

# AP-4, a Novel Protein Complex Related to Clathrin Adaptors\*

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**Here we report the identification and characterization of AP-4, a novel protein complex related to the heterotetrameric AP-1, AP-2, and AP-3 adaptors that mediate protein sorting in the endocytic and late secretory pathways. The key to the identification of this complex was the cloning and sequencing of two widely expressed, mammalian cDNAs encoding new homologs of the adaptor  $\beta$  and  $\sigma$  subunits named  $\beta 4$  and  $\sigma 4$ , respectively. An antibody to  $\beta 4$  recognized in human cells an ~83-kDa polypeptide that exists in both soluble and membrane-associated forms. Gel filtration, sedimentation velocity, and immunoprecipitation experiments revealed that  $\beta 4$  is a component of a multisubunit complex (AP-4) that also contains the  $\sigma 4$  polypeptide and two additional adaptor subunit homologs named  $\mu 4$  ( $\mu$ -ARP2) and  $\epsilon$ . Immunofluorescence analyses showed that AP-4 is associated with the *trans*-Golgi network or an adjacent structure and that this association is sensitive to the drug brefeldin A. We propose that, like the related AP-1, AP-2, and AP-3 complexes, AP-4 plays a role in signal-mediated trafficking of integral membrane proteins in mammalian cells.**

Sorting of integral membrane proteins at various stages of the endocytic and secretory pathways is mediated by interactions of signals contained within the cytosolic domains of these proteins with organellar coats associated with the cytosolic face of membranes (reviewed in Refs. 1–3). Two types of signals referred to as “tyrosine-based” and “dileucine-based” have been implicated in various sorting processes in mammalian cells, including internalization from the plasma membrane, lysosomal targeting, delivery to the basolateral plasma membrane of polarized epithelial cells, transport from early to late endosomes, and localization to specialized organelles such as melanosomes and antigen-processing compartments. Both tyrosine- and dileucine-based signals have been shown to interact with heterotetrameric adaptor protein (AP)<sup>1</sup> complexes, which associate with the protein clathrin and accessory molecules to generate coated transport vesicles (1–3).

To date, three adaptor complexes, known as AP-1, AP-2, and

AP-3, have been identified (reviewed in Ref. 4). These complexes consist of two large chains ( $\gamma/\alpha/\delta$  and  $\beta 1/\beta 2/\beta 3$ , ~90–130 kDa), one medium chain ( $\mu 1/\mu 2/\mu 3$ , ~50 kDa), and one small chain ( $\sigma 1/\sigma 2/\sigma 3$ , ~20 kDa) (Fig. 1A). The analogous subunits of the complexes are all structurally related and probably fulfill similar functions. For instance, the  $\mu$  chains of the three complexes are involved in the recognition of tyrosine-based signals (5–7), and the  $\beta$  chains interact with clathrin (8–11). The functions of the  $\gamma/\alpha/\delta$  and the  $\sigma$  subunits are less clear, although AP-2  $\alpha$  has been shown to interact with other components of the protein sorting machinery such as amphiphysin, dynamin, Eps15, and epsin (12–14).

AP-1 plays a role in protein sorting from the TGN and endosomes to compartments of the endosomal/lysosomal system, while AP-2 is involved in clathrin-mediated endocytosis (1–4). AP-3 is associated with endosomes (7, 11) and/or the TGN (15) and recruits integral membrane proteins for transport to lysosomes and lysosome-related organelles (16, 17). The existence of more sorting events mediated by tyrosine- and dileucine-based signals than known AP complexes, however, suggests the existence of additional adaptors remaining to be identified (3).

In this study, we have found through searches in EST data bases several cDNAs that encode new mammalian homologs of AP subunits. Antibodies raised to these homologs and to the recently described AP  $\mu$ -related protein,  $\mu$ -ARP2 (18), allowed us to identify a novel heterotetrameric adaptor-like complex, which we named AP-4. Like other AP complexes, AP-4 is composed of two large chains ( $\epsilon$  and  $\beta 4$ ), a medium chain ( $\mu$ -ARP2, herein referred to as  $\mu 4$ ), and a small chain ( $\sigma 4$ ) (Fig. 1B). AP-4 is widely expressed in mammalian tissues and associates with membranes as a peripheral membrane protein. By immunofluorescence microscopy, AP-4 was found to localize to the TGN or a neighboring compartment and to be sensitive to brefeldin A, a drug that also affects the distribution of AP-1 and AP-3 (7, 19, 20). These properties of AP-4 are similar to those of other AP complexes, suggesting that AP-4 may be a novel component of the cellular machinery that sorts integral membrane proteins in mammalian cells.

## EXPERIMENTAL PROCEDURES

**Sequencing of  $\sigma 4$  and  $\beta 4$  cDNAs**—All EST clones used in this study were from the I.M.A.G.E. Consortium (Lawrence Livermore National Laboratory, Livermore, CA) and were purchased from the American Type Culture Collection (Rockville, MD). DNA sequencing was performed on both strands using the dideoxy method. The mouse EST clone 619775 consisted of a portion of the  $\sigma 4$  cDNA spanning nucleotide 656 to the start of the poly(A) tail, while the mouse EST clones 442436 and 572356 comprised the complete coding region of  $\sigma 4$  together with both the 5'- and 3'-untranslated regions. Human EST clones 51094 and 206232 consisted of 3' fragments of the  $\beta 4$  cDNA starting at nucleotides 1414 and 1724, respectively. The missing 5' portion of the  $\beta 4$  cDNA was obtained from a human skeletal muscle Marathon Ready™ cDNA library (CLONTECH, Palo Alto, CA) by sequential 5'-RACE PCR using primers complementary to nucleotides 1657–1683 and 1626–1651 for the first and second (nested) PCR steps, respectively. The final PCR product was cloned into the pNoTAT/7 vector (5 Prime → 3 Prime, Boulder, CO), and several independent clones were isolated and sequenced.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF092093 and AF092094.

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<sup>1</sup> The abbreviations used are: AP, adaptor protein; BSA, bovine serum albumin; EST, expressed sequence tag; GST, glutathione S-transferase; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; TGN, *trans*-Golgi network.

**Northern Blotting**—Northern blot analysis was performed on multiple tissue blots (CLONTECH) as described previously (7). Both the  $\beta 4$  and  $\sigma 4$  probes were obtained by PCR. The  $\beta 4$  probe comprised nucleotides 1776–2226 of the full-length  $\beta 4$  cDNA, while the probe for  $\sigma 4$  consisted of its complete coding sequence.

**GST Fusion Proteins**—A GST- $\beta 4_C$  fusion construct was generated by PCR amplification of a cDNA segment encoding residues 601–739 of human  $\beta 4$ , followed by cloning in frame into the *Bam*HI–*Not*I sites of the pGEX-5X-1 vector (Pharmacia Biotech, Uppsala, Sweden). The GST- $\sigma 4$ -(18–54) and GST- $\mu 4$ -(122–326) constructs, bearing residues 18–54 of mouse  $\sigma 4$  and residues 122–326 of human  $\mu 4/\mu$ -ARP2 (EMBL data bank accession no. Y08387), respectively, were prepared by PCR and subsequent cloning into the *Eco*RI–*Not*I sites of the pGEX-5X-1 vector. The constructs were verified by DNA sequencing. Overexpression of GST- $\beta 4_C$  and GST- $\sigma 4$ -(18–54) in *Escherichia coli* yielded soluble proteins that were affinity-purified on glutathione-Sepharose 4B beads (Pharmacia Biotech) according to the manufacturer's instructions. The GST- $\mu 4$ -(122–326) fusion protein was insoluble and was purified from inclusion bodies by preparative SDS-PAGE.

