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AP-3: an adaptor-like protein complex with ubiquitous expression

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We have identified two closely related human proteins (σ_{3A} and σ_{3B}) that are homologous to the small chains, σ_1 and σ_2, of clathrin-associated adaptor complexes. Northern and Western blot analyses demonstrate that the products of both the σ_{3A} and σ_{3B} genes are expressed in a wide variety of tissues and cell lines. σ_{3A} and σ_{3B} are components of a large complex, named AP-3, that also contains proteins of apparent molecular masses of 47, 140 and 160 kDa. In non-neuronal cells, the 47 kDa protein most likely corresponds to the medium chain homolog p47A, and the 140 kDa protein is a homolog of the neuron-specific protein β-NAP. Like other members of the medium-chain family, the p47A chain is capable of interacting with the tyrosine-based sorting signal YQRL from TGN38. Immunofluorescence microscopy analyses show that the σ_3-containing complex is present both in the area of the TGN and in peripheral structures, some of which contain the transferrin receptor. These results suggest that the σ_3 chains are components of a novel, ubiquitous adaptor-like complex involved in the recognition of tyrosine-based sorting signals.

Keywords: adaptors/coats/endosomes/sorting signals

Introduction

The interaction of signals present in the cytoplasmic domains of integral membrane proteins with cytoplasmic membrane coats is now regarded as a general mechanism of protein sorting at several stages of the endocytic and secretory pathways. Among the best characterized sorting signals are sequences of four to six amino acid residues having a critical tyrosine residue (reviewed by Trowbridge *et al.*, 1993; Bonifacino *et al.*, 1996). These tyrosine-based signals were first implicated in the internalization of endocytic receptors from the cell surface (Davis *et al.*, 1986), a process that involves concentration of the receptors in clathrin-coated pits of the plasma membrane (Goldstein *et al.*, 1979). The recruitment of endocytic receptors to clathrin-coated pits is mediated by an interaction between tyrosine-based signals in the cytoplasmic domains of the receptors and the clathrin-associated adaptor complex AP-2 (Pearse, 1988; Glickman *et al.*, 1989; Chang *et al.*, 1993; Sorkin and Carpenter, 1993; Sosa *et al.*, 1993; Boll *et al.*, 1995; Gilboa *et al.*, 1995; Ohno *et al.*, 1995; Sorkin *et al.*, 1995). The AP-2 complex consists of two large chains (α- and β_2- or β_1-adaptin, ~100 kDa), a medium chain (μ_2, ~50 kDa) and a small chain (σ_2, ~17 kDa) (reviewed by Keen, 1990; Pearse and Robinson, 1990; Kirchhausen, 1993). Recent studies have demonstrated that μ_2 has the ability to bind a subset of tyrosine-based signals with sequence requirements that are consistent with it being the signal-recognition component of the AP-2 complex (Ohno *et al.*, 1995).

In addition to playing a role in endocytosis, some tyrosine-based signals have been shown to function in the biosynthetic targeting of integral membrane proteins to lysosomes (Williams and Fukuda, 1990; Harter and Mellman, 1992; Mathews *et al.*, 1992). Targeting to lysosomes is thought to occur largely through clathrin-coated areas of the *trans*-Golgi network (TGN), although an indirect route through the plasma membrane has also been proposed (Braun *et al.*, 1989; Nabi *et al.*, 1991; Harter and Mellman, 1992; Mathews *et al.*, 1992). TGN clathrin coats contain an adaptor complex known as AP-1, which is structurally similar but distinct from AP-2 (Keen, 1990; Pearse and Robinson, 1990; Kirchhausen, 1993). AP-1 is also composed of two large chains (γ- and β_1- or β_2-adaptin, ~100 kDa), a medium chain (μ_1, ~47 kDa) and a small chain (σ_1, ~19 kDa). Like μ_2, μ_1 has been shown to be capable of interacting with tyrosine-based signals (Ohno *et al.*, 1995).

The mechanisms by which tyrosine-based signals mediate transport to distinct compartments (i.e. endosomes or lysosomes) are still poorly understood. It is likely that different tyrosine-based signals interact preferentially with either AP-2 or AP-1 and that this preferential interaction determines the sorting processes in which particular signals are involved. The problem of fine specificity in the recognition of tyrosine-based signals is certain to be more complex than originally anticipated, as it is now clear that there are additional sorting processes, distinct from internalization and lysosomal targeting, that also rely on tyrosine-based signals. These processes include sorting from early to late endosomes (Rohrer *et al.*, 1996), delivery to specialized endosomal/lysosomal organelles such as MHC class II antigen-processing compartments (Lindstedt *et al.*, 1995; Marks *et al.*, 1995), retrieval to the TGN (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Ponnambalam *et al.*, 1994), and targeting to the basolateral plasma membrane of polarized epithelial cells (reviewed by Matter and Mellman, 1994). The growing diversity of the processes mediated by tyrosine-based sorting signals makes it difficult to explain the function of the signals solely on the basis of interactions with the AP-1 and AP-2 adaptors. It is thus conceivable

