Woods Hole, MA) were dissected, frozen in liquid nitrogen, and stored at −80°C. *Hermissenda crassicornis* were obtained live from Sea Life Supply, Sand City, CA.

Purification of cp20. One hundred fifty squid optic lobes were added to 100 ml of buffer (10 mM Tris·HCl, pH 7.4/20 μg of leupeptin per ml/20 μg of pepstatin per ml/50 mM NaF/1 mM EDTA/1 mM EGTA). Phenylmethylsulfonyl fluoride and dithiothreitol (DTT) were added to 0.1 mM and 200 mM, respectively, and the optic lobes were homogenized at 4°C in a high-speed homogenizer followed by sonication. The homogenate was centrifuged (100,000 × *g*, 90 min) and the supernatant was filtered through a 0.22-μm filter and passed through an Amicon filter (30-kDa cutoff). The low molecular weight fraction was then concentrated on a second filter (3-kDa cutoff) followed by concentration to 100 μl in a Centricon concentrator pretreated with bovine serum albumin. Use of untreated Centricon concentrators led to complete loss of protein.

The retained fractions were injected onto an AX-300 anion-exchange HPLC column (1 × 25 cm, Synchrom, Lafayette, IN). The column was eluted at 2 ml/min and 10°C with a gradient of 0–0.6 M buffer (1 M KOAc, pH adjusted to 7.4 with HOAc) for 20 min followed by 0.6 M buffer for 40 min. Each chromatogram was statistically analyzed by creating a correlation curve with the retention time (t_R) of each peak plotted against the t_R of all peaks in a reference chromatogram, a chromatogram of proteins from five eyes dissected from a group of *Hermissenda* conditioned in a previous experiment, as described (2). A candidate cp20 peak was considered to match only if its t_R fit within ±0.2% to the expected t_R and if 10 or more other peaks could also be matched with the same precision. If the cp20 peak could not be unequivocally identified, or a unique correlation curve could not be constructed, the preparation was discarded. Fractions were collected in polypropylene tubes containing Triton X-100 at a final concentration of 0.2 mM.

A portion of each HPLC fraction surrounding the final cp20 peak was analyzed by SDS gel, blotted, stained with colloidal gold (CG), and enhanced with silver (IntenSE BL, Amersham). If densitometry of the blot indicated <85% purity, the preparation was repurified or discarded.

Cation-Exchange HPLC. In several experiments, the cp20 was further purified by cation-exchange HPLC (S-300, 4.6 × 250 mm, Synchropak). The column was eluted at 0.5 ml/min for 10 min with 0.02 M LiCl (pH 6.0) followed by a gradient of 0.02–0.7 M LiCl over 60 min. Each fraction was analyzed for GTPase and analyzed by SDS gel. Some samples were analyzed by CM-300 HPLC (Synchropak) with a gradient of 0–1 M KOAc over 30 min.

Reversed-Phase HPLC (RP-HPLC). The C_{18} column (Macrosphere 300, 5 μm) was eluted at 0.35 ml/min with 20–100%

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Abbreviations: t_R, retention time; DTT, dithiothreitol; ARF, ADP-ribosylation factor; mAb, monoclonal antibody; RP-HPLC, reversed-phase HPLC; CG, colloidal gold.

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acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) over 90 min followed by 100% ACN/0.1% TFA for 90 min.

GTPase was measured as described (2).

Peptides and proteins were quantitated using CG reagent (Aurodye, Amersham) (12) as modified in ref. 2.

Photoaffinity Labeling. Samples were incubated in a closed 0.5-ml tube for 30 min at 25°C with [α-^{32}P]GTP, irradiated with UV light, and analyzed by SDS gels as described (13) followed by autoradiography.

mAbs. cp20 from 20 squid optic lobes was injected into mouse spleen. The spleen lymphocytes were fused with mouse myeloma cells X63-AG8-653. Hybridoma cells were selected by ELISA using plates coated with optic lobe extract. The hybridoma was cloned by limiting dilution and cultivated in serum-free medium. The IgM fraction was purified by precipitation with $(NH_4)_2SO_4$ and dialyzed against phosphate-buffered saline.