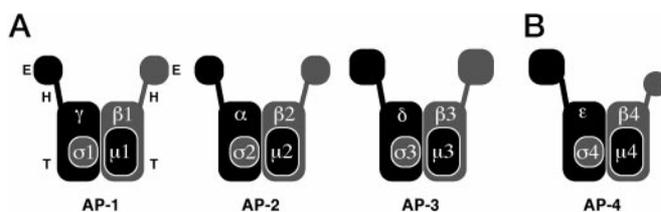
**Antibodies**—Polyclonal antibodies to  $\beta 4$  and  $\sigma 4$  were raised in rabbits by immunization with purified GST- $\beta 4_C$  and GST- $\sigma 4$ -(18–54) fusion proteins, respectively. The antibodies were affinity-purified (7) and subsequently absorbed with immobilized GST (Pierce). An antiserum to  $\mu 4$  was generated by immunizing rabbits with the GST- $\mu 4$ -(122–326) fusion protein that had been purified on polyacrylamide gels. The anti- $\epsilon$  antiserum was raised in rabbits using as the immunogen the peptide TALTSKHEEEKLIQQLSSL (Zymed Laboratories Inc., San Francisco, CA). The anti-AP-3 mouse antiserum was generated by immunization with a mixture of GST fusion proteins bearing portions of all of the AP-3 subunits; preparation of these GST-fusion proteins is described elsewhere (7, 21, 22). Preparation of rabbit polyclonal antibodies to the AP-3  $\sigma 3$  subunit and to GST has been described previously (7, 11). The 58K-9 monoclonal antibody to the Golgi 58-kDa protein was purchased from Sigma. The sources of the remaining antibodies used in this study are indicated elsewhere (7, 17).

**Biochemical Procedures**—All of the biochemical procedures used in this study have been described in a previous report (7). Further details on the immunoprecipitation-recapture method are given elsewhere (23). The sedimentation coefficient of AP-4 was estimated from sedimentation velocity experiments using the following protein markers ( $S_{20,w}$  values given in parentheses): horse spleen ferritin (16.5 S), bovine catalase (11.3 S), BSA (4.6 S), and chicken ovalbumin (3.6 S) (24). The gel filtration markers used to determine Stokes radii were those described previously (7). The molecular mass of the AP-4 complex was calculated on the basis of its Stokes radius and sedimentation coefficient, assuming a partial specific volume of 0.72–0.75 cm<sup>3</sup>/g (24).

**Immunofluorescence Microscopy**—HeLa cell monolayers were grown on glass coverslips and in some cases treated with brefeldin A or transiently transfected with HA epitope-tagged constructs of TGN38 or furin (7, 25). Cells were fixed in methanol/acetone (1:1, v/v) for 10 min at –20 °C and subsequently air-dried. Incubation with the primary antibody diluted in PBS, 0.1% (w/v) saponin, 1 g/liter BSA, 50 mg/liter GST was carried out for 1 h at room temperature. Unbound antibodies were removed by rinsing with PBS for 5 min, and cells were subsequently incubated with secondary antibodies (Cy3-conjugated donkey anti-rabbit Ig or Alexa 448-conjugated donkey anti-mouse Ig) diluted in PBS, 0.1% (w/v) saponin, 1 g/liter BSA, for 30–60 min at room temperature. After a final rinse with PBS, coverslips were mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Fluorescence images were acquired on a Zeiss LSM 410 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

## RESULTS

**Identification of  $\beta 4$  and  $\sigma 4$** —Complementary DNAs encoding two novel mammalian proteins related to subunits of the AP-1, AP-2, and AP-3 complexes were identified through BLAST searches of EST data bases. One of the proteins was named  $\beta 4$  on the basis of its homology to the AP  $\beta$  subunits. Human  $\beta 4$  was predicted to be a protein of 739 amino acids and a molecular mass of 83,262 Da. Homology of  $\beta 4$  to the other AP  $\beta$  subunits was restricted to the so-called “trunk” amino-terminal domain, with the first ~580 residues of  $\beta 4$  sharing 28% identical amino acids with the trunk domain of mammalian  $\beta 1$  and  $\beta 2$ , and 21% identical amino acids with the corresponding segment in human  $\beta 3A$  and  $\beta 3B$  (Fig. 2A and data not shown). In these molecules, the trunk domain is linked to a carboxyl-



**FIG. 1. Schematic depiction of the subunit composition and possible domain organization of the AP-1, AP-2, and AP-3 complexes (A) and the related AP-4 complex described in this study (B).** Each complex consists of four different subunits, the analogous subunits of the complexes displaying significant homology to one other at the amino acid level. The complexes are believed to adopt a mouse head-like structure with a “head,” comprising the  $\mu$  and  $\sigma$  subunits and the amino-terminal “trunk” domain (T) of the two large subunits, and two “ears” (E) that correspond to the carboxyl-terminal domains of the large subunits and are linked to the head by flexible “hinges” (H). Only a portion of the primary structure of  $\epsilon$  has been determined to date; its putative domain organization has been modeled after those of its counterparts in the AP-1, AP-2, and AP-3 complexes.

terminal “ear” domain by a flexible, solvent-accessible “hinge” region (26). Interestingly, secondary structure and accessibility predictions (27, 28) revealed that residues ~580–600 of  $\beta 4$  would correspond to a solvent-accessible, random coil structure preceding a carboxyl-terminal region with a high  $\alpha$ -helix content (not shown). It is therefore plausible that the  $\beta 4$  molecule has a domain organization similar to that of the other AP  $\beta$  subunits, albeit with smaller hinge and ear domains (Fig. 1B).

The other novel protein was named  $\sigma 4$  based on its homology to previously known AP  $\sigma$  subunits (Fig. 2B). The mouse  $\sigma 4$  cDNA encodes a 144-residue protein with a calculated molecular mass of 16,818 Da. Homology of  $\sigma 4$  to the other AP  $\sigma$  polypeptides was significant throughout its entire amino acid sequence (37–43% identity; Fig. 2B and data not shown).

Northern analyses revealed expression of human  $\beta 4$  and mouse  $\sigma 4$  mRNA species in all tissues examined, although some differences in the expression patterns were observed (Fig. 3). The sizes of the major ~2.5-kb and ~1.2–1.4-kb species detected with the  $\beta 4$  and  $\sigma 4$  probes were consistent with those of the isolated  $\beta 4$  and  $\sigma 4$  cDNAs, respectively. A minor ~6-kb species was also detected in some human tissues with the  $\beta 4$  probe; the nature of this species is unknown, although it could correspond to an unprocessed or alternatively spliced form of the  $\beta 4$  mRNA.

**Detection of Endogenous  $\beta 4$  with a Specific Antibody**—To characterize the  $\beta 4$  protein, we raised a polyclonal antibody to its carboxyl-terminal region (anti- $\beta 4_C$ ). This antibody immunoprecipitated under nondenaturing conditions several proteins from detergent lysates of [<sup>35</sup>S]methionine-labeled cells (Fig. 4A). In order to identify unambiguously the endogenous  $\beta 4$  protein, the immunoprecipitate was denatured with SDS and then subjected to a second immunoprecipitation step using the same antibody. This immunoprecipitation-recapture procedure (7, 23) resulted in the isolation of a single <sup>35</sup>S-labeled polypeptide that migrated on SDS-PAGE with an apparent molecular mass of ~83-kDa (Fig. 4B, lane 1), a value that was in close agreement with the calculated molecular mass of  $\beta 4$ . The specificity of the immunoprecipitation was corroborated by competition with excess GST- $\beta 4_C$  (Fig. 4B, lane 2) and by using an irrelevant antibody (to BSA) in the recapture step (Fig. 4B, lane 3). The same immunoprecipitation-recapture procedure was used to establish that  $\beta 4$  was present in both cytosolic (C) and postnuclear membrane (M) fractions (Fig. 4C). The relative yields of soluble and membrane-associated forms of  $\beta 4$  were influenced by the composition of the homogenization buffer (Fig. 4C), as previously observed for the  $\sigma 3$  subunit of AP-3 (7). In addition, the membrane-associated form could be partially