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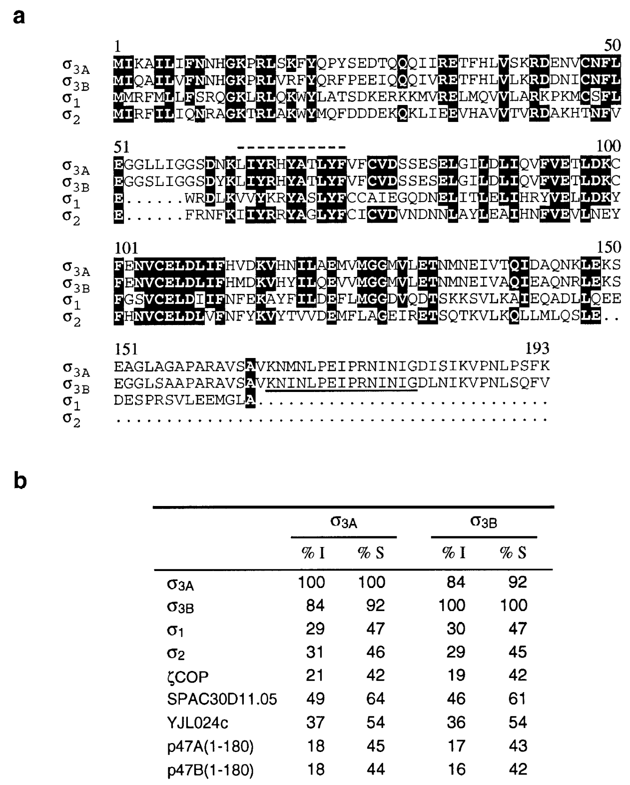
that there may be other adaptor-like complexes that mediate some of the additional sorting functions attributed to tyrosine-based signals.

Ultrastructural studies have provided evidence for the existence of novel membrane coats on endosomes (Whitney *et al.*, 1995; Aniento *et al.*, 1996; Stoorvogel *et al.*, 1996) and on the TGN (Ladinsky *et al.*, 1994), two stations where some of the additional sorting events mentioned above might take place. Some endosomal coats have been shown to contain clathrin but no AP-1 or AP-2 (Stoorvogel *et al.*, 1996). Other endosomal coats seem to be related to the coatomer or COPI (Whitney *et al.*, 1995; Aniento *et al.*, 1996), a non-clathrin coat (Orci *et al.*, 1986). Finally, a novel non-clathrin, adaptor-related complex has been shown to exist in association with the TGN and the plasma membrane of cells of neuronal origin (Newman *et al.*, 1995; Simpson *et al.*, 1996). Biochemical characterization of this complex has revealed a heterotetrameric structure reminiscent of AP-1 and AP-2; the apparent molecular masses of the subunits of this complex are 160, 120, 47 and 25 kDa (Simpson *et al.*, 1996). The ~120 kDa subunit corresponds to β-NAP, a recently described neuron-specific homolog of β_1- and β_2-adaptin (Newman *et al.*, 1995), while the ~47 kDa subunit corresponds to either p47A or p47B, homologs of the adaptor medium chains μ_1 and μ_2 (Pevsner *et al.*, 1994). The 160 and 25 kDa subunits were not identified, although they are presumably related to α- and γ-adaptin and to the adaptor small chains σ_1 and σ_2, respectively. The antibodies to β-NAP and to the p47 isoforms used in the characterization of this complex detected its presence almost exclusively in neuronal or neuroendocrine cells, suggesting that the complex must play a role in the function of the nervous system (Newman *et al.*, 1995; Simpson *et al.*, 1996). The fact that p47A is also expressed in non-neuronal tissues (Pevsner *et al.*, 1994), however, suggests that a similar complex might exist in other cell types.

In this paper, we report the existence of a ubiquitously expressed adaptor-like protein complex, named AP-3, that shares with AP-1 and AP-2 the property of interacting with tyrosine-based signals. The identification of this complex was made possible by the cloning of two novel cDNAs encoding proteins that have ~30% sequence identity to the adaptor small chains σ_1 and σ_2. The two proteins, named σ_{3A} and σ_{3B}, are very closely related to each other (84% identity at the amino acid level) and are expressed in all cells and tissues examined. An antibody that recognizes both σ_3 proteins immunoprecipitates from non-neuronal cells a complex similar to the β-NAP-containing complex previously described in cells of neuronal origin. The complex consists of an as yet unidentified ~160 kDa protein, a ~140 kDa protein related to β-NAP, the medium-chain homolog p47A, and a low-molecular-mass protein corresponding to either σ_{3A} or σ_{3B}. Analyses using the yeast two-hybrid system demonstrate that the p47A subunit of this complex interacts specifically with tyrosine-based signals. Immunofluorescence microscopy analyses suggest that the complex is associated, at least in part, with an endosomal compartment. Thus, this complex may be responsible for the recognition of tyrosine-based signals in sorting processes that do not involve AP-1 or AP-2.