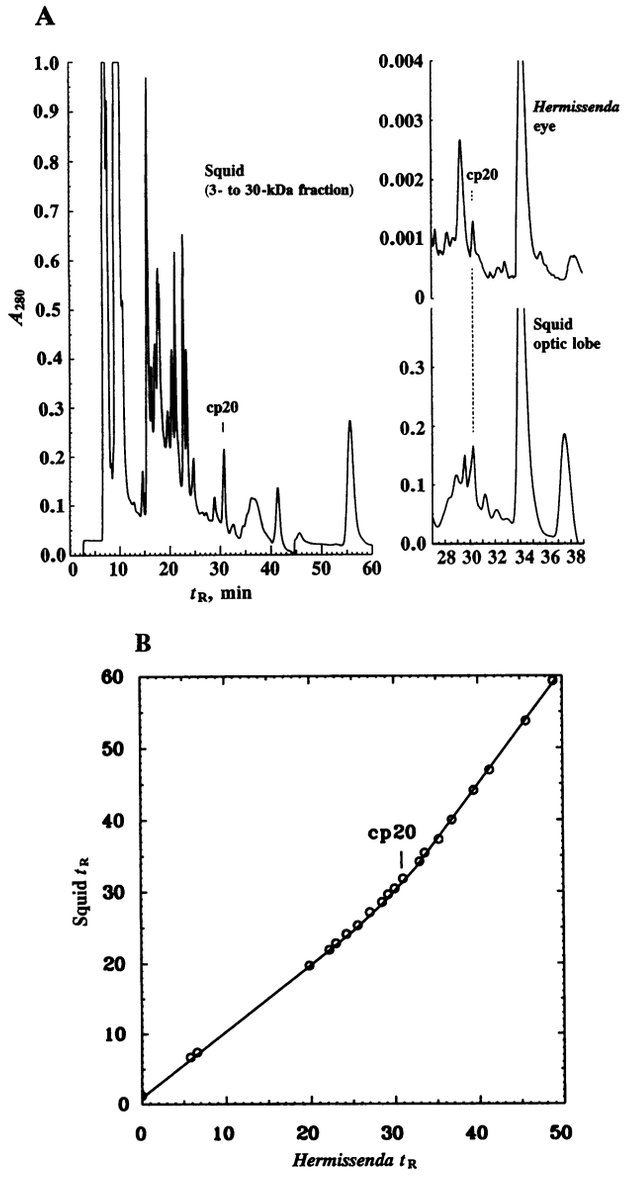

FIG. 1. (*A*) A_{280} HPLC tracings of proteins from *Hermissenda* eye, squid optic lobe, and squid 3- to 30-kDa fraction. Thirty-six eyes from *Hermissenda* trained to associate light and rotation or 1/10 squid optic lobe were analyzed by anion-exchange HPLC. In unconditioned *Hermissenda*, the cp20 peak is three to four times smaller than the cp20 peak from conditioned animals shown here. (*B*) Correlation curve of t_R values from HPLC tracing from squid optic lobe proteins vs. t_R values from reference chromatogram of proteins from trained *Hermissenda* eye.

RESULTS

Because a single *Hermissenda* central nervous system contains only 8 μg of total protein and subnanogram quantities of cp20, it was necessary to use a different source (squid optic lobe) in order to obtain adequate quantities of cp20 for characterization. Computer-assisted pattern matching of the HPLC profiles demonstrated that the HPLC profiles of cytosolic proteins from squid optic lobe and *Hermissenda* eye were quite similar (Fig. 1), with the exception of the cp27 peak (29.5 min), which was much smaller in squid than *Hermissenda*, and two or three other peaks, which were larger in squid.

To determine whether the AX-300 HPLC column adequately separates G proteins, squid homogenate was chromatographed on AX-300 and the molecular weights of all GTPases were determined. Eighty four percent of the GTPase activity from squid eluted in large unresolved peaks at 12–18 and 19–21 min. Ras, Rap, and Sar1p, measured by Western blotting of HPLC fractions, eluted at 22.8, 20.5, and 19.4 min, respectively (not shown). Thus, the HPLC column was highly efficient at separating cp20 (t_R, 30 min) from other GTP-binding proteins. Interestingly, no G proteins were detected in the large nonretained peak (6–10 min) (see Fig. 1*A*).