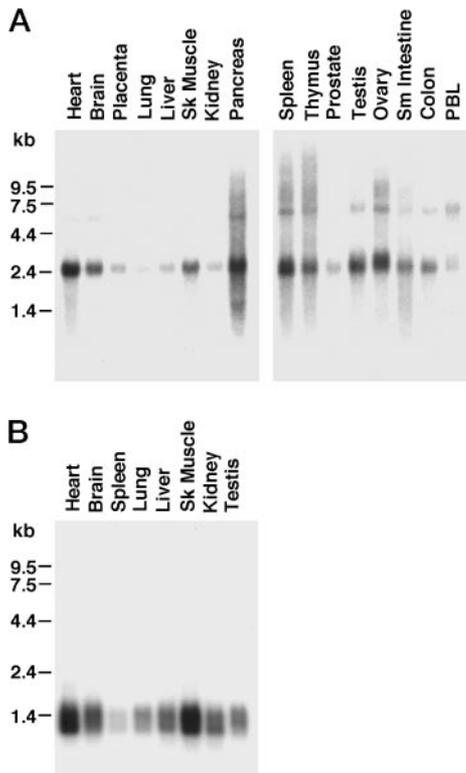
## A

β4	.....MPYL <b>G</b> SEDEVVKEL <b>KKAL</b> CNPHIQADRLRYRNV <b>IQRV</b> IRYM	40
β2	.....MTDSKYFTTN <b>KKGEI</b> FEL <b>KAEL</b> NN...EKKEKR <b>KEAV</b> KK <b>VIA</b> AM	41
β3A	MSSNSFPYNEQSGG <b>EATE</b> LG <b>Q</b> EATSTISPS <b>GAF</b> <b>G</b> LFSSDL <b>KKNE</b> DL <b>KQML</b> ES <b>NK</b> DSAK <b>LDA</b> M <b>KR</b> IVGMI	70
β4	<b>TQGLD</b> MS <b>GV</b> MEMV <b>KAS</b> ATVD <b>I</b> V <b>Q</b> KKLVYLY <b>MCT</b> Y <b>AP</b> L <b>K</b> PD <b>L</b> ALL <b>AIN</b> TL <b>CK</b> DCSD <b>PN</b> MP <b>VR</b> GL <b>AL</b> RS <b>MC</b>	110
β2	<b>TVGKDV</b> SS <b>LF</b> PD <b>V</b> NC <b>M</b> Q <b>T</b> DN <b>LE</b> L <b>KK</b> LVYLY <b>LM</b> NY <b>AKS</b> Q <b>PD</b> MA <b>IM</b> AV <b>NS</b> F <b>V</b> K <b>DC</b> ED <b>PN</b> PL <b>IR</b> AL <b>AV</b> RT <b>MG</b>	111
β3A	<b>AKG</b> KN <b>AS</b> EL <b>FP</b> AV <b>V</b> KN <b>V</b> AS <b>KN</b> IE <b>I</b> KKLVY <b>V</b> LV <b>RY</b> AE <b>EQ</b> DL <b>ALL</b> S <b>IS</b> TF <b>OR</b> AL <b>K</b> DP <b>N</b> Q <b>L</b> IR <b>AS</b> AL <b>RV</b> LS	140
β4	<b>SLR</b> MP <b>GV</b> Q <b>EY</b> I <b>QQ</b> PI <b>L</b> NG <b>L</b> R <b>DK</b> AS <b>Y</b> VR <b>RV</b> V <b>L</b> GC <b>AK</b> MN <b>L</b> H <b>GD</b> SE <b>VD</b> GAL <b>V</b> NE <b>L</b> YS <b>LL</b> R <b>D</b> Q <b>DP</b> I <b>V</b> V <b>V</b> N <b>CL</b>	180
β2	<b>C</b> IR <b>VD</b> K <b>I</b> TE <b>YL</b> CE <b>PL</b> R <b>K</b> CL <b>K</b> DE <b>D</b> PY <b>VR</b> KT <b>AA</b> VC <b>V</b> AK <b>L</b> HD <b>INA</b> Q <b>M</b> VE <b>D</b> Q <b>G</b> FL <b>D</b> SL <b>R</b> DL <b>I</b> AD <b>SN</b> PM <b>V</b> AV <b>NAV</b>	181
β3A	<b>S</b> IR <b>VP</b> I <b>I</b> V <b>P</b> IM <b>ML</b> A <b>IK</b> EAS <b>AD</b> LS <b>P</b> Y <b>VR</b> KN <b>AA</b> HAI <b>Q</b> K <b>L</b> Y <b>SL</b> ... <b>D</b> PE <b>Q</b> KE <b>M</b> L <b>I</b> EV <b>IE</b> K <b>L</b> L <b>K</b> D <b>K</b> ST <b>L</b> V <b>AG</b> SV <b>V</b>	208
β4	RS <b>LEE</b> IL <b>K</b> Q <b>E</b> GG...VV <b>I</b> N <b>K</b> PIA <b>H</b> LL <b>N</b> R <b>MS</b> K <b>L</b> D <b>Q</b> W <b>G</b> Q <b>AE</b> V <b>L</b> N <b>F</b> LL <b>RY</b> .....	225
β2	A <b>AL</b> SE <b>I</b> SE <b>SH</b> PN <b>S</b> N <b>LL</b> DL <b>N</b> P <b>Q</b> N <b>I</b> N <b>K</b> LL <b>T</b> AL <b>NE</b> CT <b>EW</b> Q <b>I</b> F <b>I</b> L <b>D</b> CL <b>S</b> NY.....	229
β3A	MA <b>FE</b> EV <b>C</b> .....P <b>D</b> R <b>I</b> DL <b>I</b> H <b>K</b> N <b>Y</b> R <b>K</b> L <b>C</b> N <b>L</b> L <b>V</b> D <b>VE</b> W <b>G</b> Q <b>V</b> VI <b>I</b> H <b>ML</b> TR <b>Y</b> ART <b>Q</b> F <b>V</b> SP <b>W</b> KE <b>G</b> DE <b>L</b> ED <b>NG</b> K <b>N</b> F	273
β4	Q <b>P</b> R <b>SE</b> EL <b>F</b> DL <b>LN</b> LL <b>S</b> FL <b>K</b> SS <b>SP</b> GV <b>M</b> G <b>AT</b> KL <b>FL</b> I...L <b>AK</b> MF <b>PH</b> V <b>Q</b> T...D <b>V</b> L <b>V</b> R...V <b>K</b> GP <b>L</b> LA <b>ACS</b>	286
β2	N <b>PK</b> DD <b>RE</b> A <b>Q</b> SI <b>C</b> ER <b>VT</b> PR <b>L</b> SH <b>AN</b> SA <b>V</b> VL <b>S</b> AV <b>K</b> V <b>LM</b> K...F <b>LE</b> LL <b>PK</b> DS <b>D</b> Y <b>N</b> ML <b>L</b> K...K <b>L</b> AP <b>PL</b> V <b>T</b> LL	292
β3A	Y <b>ES</b> DD <b>D</b> Q <b>KE</b> KT <b>DK</b> KK <b>K</b> PY <b>T</b> MD <b>P</b> DR <b>LL</b> IR <b>NT</b> TK <b>PL</b> L <b>Q</b> SR <b>NA</b> AV <b>M</b> VA <b>Q</b> LY <b>WH</b> I <b>SP</b> K <b>SE</b> AG <b>I</b> IS <b>K</b> SL <b>V</b> R <b>LL</b>	343
β4	<b>SE</b> S <b>RE</b> LC <b>F</b> VAL <b>CH</b> VR <b>Q</b> IL <b>H</b> SL <b>PG</b> H <b>SS</b> HY <b>KK</b> FF <b>CS</b> Y <b>SE</b> PH <b>Y</b> IK <b>L</b> Q <b>VE</b> VL <b>CE</b> L <b>V</b> ND <b>EN</b> V <b>Q</b> VL <b>E</b> EL <b>RG</b> Y <b>C</b>	356
β2	<b>S</b> GE <b>PE</b> V <b>Q</b> Y <b>VA</b> L <b>R</b> NI <b>N</b> L <b>I</b> V <b>Q</b> K <b>R</b> PE <b>IL</b> K <b>Q</b> E <b>IK</b> VE <b>F</b> V <b>K</b> Y <b>ND</b> PI <b>Y</b> V <b>K</b> L <b>E</b> K <b>L</b> DI <b>M</b> IR <b>LA</b> S <b>Q</b> AN <b>IA</b> Q <b>V</b> LA <b>E</b> L <b>KEY</b> A	362