	σ_{3A}		σ_{3B}	
	% I	% S	% I	% S
σ_{3A}	100	100	84	92
σ_{3B}	84	92	100	100
σ_1	29	47	30	47
σ_2	31	46	29	45
ζCOP	21	42	19	42
SPAC30D11.05	49	64	46	61
YJL024c	37	54	36	54
p47A(1-180)	18	45	17	43
p47B(1-180)	18	44	16	42

Fig. 1. Comparison of the primary structures of σ_{3A} and σ_{3B} with those of other members of the adaptor small chain family and of members of the adaptor medium-chain family. The cDNA sequences of σ_{3A} and σ_{3B} were deposited in the EMBL Nucleotide Sequence Database with accession numbers X99458 and X99459, respectively. (**a**) Multiple sequence alignment of human σ_{3A}, human σ_{3B}, mouse σ_1 and rat σ_2. Sequences were aligned using the PILEUP program. Residues that are conserved in at least three of the four sequences are highlighted on a black background. A dashed line marks the segment corresponding to the adaptor complex small chain signature (PROSITE accession code PS00989). The sequence of σ_{3B} that was used to raise a polyclonal antibody is underlined. (**b**) Identity (I) and similarity (S) values obtained by comparing the primary structures of σ_{3A} and σ_{3B} with the following amino acid sequences [SWISS-PROT (sp) or GeneBank (gb) accession codes are given in parentheses]: mouse σ_1 (sp: Q00382), rat σ_2 (sp: Q00380), bovine ζ-COP (sp: P35604), *S.pombe* open reading frame SPAC30D11.05 (sp: Q09905), *S.cerevisiae* open reading frame YJL024c (sp: P47064), rat p47A (gb: L07073) and rat p47B (gb: L07074).

Results

Identification of cDNAs encoding two novel adaptor small chain homologs, σ_{3A} and σ_{3B}

A search of the dBEST database of expressed sequence tags for homologs of the known adaptor subunits revealed the existence of two distinct groups of human sequences that encode polypeptides with significant homology to the adaptor complex small chains, σ_1 and σ_2. One cDNA clone from each group was obtained from public repositories and sequenced. Alignment of the translated sequences with σ_1 and σ_2 indicated that both clones lacked portions of their 5′ open reading frames; the missing segments were isolated by a 5′ RACE procedure. The compiled sequences predict two proteins that display 84% identity and 92% similarity relative to each other (Figure 1). The two novel proteins

were named σ_{3A} and σ_{3B} because of their homology to σ_1 and σ_2 and because they are so closely related to each other. Both σ_{3A} and σ_{3B} share ~30% identity and ~47% similarity with mouse σ_1 and rat σ_2 over almost the entire length of their sequences (Figure 1). The sequences of the small chains diverge towards the carboxy-terminus, with σ_{3A} and σ_{3B} having an additional extension that makes the predicted proteins larger than σ_1 and σ_2. The predicted molecular masses of σ_{3A} and σ_{3B} are 21 732 and 22 017 Da, and their calculated isoelectric points are 5.2 and 5.1, respectively.

Additional database searches revealed that σ_{3A} and σ_{3B} display significant homology not only to σ_1 and σ_2 but also to the coatomer subunit ζ-COP (Kuge *et al.*, 1993) and to the conceptual translations of two open reading frames from the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Figure 1b). All of these sequences contain a segment conforming to the consensus [L, I, V, M]$_2$Y[K, R]X$_4$LYF (indicated by a dashed line in Figure 1a), which is considered a signature for the small chains of adaptor-like complexes (according to the PROSITE database). In addition, the amino acid sequences of σ_{3A} and σ_{3B} exhibit a low but significant degree of similarity to the amino-terminal half of members of the medium chain family, particularly to p47A and p47B (Figure 1b); this relatedness was previously noted in a comparison of other small and medium chains (Cosson *et al.*, 1996). These observations suggest that all of the above proteins evolved from an ancestral precursor and that they are likely to share some structural or functional features.