To test the purity of the cp20, squid cp20 was reanalyzed by RP-HPLC. After the large nonretained peak caused by DTT and salts, a single peak was observed (Fig. 2). Its GTPase activity was difficult to measure, presumably due to the harsh conditions (100% ACN/0.1% TFA). No activity was seen at other positions. The t_R is comparable to that seen previously with cp20 from *Hermissenda* eye and central nervous system (2).

cp20 from both squid optic lobes and *Hermissenda* central nervous system was also rechromatographed by S-300 and CM-300 cation-exchange HPLC (Fig. 3 *A* and *B*). Each fraction was tested for GTPase activity and analyzed on SDS gels. In both cases, two peaks of GTPase activity were detected, with molecular weights of 20 and 40 kDa, suggesting a homodimeric structure. In a similar experiment, cp20 purified in the absence of DTT was fractionated on a nondenaturing gel. When the 40-kDa section of the gel was

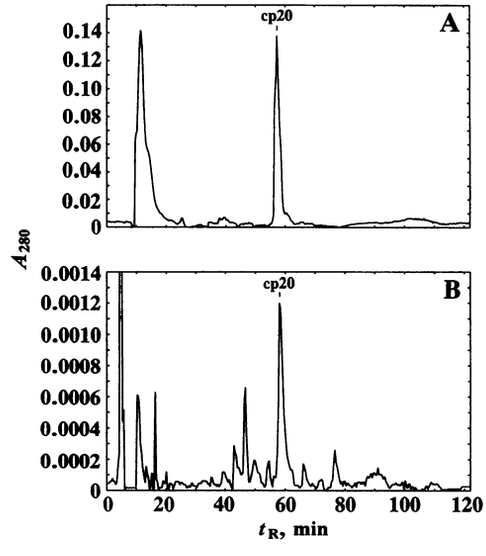

FIG. 2. (*A*) RP-HPLC A_{280} profile of purified squid cp20. The peak at 15 min is the non-retained fraction, containing DTT and buffer components. (*B*) RP-HPLC rechromatography of a cp20 peak from one *Hermissenda* CNS from an earlier experiment (2). Peaks at 4, 12, 15, 42, 46, and 78 min are buffer components. Flow rate, 0.5 ml/min.

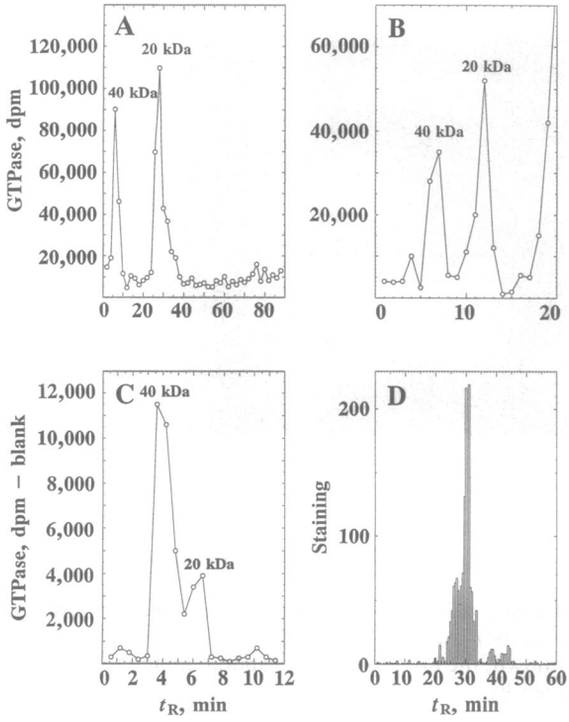

FIG. 3. S-300 (*A*) and CM-300 (*B*) cation-exchange HPLC GTPase profiles of purified squid cp20. Half of each fraction was analyzed for GTPase activity and half was analyzed on SDS gels. After 18 min in *B*, the GTPase baseline increased dramatically due to interference in the assay by the HPLC solvent. (*C*) GPC-100 size-exclusion HPLC GTPase profile of squid cp20 purified in the absence of DTT. By this stage, most of the cp20 has dimerized. (*D*) Specificity of anti-cp20. Supernatant from 10 *Hermissenda* central nervous systems was applied to an AX-300 column. Each fraction was blotted, allowed to react with mouse anti-cp20, and developed with alkaline phosphatase/5-bromo-4-chloro-3-indolyl phosphate. The blot was scanned, converted to OD, and integrated by computer. The large peak at 31 min coincided with the cp20 peak in the A_{280} profile.