β3A	<b>R</b> SN <b>RE</b> V <b>Q</b> Y <b>IV</b> L <b>Q</b> NI <b>AT</b> MS <b>I</b> Q <b>R</b> K <b>G</b> MF <b>E</b> PY <b>L</b> K <b>S</b> F <b>Y</b> VR <b>ST</b> DP <b>TM</b> IK <b>TL</b> K <b>E</b> IL <b>T</b> N <b>LAN</b> E <b>ANI</b> ST <b>L</b> RE <b>F</b> Q <b>TY</b> V	413
β4	<b>TD</b> V <b>S</b> AD <b>FA</b> Q <b>AA</b> I <b>FA</b> IG <b>G</b> IA...R <b>TY</b> TD <b>Q</b> CV <b>Q</b> IL <b>T</b> EL <b>L</b> GL <b>RO</b> EH <b>IT</b> V <b>V</b> V <b>Q</b> T <b>FR</b> DL <b>V</b> WL <b>CP</b> Q <b>TE</b> AV <b>Q</b> AL	423
β2	<b>TE</b> VD <b>V</b> DF <b>VR</b> KA <b>V</b> RA <b>IG</b> RC <b>AI</b> K <b>VE</b> Q <b>SA</b> ER <b>CV</b> ST <b>LL</b> DL <b>I</b> Q <b>T</b> K <b>V</b> NY <b>V</b> Q <b>EA</b> IV <b>VI</b> RD <b>I</b> FR <b>K</b> Y <b>EN</b> K <b>Y</b> ES <b>IT</b> AT <b>L</b>	432
β3A	<b>K</b> S <b>Q</b> DK <b>Q</b> FA <b>AA</b> T <b>I</b> Q <b>T</b> IG <b>RC</b> AT <b>NI</b> LEV <b>TD</b> T <b>CL</b> NG <b>L</b> V <b>CL</b> LS <b>NR</b> DE <b>IV</b> VA <b>ES</b> V <b>V</b> V <b>I</b> KK <b>L</b> L <b>Q</b> M <b>Q</b> PA <b>Q</b> H <b>GE</b> II <b>K</b> HM	483
β4	PG <b>CE</b> EN <b>I</b> Q <b>D</b> SE <b>G</b> Q <b>AL</b> I <b>W</b> LL <b>G</b> V <b>H</b> GER <b>IP</b> N <b>AP</b> Y <b>V</b> LE <b>D</b> F <b>V</b> EN <b>V</b> K <b>SE</b> TF <b>PA</b> V <b>K</b> M <b>EL</b> L <b>T</b> AS <b>L</b> R <b>L</b> FL <b>S</b> RP <b>AE</b> C <b>Q</b>	492
β2	C <b>EN</b> LD <b>S</b> L <b>D</b> EP <b>D</b> ARA <b>AM</b> I <b>W</b> V <b>GE</b> YA <b>ER</b> ID <b>N</b> .A <b>DE</b> LE <b>SE</b> DE <b>G</b> F <b>H</b> DES.T <b>Q</b> V <b>Q</b> L <b>T</b> LL <b>T</b> A <b>IV</b> K <b>L</b> FL <b>K</b> RP <b>SE</b> T <b>Q</b>	500
β3A	<b>AK</b> LD <b>S</b> I <b>TV</b> E <b>VA</b> RA <b>S</b> I <b>L</b> W <b>L</b> I <b>GE</b> NC <b>ER</b> V <b>PK</b> I <b>AP</b> D <b>V</b> L <b>R</b> K <b>MA</b> K <b>S</b> F <b>T</b> SE <b>D</b> .DL <b>V</b> K <b>L</b> Q <b>IL</b> N <b>L</b> G <b>AK</b> L <b>YL</b> T <b>NS</b> K <b>Q</b> T <b>K</b>	552
β4	D <b>M</b> L <b>G</b> R <b>L</b> L <b>Y</b> YC <b>IE</b> E <b>K</b> DM <b>AV</b> RD <b>R</b> GL <b>F</b> Y <b>R</b> LL <b>L</b> V <b>G</b> IDE <b>V</b> K <b>R</b> IL <b>C</b> SP <b>K</b> SD <b>PT</b> L <b>G</b> L <b>LE</b> D <b>PA</b> ER <b>P</b> .V <b>NS</b> W <b>AS</b> DF <b>N</b>	561
β2	<b>EL</b> V <b>Q</b> Q <b>V</b> LS <b>L</b> AT <b>Q</b> SD <b>N</b> PD <b>L</b> DR <b>GY</b> I <b>Y</b> WR <b>LL</b> ST <b>DP</b> V <b>T</b> A <b>KE</b> V <b>V</b> L <b>SE</b> K...P <b>L</b> ISE <b>ET</b> D <b>L</b> I <b>E</b> PT <b>LL</b> DE <b>L</b> ICH <b>I</b> G	568
β3A	<b>LL</b> T <b>Q</b> Y <b>IL</b> N <b>L</b> G <b>K</b> Y <b>D</b> Q <b>NY</b> D <b>IR</b> DR <b>T</b> RE <b>I</b> .R <b>Q</b> L <b>IV</b> P <b>NE</b> K <b>S</b> G <b>AL</b> S <b>K</b> Y <b>AK</b> K <b>I</b> FL <b>A</b> Q <b>K</b> P <b>AP</b> L <b>LE</b> S <b>F</b> K <b>D</b> RD <b>H</b> F <b>Q</b> L <b>G</b>	620
β4	<b>TL</b> VP <b>V</b> Y <b>G</b> KA <b>H</b> W <b>AT</b> I <b>SK</b> Q <b>GA</b> ER <b>CD</b> PE <b>L</b> PK <b>T</b> SS <b>F</b> A <b>AS</b> GL <b>I</b> PE <b>EN</b> K <b>ER</b> V <b>Q</b> EL <b>P</b> D <b>S</b> G <b>AL</b> M <b>L</b> V <b>PN</b> R <b>Q</b> L <b>T</b> AD <b>Y</b> F	631
β2	SL <b>AS</b> V <b>Y</b> H <b>K</b> P-	577
β3A	<b>TL</b> S <b>H</b> T <b>L</b> NI <b>K</b> AT <b>G</b> Y <b>L</b> EL <b>S</b> N <b>W</b> PE <b>V</b> -	642
β4	E <b>K</b> T <b>W</b> LS <b>L</b> K <b>V</b> A <b>H</b> Q <b>Q</b> V <b>L</b> P <b>WR</b> GE <b>F</b> HP <b>DT</b> L <b>Q</b> M <b>AL</b> Q <b>V</b> V <b>NI</b> Q <b>T</b> I <b>AM</b> S <b>R</b> AG <b>S</b> RP <b>W</b> K <b>AY</b> LS <b>A</b> Q <b>DD</b> T <b>G</b> CL <b>FL</b> TE <b>LL</b> EP	701
β4	G <b>N</b> SE <b>M</b> Q <b>I</b> SV <b>K</b> Q <b>NE</b> ART <b>ET</b> L <b>NS</b> F <b>I</b> SV <b>L</b> ET <b>V</b> IG <b>T</b> IE <b>E</b> IK <b>S</b>	739