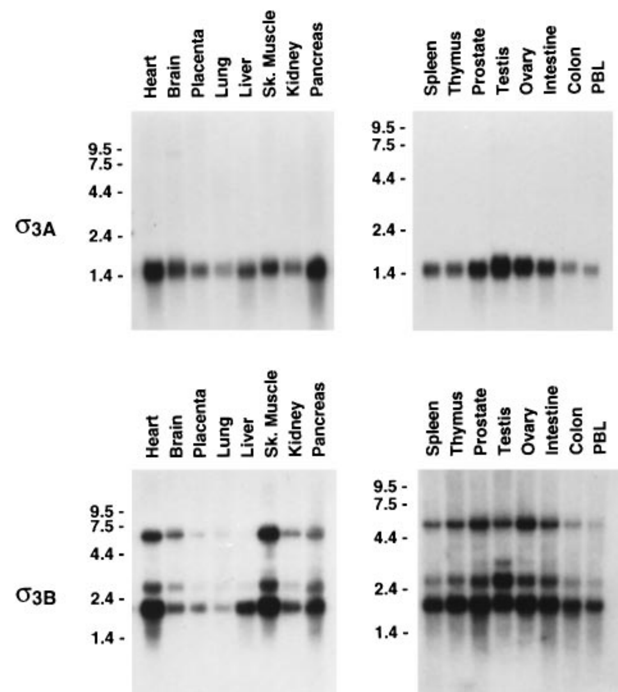


Fig. 2. Northern blot analysis of the expression of σ_{3A} and σ_{3B} mRNAs in various human tissues. Multiple tissue RNA blots were analyzed with ^{32}P-labeled probes for σ_{3A} and σ_{3B} mRNAs. The migration of RNA size markers (in kb) is indicated on the left of each blot. One mRNA species was observed for σ_{3A} and three for σ_{3B} in all tissues examined. The same three RNA species were observed using two different probes derived from either the 3′ untranslated region (shown in the figure) or from the open reading frame (not shown) of the σ_{3B} cDNA.

Expression of the σ_{3A} and σ_{3B} mRNAs in multiple tissues and cells

Because some of the subunits of adaptor-related complexes are expressed in all cells while others are neuron-specific, it was of interest to examine the pattern of tissue expression of the σ_{3A} and σ_{3B} mRNAs. Northern analyses of multiple tissue blots revealed the presence of mRNA species for both proteins in all tissues examined, although their expression levels varied widely (Figure 2). For both σ_{3A} and σ_{3B}, the mRNAs were particularly abundant in heart, skeletal muscle, pancreas, testis and ovary. The σ_{3A} mRNA consisted of a single species of 1.5 kb while three mRNA species of 2.0, 2.9 and 6.0 kb were observed for σ_{3B}. The same species of σ_{3A} and σ_{3B} mRNAs were detected in all human cell lines examined, including M1 (fibroblast), MOLT-4 and Jurkat (T-cell lymphomas), HeLa (epitheloid carcinoma), H4 (neuroglioma), RD4 (rhabdomyosarcoma) and NTera2 (embryonal carcinoma) (data not shown). These results suggest that expression of σ_{3A} and σ_{3B} is widespread among different cell types rather than restricted to a particular type, as is the case for β-NAP (Newman *et al.*, 1995) and p47B (Pevsner *et al.*, 1994).

Detection of the σ_{3A} and σ_{3B} proteins using a specific antibody

In order to study the properties of the σ_{3A} and σ_{3B} proteins, we prepared a rabbit polyclonal antibody to a sequence near the carboxy-terminus of σ_{3B} (underlined in Figure 1a); this sequence is almost identical to the analogous sequence in σ_{3A} but has no correlate in either σ_1 or σ_2. The antibody reacted with both GST–σ_{3A}(113–193) and GST–σ_{3B}(113–193) fusion proteins on Western blot analyses (data not shown); we therefore refer to the species detected by this antibody generically as σ_3. As expected, the antibody did not cross-react with components of the AP-1 and AP-2 adaptor complexes (data not shown). Western blot analyses revealed a major protein species of ~20 kDa in human cells of both non-neuronal (M1, RD4, HeLa) and neuronal (H4, SK-N-MC, SK-N-SH) origins (Figure 3a), thus confirming the predicted size and the widespread distribution of these proteins. A small amount ($<5\%$) of faster-migrating species was observed in some cell lines, most notably in RD4 and HeLa cells (Figure 3a). Recognition of both the major and the minor species by the antibody was specific, as demonstrated by inhibition of antibody binding by the corresponding σ_{3B} peptide (Figure 3a). The apparent molecular mass of σ_3 was dependent on the type of gel system used. The values obtained for the major species in Tricine and Laemmli gels were ~20 and 23 kDa, respectively. This behavior was also observed for *in vitro*-translated σ_{3A} and σ_{3B} (data not shown).

Western blot analyses were used to estimate the amount of σ_3 present in M1 cells. To this end, we compared the signal generated from a known number of cells with a calibration curve obtained using known amounts of the GST–σ_{3B}(113–193) fusion protein (data not shown). This method yielded a quantity of ~1×10^5 copies of σ_3 per cell. We were also interested in determining which of the two forms of σ_3 (σ_{3A} or σ_{3B}) was the predominant species in M1 cells. Fortunately, *in vitro*-translated σ_{3A} and