eluted, allowed to react with DTT, and analyzed by SDS/PAGE, a 20-kDa band was observed. In contrast, in the absence of DTT, only a 40-kDa protein band was observed (Fig. 4, lanes A and B). Thus, the 40-kDa protein is not an impurity, but dimerized cp20.

Further evidence of dimerization was obtained by photoaffinity-labeling the 20- and 40-kDa peaks with [^{32}P]GTP and analyzing by SDS/PAGE (Fig. 3*C*). ^{32}P-labeled bands of 40 and 20 kDa were found in the lanes corresponding to both the 40- and 20-kDa HPLC peaks (not shown). Thus, the 40-kDa band is not an artifact of photolabeling but is caused by natural dimerization. However, it is not yet known whether dimerization occurs under physiological conditions.

A mAb prepared against purified squid cp20 also recognized 20-kDa and 40-kDa bands in squid supernatant and a 20-kDa band in *Hermissenda* (Fig. 4, lanes D and E). The proportion of staining at 40 kDa increased if the samples were allowed to stand at 4°C before analysis. Despite the fact that the antibody was raised against squid protein, it reacted more strongly with *Hermissenda* cp20. cp20 was also detected in rabbit hippocampus particulate fraction, but not in the supernatant (Fig. 4, lanes F and G).

Western blots of HPLC fractions from *Hermissenda* supernatant revealed a large peak at 31 min coinciding with cp20, and a smaller peak at 28 min, possibly the dephosphorylated form of cp20 (Fig. 3*D*).

Squid cp20 did not cross-react with pan-Ras, anti-ARF, or anti-Rap mAbs (not shown). cp20 weakly cross-reacted with anti-$G_{i\alpha}$, an antibody against the GTPase active site (14) (Fig.