## B

σ4	<b>MI</b> K <b>FF</b> LM <b>VN</b> K <b>Q</b> Q <b>T</b> RL <b>SK</b> Y <b>EH</b> VD <b>I</b> N <b>K</b> R <b>AL</b> LE <b>T</b> D <b>V</b> SK <b>S</b> CL <b>S</b> RS <b>SE</b> Q <b>CS</b> F <b>IE</b> Y..... <b>K</b> DE <b>K</b> L <b>I</b> Y <b>Q</b> Y <b>AA</b>	64
σ1	<b>MM</b> R <b>F</b> ML <b>L</b> FS <b>R</b> Q <b>G</b> KL <b>R</b> L <b>Q</b> W <b>Y</b> L <b>AT</b> SD <b>K</b> ER <b>K</b> M <b>V</b> RE <b>LM</b> Q <b>V</b> L <b>AR</b> K <b>PK</b> M <b>C</b> S <b>F</b> LE <b>W</b> ..... <b>R</b> DL <b>K</b> V <b>V</b> Y <b>K</b> R <b>Y</b> AS	64
σ2	<b>M</b> IR <b>F</b> IL <b>I</b> Q <b>N</b> RA <b>G</b> K <b>T</b> RL <b>A</b> K <b>W</b> Y <b>M</b> Q <b>F</b> DD <b>E</b> K <b>Q</b> K <b>L</b> IE <b>V</b> H <b>AV</b> V <b>T</b> VR <b>DA</b> K <b>H</b> T <b>N</b> F <b>VE</b> F..... <b>R</b> N <b>F</b> K <b>I</b> T <b>Y</b> RR <b>Y</b> AG	64
σ3A	<b>MI</b> K <b>AI</b> L <b>I</b> F <b>N</b> N <b>H</b> G <b>K</b> P <b>RL</b> S <b>K</b> F <b>Y</b> Q <b>P</b> Y <b>SE</b> DT <b>Q</b> Q <b>I</b> I <b>RE</b> TF <b>HL</b> V <b>SK</b> R <b>DE</b> NV <b>C</b> N <b>F</b> LE <b>G</b> GL <b>L</b> I <b>G</b> GS <b>DN</b> K <b>L</b> I <b>Y</b> R <b>HY</b> AT	70
σ4	<b>LF</b> V <b>V</b> V <b>G</b> V <b>ND</b> T <b>EN</b> E <b>MA</b> I <b>Y</b> E <b>F</b> I <b>H</b> N <b>F</b> VE <b>V</b> LD <b>G</b> Y <b>F</b> SR <b>V</b> SE <b>L</b> D <b>IM</b> F <b>N</b> LD <b>K</b> V <b>HI</b> IL <b>DE</b> M <b>V</b> L <b>NC</b> IV <b>ET</b> N <b>R</b> AR <b>I</b> L <b>AP</b>	134
σ1	<b>LY</b> F <b>CA</b> IE <b>G</b> Q <b>DN</b> EL <b>I</b> TE <b>L</b> I <b>H</b> RY <b>VE</b> LL <b>D</b> K <b>Y</b> F <b>GS</b> V <b>CE</b> LD <b>I</b> IF <b>N</b> F <b>E</b> K <b>AY</b> F <b>IL</b> DE <b>FL</b> M <b>G</b> GD <b>V</b> Q <b>D</b> T <b>SK</b> K <b>S</b> V <b>L</b> KA	134
σ2	<b>LY</b> F <b>C</b> IC <b>V</b> D <b>V</b> ND <b>N</b> L <b>AY</b> LE <b>AI</b> H <b>N</b> F <b>VE</b> VL <b>NE</b> Y <b>F</b> H <b>N</b> V <b>CE</b> LD <b>L</b> V <b>F</b> N <b>F</b> Y <b>K</b> V <b>Y</b> T <b>V</b> DE <b>M</b> FL <b>AG</b> E <b>IR</b> ET <b>S</b> Q <b>T</b> K <b>V</b> L <b>K</b> Q	134
σ3A	<b>LY</b> F <b>V</b> FC <b>V</b> D <b>SS</b> E <b>SE</b> L <b>G</b> IL <b>DL</b> I <b>Q</b> V <b>ET</b> LD <b>K</b> CF <b>EN</b> V <b>CE</b> LD <b>L</b> IF <b>H</b> V <b>D</b> K <b>V</b> H <b>N</b> IL <b>A</b> EM <b>V</b> M <b>G</b> M <b>V</b> LE <b>T</b> N <b>M</b> NE <b>IV</b> T <b>Q</b>	140
σ4	LL <b>I</b> LD <b>K</b> L <b>S</b> ES.....	144
σ1	IE <b>Q</b> AD <b>L</b> L <b>Q</b> E <b>E</b> DES <b>PR</b> SV <b>L</b> E <b>E</b> M <b>G</b> LA.....	158
σ2	LL <b>M</b> L <b>Q</b> S <b>L</b> E.....	142
σ3A	IDA <b>Q</b> N <b>K</b> L <b>E</b> K <b>S</b> E <b>AG</b> L <b>AG</b> AP <b>AR</b> AV <b>SA</b> V <b>K</b> N <b>M</b> N <b>L</b> PE <b>I</b> PR <b>N</b> IN <b>I</b> G <b>D</b> IS <b>I</b> K <b>V</b> PN <b>L</b> PS <b>F</b> K	193

Fig. 2. Sequence alignments of β4 and σ4 with selected members of the AP β and AP σ protein families. Sequences were aligned using the PILEUP program. A, comparison of the predicted amino acid sequence of human β4 to residues 1–577 (trunk domain) of rat β2 (GenBank™ accession no. M77245) and to residues 1–642 (trunk/A domain) of human β3A (GenBank™ accession no. U81504). Amino acid residues conserved in at least two of the three sequences are highlighted on a black background. B, alignment of the primary structure of mouse σ4 with those of mouse σ1A (GenBank™ accession no. M62418), rat σ2 (GenBank™ accession no. M37194), and human σ3A (EMBL Nucleotide Data Base accession no. X99458). Residues conserved in at least three of the four sequences are highlighted.

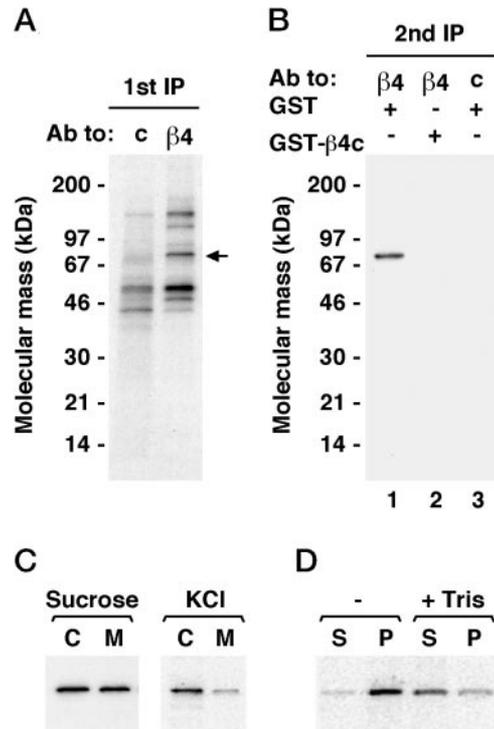


**FIG. 3. Northern blot analysis of the expression of  $\beta 4$  (A) and  $\sigma 4$  (B) mRNAs in normal tissues.** Multiple tissue blots were incubated with specific  $^{32}\text{P}$ -labeled probes as described elsewhere (7). The positions of molecular size markers (in kb) are indicated on the left. Notice the presence of a  $\sim 2.5$ -kb band corresponding to the full-length, mature  $\beta 4$  mRNA in all lanes in A and that of a  $\sim 1.2$ – $1.4$ -kb band corresponding to the  $\sigma 4$  mRNA in all lanes in B.

extracted with either 0.5 M Tris-HCl (Fig. 4D) or 1 M NaCl (data not shown), thus suggesting that  $\beta 4$  associated with membranes as a peripheral membrane protein.

**$\beta 4$  Is a Component of a Large Complex**—Upon fractionation of human fibroblast cytosol by gel filtration under nondenaturing conditions,  $\beta 4$  could be detected by immunoblotting in fractions corresponding to a Stokes radius of  $\sim 65$  Å (Fig. 5A). This size was significantly larger than that predicted for monomeric  $\beta 4$  and only slightly smaller than those of the AP-1, AP-2, and AP-3 complexes (Fig. 5A), thus suggesting that endogenous  $\beta 4$  was part of a complex.

To gain further insight into the physical properties and composition of the  $\beta 4$ -containing complex, we performed sedimentation analyses on a sucrose gradient of a cytosolic extract from [ $^{35}\text{S}$ ]methionine-labeled HeLa cells. Gradient fractions were subjected to immunoprecipitation with anti- $\beta 4$  and irrelevant antibodies. Using this approach, we observed co-immunoprecipitation of the  $\sim 83$ -kDa protein (corresponding to endogenous  $\beta 4$ ) with three other polypeptides of apparent molecular masses  $\sim 140$ ,  $\sim 50$ , and  $\sim 17$  kDa (Fig. 5B). These polypeptides were not detected in the control immunoprecipitation with the irrelevant antibody (to GST) and were found to co-sediment with  $\beta 4$  on the sucrose gradient (Fig. 5B, fractions 7 and 8). The position of  $\beta 4$  and its associated polypeptides on the gradient corresponded to a species with a sedimentation coefficient of  $\sim 10$  S. Using this value and the estimated Stokes radius (see above), we calculated a molecular mass for the  $\beta 4$ -containing complex of  $280 \pm 15$  kDa. This value fits within the experimental error the predicted molecular mass of a complex containing one molecule each of  $\beta 4$  and the three associated polypeptides (*i.e.*  $\sim 290$  kDa). Taken together, these results indicated that  $\beta 4$  is a component of a multisubunit protein complex, which we