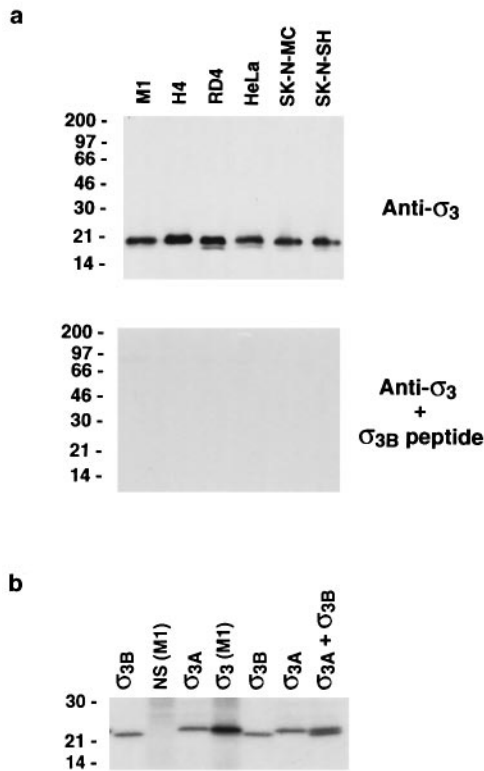


Fig. 3. Analysis of the expression of σ_3 species in various human cell lines and comparison of its electrophoretic mobility with those of *in vitro*-translated σ_{3A} and σ_{3B}. (**a**) Western blot analysis. Whole-cell lysates from the human cell lines indicated in the figure were resolved by Tricine –SDS–PAGE (10% acrylamide) and subsequently blotted onto PVDF membranes. The blots were probed with an affinity-purified antibody to σ_3, in the absence or the presence of a competing peptide derived from σ_{3B} (0.25 mM). (**b**) Comparison of the electrophoretic mobilities of *in vitro*-translated σ_{3A} and σ_{3B} with that of σ_3 from M1 cells. Endogenous σ_3 was immunoprecipitated from ^{35}S-labeled M1 cells with the anti-σ_3 antibody. A non-specific (NS) control for the immunoprecipitation from M1 cells was performed by using an irrelevant rabbit antibody. Samples were fractionated on a 4–20% gradient SDS–PAGE gel (Laemmli system) and protein bands were detected by fluorography. In both (a) and (b), the positions of molecular mass standards (in kDa) are indicated on the left.

σ_{3B} displayed different electrophoretic mobilities on both Laemmli (Figure 3b) and Tricine (not shown) gels, with σ_{3A} migrating more slowly than σ_{3B}. Endogenous σ_3 isolated from metabolically labeled M1 cells co-migrated with σ_{3A} (Figure 3b), thus suggesting that σ_{3A} is the predominant species in M1 cells.

σ_3 exists as part of both a cytosolic and a membrane-bound pool

A characteristic of both clathrin and non-clathrin coats is that they cycle between cytosolic and membrane-bound pools (Rothman and Wieland, 1996; Schekman and Orci, 1996). To assess whether σ_3 could potentially share this property with other coat proteins, we determined the relative amounts of σ_3 in cytosol (C) and total membrane (M) fractions. After homogenization of M1 cells in either a KCl-containing (Figure 4a, A) or a sucrose-containing buffer (Figure 4a, B), σ_3 partitioned into both the cytosolic and membrane fractions. The amount of membrane-associated σ_3 was higher in lysates prepared with the sucrose-containing buffer (~80%) than in those obtained with the KCl-containing buffer (~65%), presumably because KCl caused partial dissociation of the protein from membranes. To study the nature of the association of σ_3 with membranes, microsomes from M1 cells were incubated with different concentrations of urea or salts; after centrifugation, we tested for the presence of the protein in supernatant (S) and pellet (P) fractions. The membrane-associated form of σ_3 was resistant to 3 M urea but could be extracted with 0.2 M Na_2CO_3 and with various concentrations of NaCl and Tris–HCl (Figure 4b), thus indicating that σ_3 behaves as a peripheral membrane protein. Therefore, the distribution of σ_3 and the nature of its association with membranes are consistent with it being a component of a membrane coat. Additional Western blot analyses revealed that σ_3 was barely detectable in preparations of purified clathrin-coated vesicles, in contrast to α-adaptin which was highly enriched in such preparations (data not shown). This observation suggests that σ_3 is not associated with clathrin-coated vesicles.

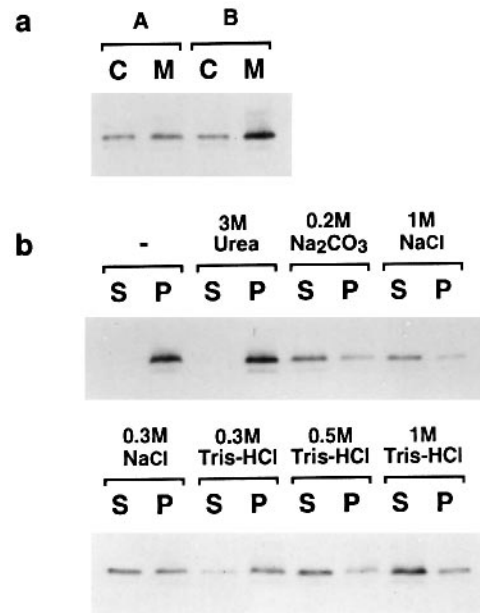


Fig. 4. Analysis of the association of σ_3 with membranes. (**a**) M1 cells were lysed in either buffer A (10 mM HEPES, pH 7.0, 0.15 M KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM dithiothreitol) or buffer B (10 mM HEPES, pH 7.0, 0.25 M sucrose, 1 mM EGTA, 0.5 mM $MgCl_2$). Cytosol (C) and membrane (M) fractions were prepared by centrifugation. (**b**) Membranes prepared in buffer A were extracted with 0.2× buffer A containing either no additions (–) or the additives indicated in the figure. Supernatant (S) and pellet (P) fractions were prepared by centrifugation. In both (a) and (b), the presence of σ_3 was determined by Western blot analysis.