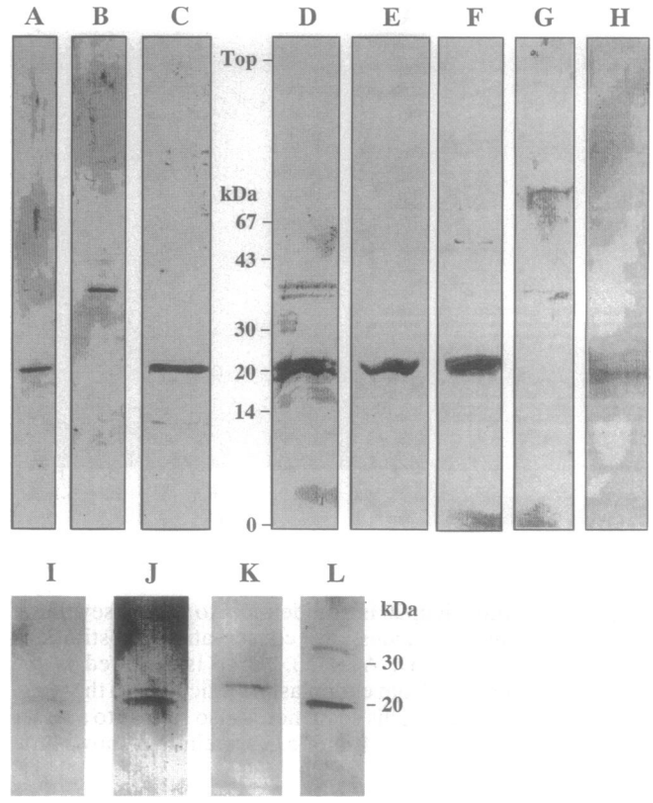

FIG. 4. Lanes A and B, interconversion of the 20-kDa and 40-kDa forms of cp20 by DTT. cp20 purified by anion-exchange HPLC in the absence of DTT was fractionated on a nondenaturing gel. The 40-kDa region of the gel was eluted, allowed to react with DTT (lane A) or water (lane B), and analyzed by SDS/PAGE. Lane C, SDS gel of purified squid cp20. Lanes D–G, Western blots of squid supernatant (lane D), *Hermissenda* supernatant (lane E), and rabbit hippocampus particulate (lane F) and supernatant fraction (lane G) reacted with anti-cp20 mAb. Lane H, Western blot of cross-reaction of purified squid cp20 with anti-$G_{i\alpha}$. (Staining: lanes A–C, CG; lanes D–G, alkaline phosphatase/5-bromo-4-chloro-3-indolyl phosphate; lanes H–L, horseradish peroxidase/diaminobenzidine.) Lanes I–L, Western blots of ARF (lanes I and J), yeast Sar1p (lane K), and squid cp20 (lane L) reacted with anti-cp20 polyclonal antibody (Staining, horseradish peroxidase/diaminobenzidine). Lane J has been contrast-enhanced to more clearly show the ARF band in lane I.

4, lane H). This antibody did not cross-react with a sample of cloned Ras, suggesting that cp20 is more closely related to the trimeric G proteins than to Ras.

A polyclonal antibody against the largest tryptic peptide of cp20 (t_R = 40 min in Fig. 6) cross-reacted with cp20 and Sar1p and weakly cross-reacted with cloned ARF, but not with Ras (Fig. 4, lanes I–L), also consistent with the conclusion that cp20 is more closely related to ARF family proteins than to Ras.

Using the ability of DTT to convert cp20 into monomers, it was possible to purify cp20 to apparent homogeneity with two ultrafiltration steps followed by a single HPLC column step (Figs. 4, lane C, and 5*A*). The stoichiometry of [^{32}P]GTP binding to purified squid cp20 in several preparations ranged from 0.90 to 0.95, indicating that the protein was 90–95% pure. The protein, when pure, adsorbed to concentrators and polypropylene test tubes unless Triton X-100 was added. The pI of squid cp20 was 5.2 by electrophoresis and 5.86 by chromatofocusing. *Hermissenda* cp20 was identical to squid in both molecular weight and pI (Fig. 5*B*).

Sequencing of five tryptic peptides from squid cp20 revealed an overall 50% identity (23/46 amino acids) with Sar1p, a 21-kDa GTP-binding protein in the ARF family (15)

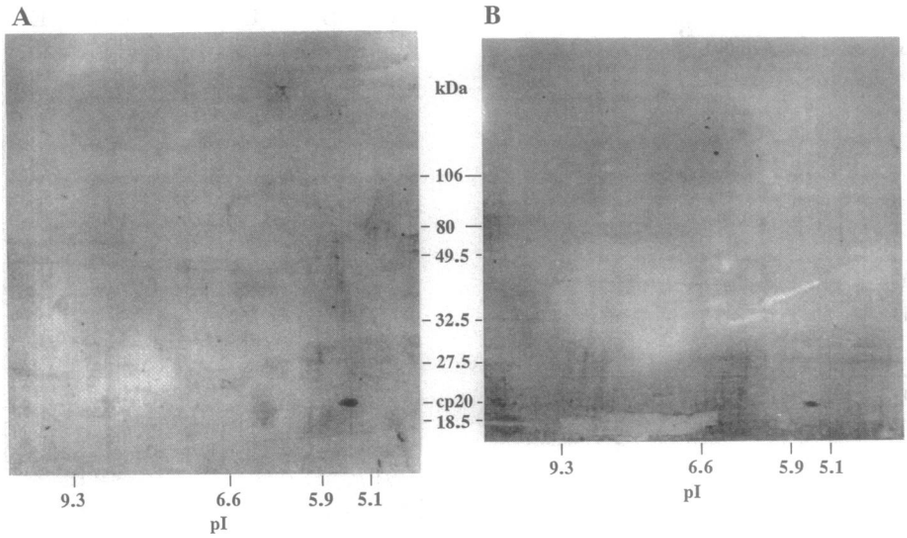

FIG. 5. Two-dimensional gel of squid cp20 (*A*) and *Hermissenda* cp20 (*B*), purified in the presence of DTT. (CG stain.)