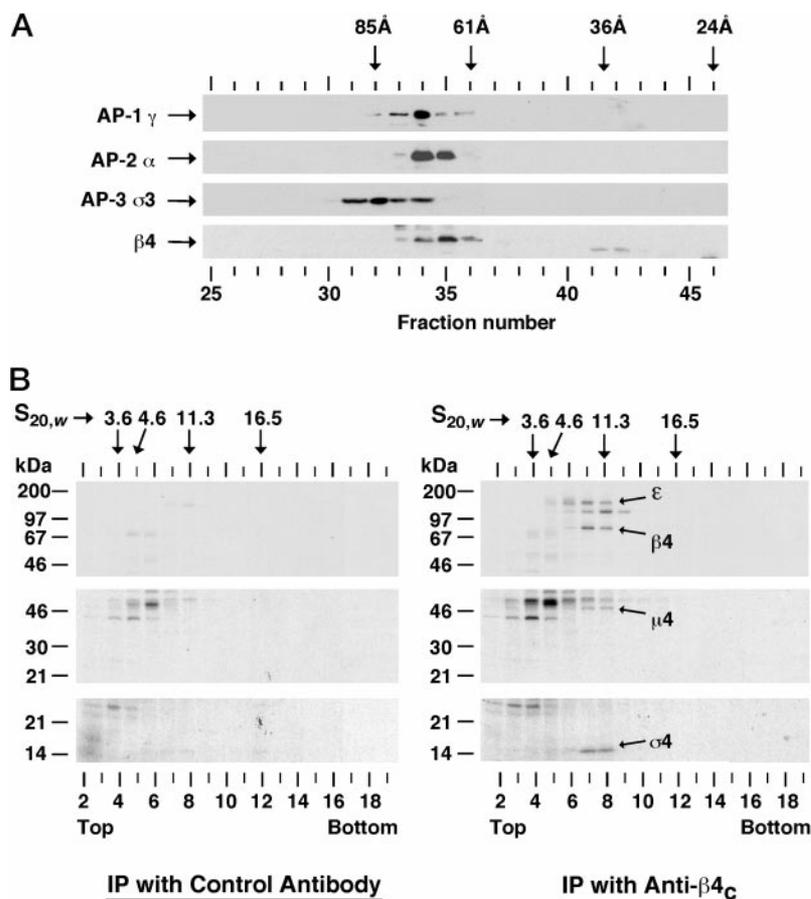
**FIG. 4. Detection and partial membrane association of endogenous  $\beta 4$  from HeLa cells.** A, a Triton X-100 extract prepared from HeLa cells metabolically labeled with [ $^{35}\text{S}$ ]methionine was subjected to immunoprecipitation (IP) using an antibody to  $\beta 4$  or an irrelevant control (c) antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. The arrow points to an  $\sim 83$ -kDa protein that was present in the immunoprecipitate obtained with the anti- $\beta 4_C$  antibody and was absent in the control immunoprecipitate. B, isolation of endogenous  $\beta 4$  by immunoprecipitation-recapture (23). The immunoprecipitate obtained with the anti- $\beta 4_C$  antibody was denatured in the presence of SDS, diluted, and then subjected to a second immunoprecipitation step using either the same specific antibody ( $\beta 4$ , lanes 1 and 2) or an irrelevant control antibody (c, lane 3). The resulting samples were analyzed by SDS-PAGE and fluorography. Notice the presence of the  $\sim 83$ -kDa protein band that was recognized by the antibody to  $\beta 4$  in the presence of GST but not in the presence of the competing antigen (GST- $\beta 4_C$ ). C, immunoprecipitation of  $\beta 4$  from cytosolic (C) and post-nuclear membrane (M) fractions that were obtained by ultracentrifugation of [ $^{35}\text{S}$ ]methionine-labeled HeLa cell extracts containing either 0.25 M sucrose or 0.15 M KCl (7). D, immunoprecipitation of  $\beta 4$  from supernatants (S) or pellets (P) obtained by ultracentrifugation of membranes that had been incubated overnight at 4 °C with a low salt buffer (7) containing no additive (–) or 0.5 M Tris-HCl (+Tris).

named AP-4 based on its structural similarity to the previously characterized AP-1, AP-2, and AP-3 adaptors.

**Identification of the Other Subunits of AP-4**—We hypothesized that the polypeptides associated with  $\beta 4$  could correspond to other homologs of known AP subunits. Likely candidates for the  $\sim 17$ - and  $\sim 50$ -kDa polypeptides were the  $\sigma 4$  protein identified in this study and the previously described AP  $\mu$  subunit homolog  $\mu 4/\mu$ -ARP2 (18). In addition, a search of EST data bases resulted in the identification of a human cDNA clone (I.M.A.G.E. clone ID 1031294) containing a  $\sim 0.4$ -kb insert that encodes a novel protein sequence with significant homology to the trunk region of the AP subunits  $\alpha$ ,  $\gamma$ , and  $\delta$  (25–30% identical amino acids over a 132-residue overlap; data not shown). Northern blot analysis indicated that this cDNA fragment corresponded to a ubiquitously expressed mRNA species of about 7 kb (data not shown). Based on this information, we hypothesized that this as yet uncharacterized protein, herein referred to as  $\epsilon$ , could correspond to the  $\sim 140$ -kDa subunit of the AP-4 complex.

To test these predictions, we raised antibodies to these putative AP-4 subunits and used these antibodies in immunoprecipitation

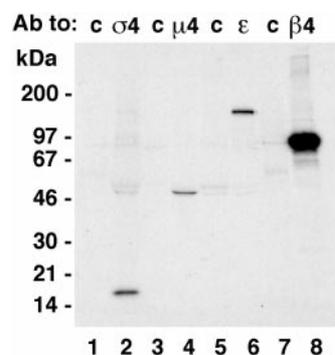
**FIG. 5. Association of endogenous  $\beta 4$  into a complex.** *A*, Superose 6 gel filtration analysis of a cytosolic fraction from unlabeled M1 fibroblasts (7). Eluted fractions were analyzed by immunoblotting using antibodies to AP-1  $\gamma$  (100/3), AP-2  $\alpha$  (100/2), AP-3  $\sigma 3$ , and  $\beta 4$ . The elution positions of molecular size markers (Stokes radii given in Ångstroms) are indicated by the arrows. *B*, fractionation of a cytosolic preparation from [ $^{35}$ S]methionine-labeled HeLa cells by velocity sedimentation on a 5–20% (w/v) sucrose gradient. Fractions were immunoprecipitated (IP) with an irrelevant control antibody (to GST) or with the anti- $\beta 4_C$  antibody, and the resulting precipitates were analyzed by SDS-PAGE and fluorography. Three overlapping portions of the fluorograms, corresponding to different exposure times, are shown. The migration of standard proteins on the sucrose gradient ( $s_{20,w}$  values given in Svedberg units) are indicated. Notice the specific co-precipitation of four polypeptides (labeled  $\epsilon$ ,  $\beta 4$ ,  $\mu 4$ , and  $\sigma 4$ ) by the anti- $\beta 4_C$  antibody in fractions 7 and 8 of the sucrose gradient.



cipitation-recapture experiments. AP-4 was first isolated from [ $^{35}$ S]methionine-labeled HeLa cells by immunoprecipitation with the anti- $\beta 4_C$  antibody, and the immunoprecipitate was then denatured and subjected to reprecipitation with the same antibody or with antibodies to the other putative subunits (Fig. 6). As mentioned above, reprecipitation with the anti- $\beta 4_C$  antibody yielded the  $\sim 83$ -kDa polypeptide (Fig. 6, lane 8). In addition, reprecipitation with antibodies to  $\sigma 4$ ,  $\mu 4$ , and  $\epsilon$  resulted in the isolation of the  $\sim 17$ -,  $\sim 50$ -, and  $\sim 140$ -kDa subunits of the complex, respectively (Fig. 6). Specificity was verified by control reprecipitations using irrelevant rabbit immunoglobulins or preimmune sera (Fig. 6) or by competition experiments using purified antigens (data not shown). Furthermore, none of our antibodies to AP-4 subunits recognized components of AP-1, AP-2, or AP-3 on similar immunoprecipitation-recapture experiments (data not shown).