σ_3 is a component of a large-sized complex

To determine whether σ_3 is part of a complex, cytosol from M1 cells was fractionated by gel filtration on Superose 6 and σ_3 was detected by Western blotting (Figure 5a). The vast majority of cytosolic σ_3 was found to elute as a homogeneous, large-sized species with a Stokes' radius of ~85 Å. A small amount of σ_3 peaked at later fractions, corresponding to a Stokes' radius of ~28 Å (Figure 5a). σ_3 extracted from membranes behaved similarly to cytosolic σ_3, except for the absence of the smaller species (Figure 5b). Thus, both the soluble and membrane-associated forms of σ_3 are components of a large complex.

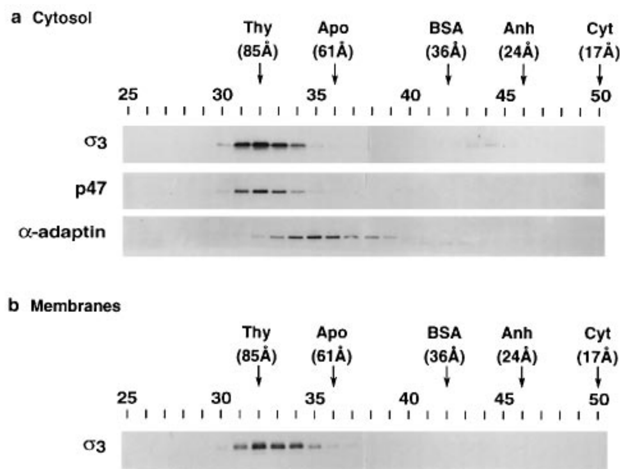


Fig. 5. Gel filtration analysis of σ_3. (**a**) Cytosol from M1 cells was fractionated by gel filtration on Superose 6. Fractions 25 to 50 were analyzed by SDS–PAGE and Western blotting. The blots were divided into three horizontal strips that were probed with antibodies to σ_3, p47 or α-adaptin, as indicated in the figure. (**b**) A membrane pellet from M1 cells was extracted with 0.2× buffer A containing 0.5 M Tris–HCl, and the solubilized material was fractionated on a Superose 6 column. The column fractions were analyzed for the presence of σ_3 as described above. The elution positions of thyroglobulin (Thy), apoferritin (Apo), bovine serum albumin (BSA), carbonic anhydrase (Anh) and cytochrome c (Cyt) are indicated; their Stokes' radii are given in Ångstroms.

The σ_3-containing complex appears to be larger than AP-2 (Stokes' radius ~66 Å), as demonstrated by probing of the same blots with an antibody to α-adaptin (Figure 5a).

Components of the σ_3-containing complex

To identify other components of the σ_3-containing complex, we took advantage of the fact that our antibody to σ_3 is capable of immunoprecipitating the metabolically labeled protein from M1 cells (Figure 3b). In order to separate background bands, cytosol from metabolically labeled M1 cells was fractionated on a linear sucrose gradient before immunoprecipitation of σ_3 from each fraction. Using this method, we could clearly see that the anti-σ_3 antibody immunoprecipitated, in addition to σ_3, three other species of apparent molecular masses 47, 140 and 160 kDa (Figure 6, lower panel). Immunoprecipitation of the three new species was specific, as demonstrated by their absence in the non-specific control immunoprecipitation (Figure 6, upper panel). These three species co-sedimented with σ_3 on the gradients, further demonstrating that they are all part of a single complex. Thus, the subunit composition of the σ_3-containing complex resembles that of AP-1 and AP-2 as well as that of the neuron-specific, β-NAP-containing complex (Simpson *et al.*, 1996).

What are the other components of the σ_3-containing complex? The recently described medium-chain homolog, p47A, is a likely candidate for the ~47 kDa species because it has a predicted molecular mass in the region of 47 kDa and, unlike its closely related homolog p47B, it is expressed in all tissues examined so far (Pevsner *et al.*, 1994). To address this issue experimentally, we prepared a polyclonal antibody to residues 393–404 of p47A. The affinity-purified antibody reacted on Western blots with both GST–p47A and GST–p47B but did not cross-react with the adaptor medium chains μ_1 and μ_2