(Fig. 6*A*). Although cp20 is not identical to Sar1p, several of the nonmatching residues are conservative substitutions (e.g., D → E, N → D, A → L). Sar1p is involved in the transport of proteins from endoplasmic reticulum to the Golgi apparatus (15, 21, 22). This sequence is also similar to a lesser degree to ARF and the $G_{i\alpha}$ trimeric G proteins but shows little similarity to Ras.

DISCUSSION

Injection of cp20 into *Hermissenda* photoreceptors causes a marked reduction of the K^+ currents I_A and $I_{K+Ca^{2+}}$, both of which are known to be reduced after associative learning (23). Injection of cp20 also reproduces the structural changes in neuronal architecture previously observed after associative learning (7).

Several other GTP-binding proteins, including Ras (24), are known to form homodimers. In squid, Rap also exists predominantly as a 46-kDa dimer (D. McPhie, personal communication). Although the complete sequence of cp20 is not yet known, the probability of a random match to our peptides is given by:

$$p_t = \prod_{i=1}^{t} \text{prob}\left\{ C\binom{n_i}{m_i}, \text{prob}[(1 + L - n_i - s_i), p^{m_i}] \right\}.$$

A derivation of this formula, and definitions of variable names, can be found in *Appendix*. This formula takes into account the increase in probability caused by the number of peptides, as well as any mismatches and insertions. For the five peptides found, the probability of a random match in a protein selected at random is 5.4×10^{-8}. Since ≈30,000 different proteins are listed in the database, the probability $p(>t)$ of a random hit in the database with the observed number of mismatches (or less) is 0.0017. Thus, the peptide sequence provides a high degree of certainty (>99.8%) that the similarity of cp20 to Sar1p is not due to chance.

Because of the strong similarity with Sar1p and ARF, cp20 probably is a member of the ARF family of low molecular weight GTP-binding proteins. In yeast, these proteins, including Sar1p, ARF, and YPT1, are involved in several steps of vesicle transport (15, 25–27). A group of low molecular weight GTP-binding proteins has also been found to be associated with rapid axonal transport (28). Thus, the similarity between cp20 and ARF-related proteins is consistent with the observed effects of cp20 on regulation of intraaxonal particle movement (6). Association with vesicle membranes is also consistent with cp20's strong retention on C_{18} HPLC, which suggests that it has a lipophilic character. It has not yet been established which of the observed physiological effects of cp20 are directly attributable to cp20 and which are mediated by some other molecule, such as protein kinase C. Ras is also able to produce some of the effects of microinjected cp20 but is only effective at much higher concentrations (29). Like cp20, ARF is more closely related to the α-subunit of trimeric G proteins than to Ras (30). The present data clearly show cp20 is not Ras but most likely a protein closely related to Sar1p and ARF.

The unambiguous classification of cp20 within a category of proteins involved in signaling and regulation of molecules between the endoplasmic reticulum and Golgi, together with its previously established impact on neuronal function and structure and its causal implication in memory storage, provide the evidence suggesting the possibility that memory

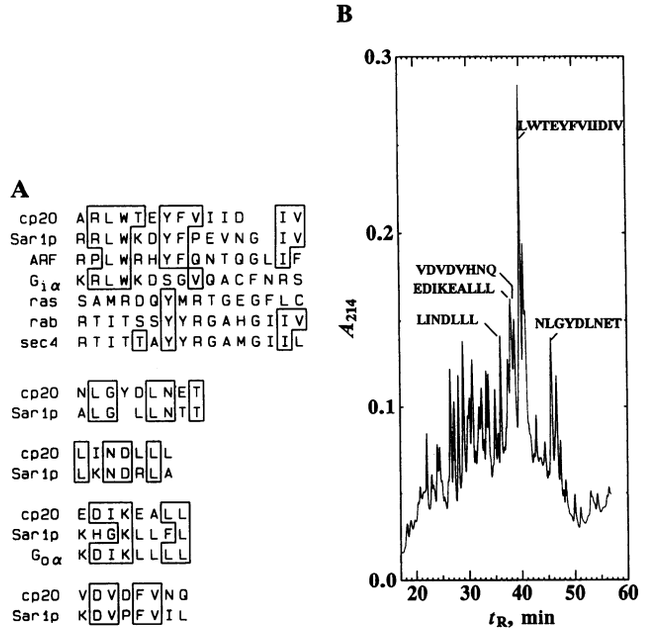

FIG. 6. (*A*) Sequence of cp20 tryptic peptides and other proteins. The top sequence is a consensus of sequences of the same peptide from three different batches of cp20. The corresponding regions in the $G_{i\alpha}$ (16), Ras (17), Rab (18), Sec4 (19), and *Drosophila* $G_{o\alpha}$ sequence (20) are shown. (*B*) RP-HPLC A_{214} profile of a tryptic digest of cp20.