**Intracellular Localization of the AP-4 Complex**—The distribution of the endogenous AP-4 complex within HeLa cells was examined by immunofluorescence microscopy using the anti- $\beta 4_C$  antibody. AP-4 was localized to a punctate juxtanuclear structure reminiscent of the Golgi complex (Fig. 7, *B*, *D*, *G*, and *J*). Staining was specific as judged by its competition with an excess of the GST- $\beta 4_C$  fusion protein (Fig. 7*A*) but not GST (Fig. 7*B*). In addition, treatment of the cells with brefeldin A, a drug that inactivates the small GTP-binding protein ARF1 (29), caused a rapid dispersal of AP-4 into the cytoplasm (Fig. 7*C*).

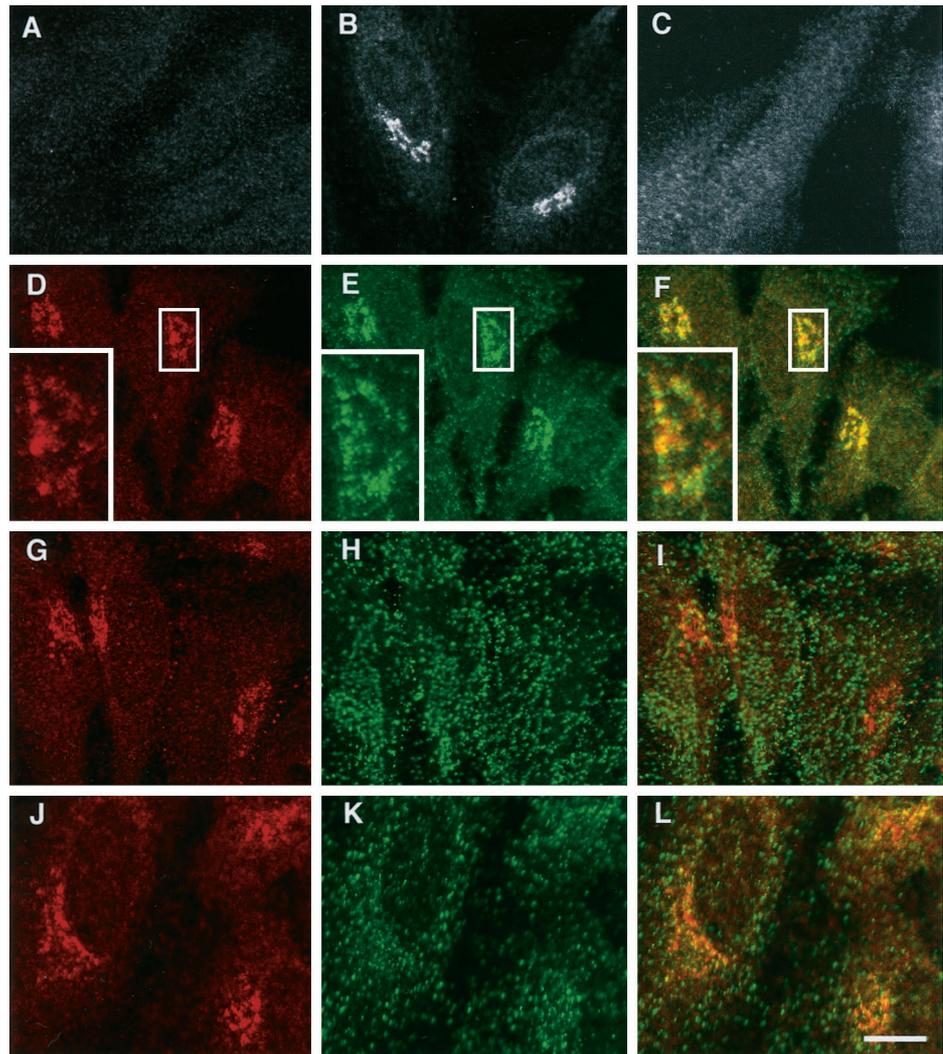
The distribution of AP-4 was next compared with those of the previously described AP complexes by double immunofluorescence staining. AP-1 is mainly associated with the TGN (4), although it has also been detected on peripheral cytoplasmic foci corresponding to endosomes (30, 31) (Fig. 7*E*). AP-2 is normally associated with coated pits at the plasma membrane (4) (Fig. 7*H*). The localization of AP-3 has not yet been estab-



**FIG. 6. Identification of the subunits of AP-4 by immunoprecipitation-recapture.** The AP-4 complex was immunoprecipitated from [ $^{35}$ S]methionine-labeled HeLa cells by using the anti- $\beta 4_C$  antibody (*Ab*). The immunoprecipitated material was denatured by heating at 95 °C in the presence of SDS and dithiothreitol, diluted 20-fold, and subsequently subjected to a second immunoprecipitation step using specific antibodies to  $\sigma 4$ ,  $\mu 4$ ,  $\epsilon$ , and  $\beta 4$ , as well as preimmune sera (lanes 3 and 5) or irrelevant rabbit Ig (lanes 1 and 7) as nonspecific controls (c).

lished with certainty, although recent evidence suggests that it is associated with a peripheral sorting compartment related to endosomes (7, 11) (Fig. 7*K*). The distribution of AP-4 most closely matched that of AP-1 in the juxtanuclear area (Fig. 7, *D–F*). A detailed inspection, however, revealed that the staining patterns for AP-4 and AP-1 were not completely overlapping (Fig. 7, *D–F*, insets), suggesting that the two complexes may be associated with different regions of the juxtanuclear structure. AP-4 exhibited little or no colocalization with AP-2 (Fig. 7, *G–I*) and AP-3 (Fig. 7, *J–L*).

The distribution of AP-4 was also compared with those of several markers of compartments of the secretory and endo-



**FIG. 7. Immunofluorescence analysis of the localization of AP-4 and other AP complexes in HeLa cells.** Fixed cells were incubated with the rabbit anti- $\beta 4_C$  antibody in the presence of either GST- $\beta 4_C$  (A) or GST (B-L) and with mouse antibodies to either AP-1 (100/3; E and F, green), AP-2 (AP.6; H and I, green), or AP-3 (K and L, green) and subsequently with Cy3-conjugated antibodies to rabbit Ig and Alexa448-conjugated antibodies to mouse Ig. C, cells were incubated for 1 min at 37 °C in the presence of 5 mg/liter brefeldin A prior to fixation. Bar, 10  $\mu$ m.

cytic pathways. The highest degree of colocalization was observed with the TGN markers furin (Fig. 8, A-C) and TGN38 (Fig. 8, D-F). The AP-4 staining pattern also resembled to some extent that of a 58-kDa *cis*-medial Golgi protein (Fig. 8, G and H). However, merging of the two images (Fig. 8I) yielded much less overlap than that observed with markers of the TGN. The distribution of AP-4 showed little colocalization with that of Lamp-1, the transferrin receptor, or the cation-dependent mannose 6-phosphate receptor, all of which are considered markers for distinct compartments of the endosomal-lysosomal system (data not shown). Taken together, the immunofluorescence microscopy analyses suggested that AP-4 is associated with the TGN or with a compartment that is immediately adjacent to it.