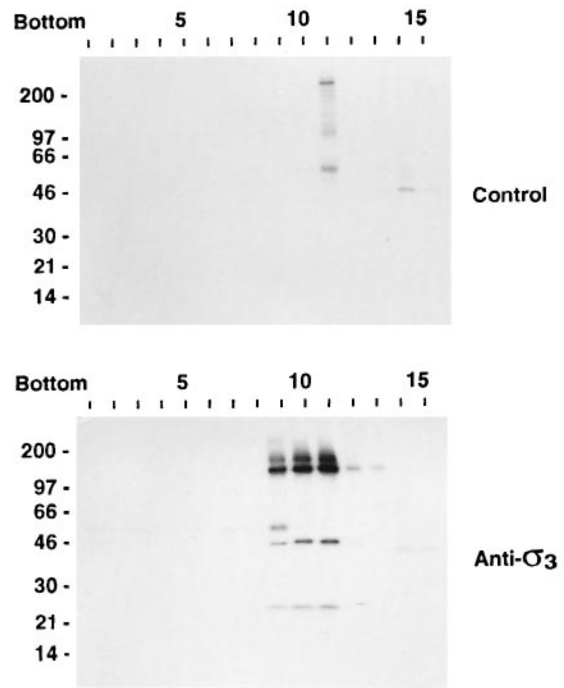


Fig. 6. Immunoprecipitation of a σ_3-containing complex from cytosolic samples fractionated on a 5–20% linear sucrose gradient. A cytosolic extract of [^{35}S]methionine-labeled M1 cells was fractionated by centrifugation on a 5–20% linear sucrose gradient. Fractions were collected from the bottom of the gradients and immunoprecipitated with either an irrelevant antibody (control) or an antibody to σ_3. Immunoprecipitates were resolved by 4–20% SDS–PAGE (Laemmli system). The positions of molecular mass markers (in kDa) are indicated on the left. Notice the co-precipitation of a complex of proteins of apparent molecular masses 160, 140, 47 and 23 kDa in fractions 9–11 of the gradient.

(data not shown). This anti-p47 antibody recognized on Western blots a ~47 kDa protein from M1 cells which co-eluted with σ_3 on Superose 6 gel filtration (Figure 5a). Further evidence that p47A is a component of the σ_3-containing complex was obtained from immunoprecipitation–recapture experiments. In these experiments, the complex was first isolated with the anti-σ_3 antibody and then denatured to induce dissociation of the complex and unfolding of the polypeptide chains. The denatured sample was subjected to re-precipitation with either irrelevant antibodies or antibodies to σ_3 or p47. The negative and positive control re-precipitations gave the expected results: no bands were seen in the lanes corresponding to the irrelevant antibodies (to TGN38 or BSA), while a 23 kDa protein band was seen in the anti-σ_3 lanes (Figure 7). Variable amounts of high-molecular-mass (140–160 kDa) species were observed in the lane corresponding to the anti-σ_3 antibody (Figure 7), presumably reflecting an incomplete dissociation of the complex during the denaturation step. As hypothesized, the anti-p47 antibody recognized the ~47 kDa subunit of the complex isolated from both M1 fibroblasts and HeLa cells (Figure 7), suggesting that p47A is a component of the σ_3-containing complex. A similar result was obtained with the neuroblastoma-derived cell line SK-N-SH (Figure 7), although in this case it is uncertain whether the ~47 kDa subunit corresponds to p47A or p47B.

In a recent report (Simpson *et al.*, 1996), p47B was shown to be a component of a protein complex with

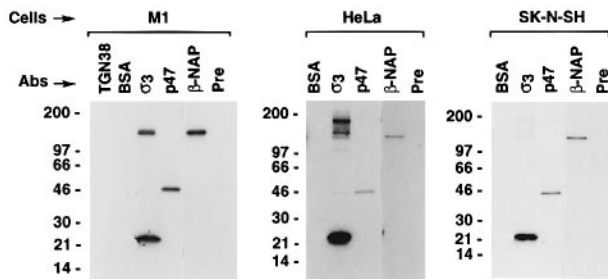


Fig. 7. Identification of components of the σ_3-containing complex by immunoprecipitation–recapture. The σ_3-containing complex was immunoprecipitated from metabolically labeled M1, HeLa and SK-N-SH cells with an antibody to σ_3. The immunoprecipitated complex was denatured by heating in the presence of SDS. After dilution with buffer, aliquots were subjected to re-precipitation with irrelevant antibodies (to TGN38 or BSA), antibodies to σ_3 or to p47, an antiserum to β-NAP or its corresponding preimmune serum (Pre). Bound proteins were resolved by 4–20% gradient SDS–PAGE (Laemmli system) and visualized by fluorography. The positions of molecular mass markers (in kDa) are indicated on the left of each panel.

the neuron-specific β-adaptin homolog, β-NAP (Newman *et al.*, 1995). It is thus possible that an as yet unknown, ubiquitous β-NAP-like protein might be a component of the σ_3- and p47A-containing complex in non-neuronal cells. We prepared an antiserum against a GST–β-NAP(647–796) fusion protein and used this antiserum to probe the identity of the σ_3-associated proteins by immunoprecipitation–recapture. As shown in Figure 7, the antiserum recognized the ~140 kDa subunit of the complex from M1, HeLa and SK-N-SH cells. The specificity of the interaction was evidenced by the absence of bands in the lanes corresponding to the preimmune serum (Figure 7), and by the fact that the immunoprecipitation of the ~140 kDa species could be blocked by an excess of GST–β-NAP(647–796) but not GST (data not shown). Thus, the ~140 kDa subunit of the σ_3-containing complex from the non-neuronal M1 and HeLa cells is likely to be a ubiquitous counterpart of β-NAP that is recognized by our anti-β-NAP antiserum. In SK-N-SH cells, the ~140 kDa polypeptide could correspond to either β-NAP or its widespread homolog.