storage could depend in part on regulation of particle trafficking among intraneuronal organelles.

APPENDIX

In determining the probability that a single peptide will match a region of a protein by chance, we first consider the probability of finding an exact match of a peptide at m_i fixed positions, $(1/a)^{m_i}$, where a is the number of different amino acids. This result is factored by the total number of positions at which the peptide could start—i.e., $L - n_i + 1 - s_i$. This quantity is the protein length (L) from which the quantities $n_i - 1$ and a summation term, s_i (defined below), have been subtracted. The first quantity, $n_i - 1$, where n_i is the length of the peptide in question, corrects for physically impossible cases such as the test peptide wrapping around the end of the protein. The summation term, described below, corrects for the decrease in freedom caused by matching of any previous peptides. (If there are two or more test peptides, this number is an approximation.)

These two terms are combined using the function "prob," which calculates the probability of an event with probability p occurring at least once in n independent trials:

$$\text{prob}(n, p) = 1 - (1 - p)^n. \quad [1]$$

This function has the property that as p approaches 0, prob(n, p) approaches $n \cdot p$. As n gets larger, prob(n, p) approaches 1. For example, prob(5, 0.01) = 0.04901 but prob(5, 0.5) = 0.968.

The resulting term for an individual probability (p_i) for one matching peptide, i.e.,

$$p_i = \text{prob}[(L - n_i + 1 - s_i), p^{m_i}] \quad [2]$$

(where $p = 1/a$ for equiprobable amino acids), describes the probability that a peptide of length n_i will exactly match the protein in question at m_i positions.

The s_i term mentioned above is the cumulative sum of the lengths of all peptides tested thus far, excluding the test peptide under consideration. After the probability of each peptide finding a match is calculated, its length is added to the s_i term for the next peptide.

To allow for mismatches or insertions, the number of combinations of n_i amino acids taken m_i at a time, $C(n_i\ m_i)$, must also be factored in, again using Eq. 1 instead of multiplication. Inclusion of this combinatorial term into Eq. 2 allows for up to $n_i - m_i$ mismatches:

$$p_i = \text{prob}\left\{C\binom{n_i}{m_i}, \text{prob}[(L - n_i + 1 - s_i), p^{m_i}]\right\}. \quad [2a]$$

The overall probability p_t—i.e., the probability of matching t peptides in the same protein—is the product of these probabilities for all t test peptides, i.e.,

$$p_t = \prod_{i=1}^{t} \text{prob}\left\{C\binom{n_i}{m_i}, \text{prob}[(1 + L - n_i - s_i), p^{m_i}]\right\}, \quad [3]$$

bearing in mind that at each step, the length of the protein available for matching is reduced by $n_i - 1$.

Because of the incorporation of the combinatorial term in Eq. 3, this formula does not compute the probability of an exact match somewhere in the protein, but rather computes the probability of one or more matches for the peptide in the protein at at least m_i amino acid positions. Multiple matches induced by sequence repeats (a frequent circumstance) will therefore not influence the result.

The accuracy of Eqs. 1–3 has been confirmed by extensive simulation experiments, which will be published elsewhere.

A refinement of Eq. 3 can be easily made in the case of proteins, to account for the frequency distribution of individual amino acids. Obviously, the probability of m_i matches at m_i fixed positions is equal to p^{m_i} when all amino acids have the same probability, i.e.,

$$p^{m_i} = p_{i1}p_{i2}p_{i3}p_{i4} \cdots p_{im}. \quad [4]$$

If the amino acids are of unequal frequency, these frequencies may be substituted into Eq. 4 on a point-for-point basis. A table of amino acid frequencies is required to use this formula.

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