#### DISCUSSION

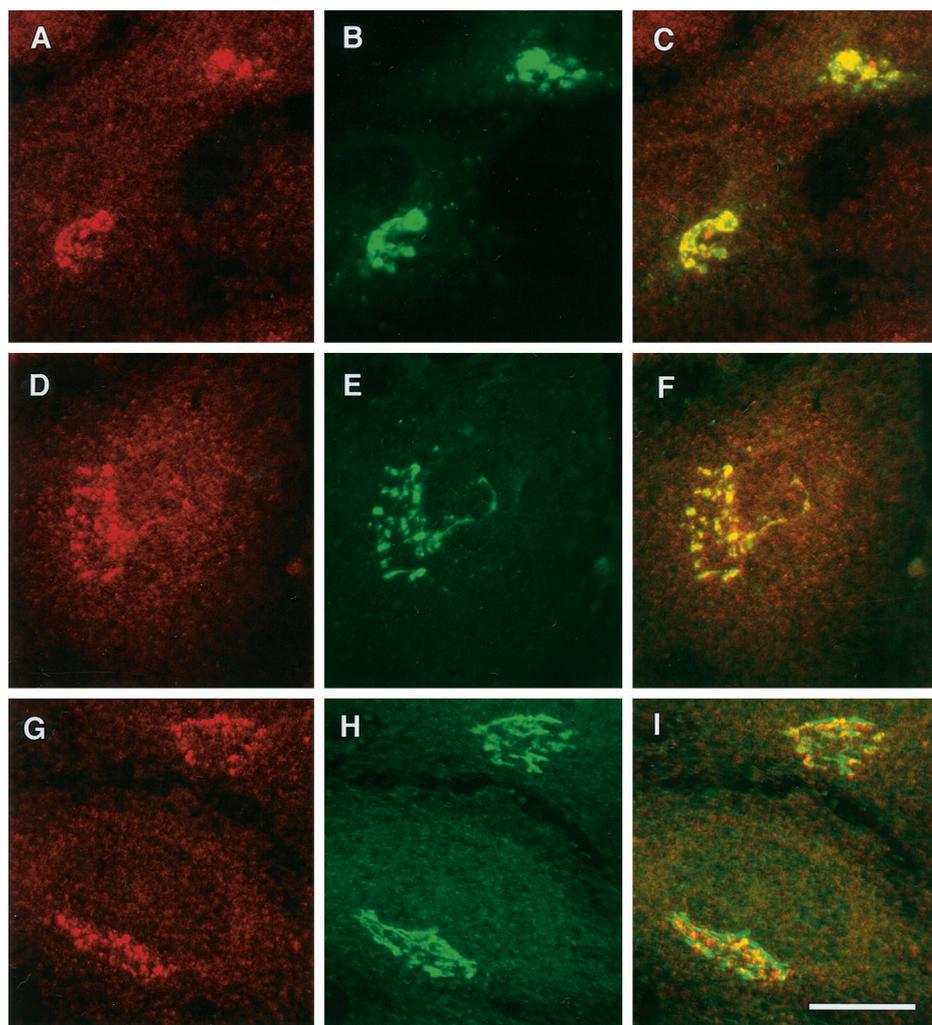
In recent years, there have been major advances in our understanding of the molecular machinery that mediates sorting of integral membrane proteins in the secretory and endocytic pathways. Our laboratory has been particularly interested in a subset of protein coats that contain heterotetrameric adaptor complexes among their major constituents. Three AP complexes have been described to date: AP-1, AP-2, and AP-3 (Fig. 1A). In this study, we describe a novel, widely expressed adaptor-like complex that we have named AP-4. In keeping with the nomenclature of heterotetrameric adaptors, the four subunits of AP-4 were named  $\epsilon$ ,  $\beta 4$ ,  $\mu 4$ , and  $\sigma 4$  (Fig. 1B). All of the AP-4 subunits are structurally related to their counterparts in other AP complexes, suggesting that AP-4 may resemble the

other complexes with regard to overall conformation and role in protein sorting.

The complete primary structures of the  $\beta 4$  and  $\sigma 4$  subunits of AP-4 are reported in this study (Fig. 2). Unlike  $\sigma 4$ , which displays a significant degree of sequence similarity to other AP  $\sigma$  chains throughout the entire molecule,  $\beta 4$  homology to other AP  $\beta$  subunits is restricted to the amino-terminal trunk domain. This domain is thought to mediate association with the other subunits of the AP complexes (32, 33). On the other hand, the hinge-ear regions of  $\beta 1$ ,  $\beta 2$ , and  $\beta 3$  (A or B) have been shown to interact with clathrin (10, 11, 34). A potential clathrin-binding motif, consisting of bulky hydrophobic and acidic residues, has recently been identified in the hinge-ear regions of  $\beta 1$ ,  $\beta 2$ , and  $\beta 3$  (11). Interestingly, although  $\beta 4$  seems to bear hinge- and ear-like domains, these regions are devoid of such a motif. This is consistent with the observations that AP-4 was not enriched in a preparation of clathrin-coated vesicles from bovine brain and that a GST- $\beta 4_C$  fusion protein failed to bind clathrin under conditions in which similar GST- $\beta 2$  or - $\beta 3A$  fusion proteins displayed significant clathrin binding activity.<sup>2</sup> Since these results are negative, however, further work will be required to establish whether *in vivo* AP-4 interacts with clathrin or is a component of a non-clathrin coat.

The  $\epsilon$  subunit of AP-4 was identified by immunoprecipitation-recapture as the product of a novel cDNA for which we

<sup>2</sup> E. C. Dell'Angelica, and J. S. Bonifacino, unpublished results.



**FIG. 8. Double immunofluorescence staining of AP-4 and Golgi markers.** HeLa cells transfected with HA epitope-tagged constructs of furin (A–C) and TGN38 (D–F), as well as untransfected cells (G–I), were fixed with methanol/acetone and incubated simultaneously with the rabbit anti- $\beta_4C$  antibody (A, C, D, F, G, and I; red) and with monoclonal antibodies to either the HA epitope (B, C, E, and F; green) or to the Golgi 58-kDa protein (H and I; green). Bound antibodies were revealed with Cy3-conjugated anti-rabbit Ig and Alexa448-conjugated anti-mouse Ig. Bar, 10  $\mu$ m.

have obtained only a partial sequence. Similar experiments also allowed us to identify the  $\mu_4$  subunit of AP-4 as a protein previously named  $\mu$ -ARP2 (18). This protein belongs to the family of related AP  $\mu$  subunits that have been shown to interact with tyrosine-based sorting signals (5–7). It is therefore tempting to speculate that  $\mu_4$ , and by extension the whole AP-4 complex, could play a role in sorting events mediated by tyrosine-based signals.

Messenger RNAs encoding the subunits of AP-4 were detected in all tissues examined (this study and Ref. 18). While the expression patterns of the human  $\mu_4$ ,  $\beta_4$ , and  $\epsilon$  are similar to each other, they differ somewhat from that of mouse  $\sigma_4$  (Fig. 3, data not shown, and Ref. 18). The significance of these differences is at present unclear, since mRNA levels do not necessarily correlate with protein levels, especially in the case of multisubunit complexes.

The existence of both cytosolic and membrane-bound pools of AP-4 supports the notion that this complex is a component of a protein coat that cycles between the cytosol and membranes, as is the case for other coats. Cycling of AP-1, AP-3, and the non-clathrin COPI coat is regulated by the small GTP-binding protein, ARF1, with the active (GTP-bound) form of this protein promoting coat association to membranes (19, 22, 35). The drug brefeldin A inhibits exchange of GDP for GTP on ARF1 (29) and therefore causes redistribution of the coats to the cytosol due to a blockage in membrane association. By immunofluorescence microscopy, we observed a dramatic effect of the drug on the subcellular localization of AP-4. Dispersal of AP-4 from a juxtanuclear structure to the cytoplasm occurred within 30–60 s

of incubation of HeLa cells with the drug at 37 °C (Fig. 7C and data not shown). At these short incubation times, the drug also elicited complete redistribution of the Golgi-associated AP-1 and COPI coats but did not affect the distribution of Golgi-associated integral membrane proteins such as galactosyl transferase or TGN46.<sup>2</sup> Although we cannot formally exclude the possibility that the observed effect of the drug on AP-4 distribution is due to the formation of small vesicles containing the complex, our results are consistent with the idea that membrane association of AP-4 may be regulated by ARF1 or a related protein.

Our immunofluorescence microscopy analyses suggest that AP-4 is associated with the TGN or a compartment in the vicinity of the TGN. AP-4 could thus be involved in one of a number of vesicle-budding/sorting events that are thought to take place in post-Golgi compartments. For example, the lysosomal membrane protein Lamp-1, the cation-dependent mannose 6-phosphate receptor, and invariant chain-containing class II molecules of the major histocompatibility complex are all sorted to endosomal or lysosomal compartments from the TGN (36–38). Some endocytic receptors, such as the transferrin receptor and the asialoglycoprotein receptors, have been shown to transit from the TGN to early endosomes prior to their delivery to the cell surface (39, 40). The TGN is also thought to be the site of formation of vesicles carrying cargo to the apical and basolateral plasma membrane of polarized epithelial cells (41). Finally, other sorting events could take place within structures located in the proximity of the TGN, such as some endosomal compartments. AP-4 could be solely responsi-

ble for one of these sorting processes or could function as an alternative adaptor for processes that are also mediated by AP-1 or AP-3. The availability of reagents for AP-4 should now allow experiments aimed at elucidating these issues.

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