Analysis of the interaction of σ_{3A}, σ_{3B}, p47A and p47B with tyrosine-based sorting signals

One of the functions of the clathrin-associated adaptor complexes AP-1 and AP-2 is to recognize tyrosine-based sorting signals present in the cytoplasmic domain of integral membrane proteins (Pearse, 1988; Glickman *et al.*, 1989; Chang *et al.*, 1993; Sorkin and Carpenter, 1993; Sosa *et al.*, 1993; Boll *et al.*, 1995; Gilboa *et al.*, 1995; Ohno *et al.*, 1995; Sorkin *et al.*, 1995). This recognition is a property of the respective medium chains, μ_1 and μ_2 (Ohno *et al.*, 1995). Since p47A and p47B are members of the medium-chain family (Pevsner *et al.*, 1994) and both σ_{3A} and σ_{3B} have significant homology to the amino-terminal half of the medium chains (this study), we decided to test whether these proteins are capable of interacting with tyrosine-based signals. This was done using a yeast two-hybrid approach (Ohno *et al.*, 1995), in which p47A, p47B, σ_{3A} and σ_{3B} were expressed as fusions with the GAL4 activation domain. Expression of the fusion proteins was confirmed by Western blot analysis using antibodies to σ_3 and p47 (data not shown). These constructs were tested for interaction with the tyrosine-based signal, YQRL, from TGN38 (Bos *et al.*, 1993; Humphrey *et al.*, 1993) and with a mutant of this signal, AQRL, both presented in the context of the GAL4 DNA binding domain. Interactions were evidenced by growth of the co-transformed yeast cells on histidine-deficient plates and by the expression of β-galactosidase activity. As shown in Figure 8, p47A, but not σ_{3A} and σ_{3B}, was able to interact specifically with the tyrosine-based signal in both assays. The magnitude of the interaction of p47A with the YQRL signal was comparable with that observed for μ_2 (Figure 8). p47B also displayed specific interaction with the YQRL signal, but only in the growth assay and not in the β-galactosidase assay (Figure 8). This different reactivity in the two assays is probably due to the fact that the growth assay is more sensitive than the β-galactosidase assay. Thus, the affinity of p47B for the YQRL signal seems to be relatively low. Nevertheless, these observations suggest that both p47A and p47B share with μ_1 and μ_2 the property of recognizing tyrosine-based sorting signals.

Immunofluorescence microscopy localization of σ_3 in cultured cells

In order to examine the subcellular localization of σ_3, we performed indirect immunofluorescent staining of fixed-permeabilized HeLa cells using an antibody to σ_3. The cells displayed a punctate staining of the cytoplasm that was more pronounced in a juxtanuclear area but extended towards the periphery of the cell (Figure 9a). Incubation with a specific σ_3 peptide but not with an irrelevant peptide inhibited staining of both the juxtanuclear and the peripheral structures, indicating that they both represent specific labeling (data not shown). A similar pattern was observed in M1 cells (data not shown). Treatment of HeLa cells with brefeldin A (BFA), a fungal metabolite that triggers dissociation of COPI and AP-1 from their target membranes (Donaldson *et al.*, 1990; Robinson and Kreis, 1992; Wong and Brodsky, 1992), resulted in substantial dissociation of σ_3 from its membranes within 5 min (Figure 9b). The dissociation was more pronounced after 15 min of BFA addition, at which time most of the protein was found distributed throughout the cytoplasm in a diffuse or finely punctate pattern (Figure 9c). The effects of BFA were reversible, as σ_3 returned to its original distribution at 40 min after BFA removal (Figure 9d).

Double staining with antibodies to σ_3 (Figure 9e) and to an epitope-tagged form of TGN38 expressed by transfection (Figure 9f) showed that the juxtanuclear concentration of σ_3 was in the area housing the TGN, although much of the σ_3-positive structures appeared to be scattered throughout the cytoplasm, well beyond the TGN. We also compared the distribution of σ_3 with that of the transferrin receptor, a marker of early endosomes. Interestingly, we observed partial co-localization of the two proteins in most cells; the co-localization was particularly noticeable in the larger σ_3-containing structures found in the periphery of the cells (Figure 9g and h). The co-localization of σ_3 with the transferrin receptor was even more evident in cells that had undergone BFA treatment for 15 min followed by removal of BFA for 40 min (Figure 9i and j) and in nocodazole-treated cells (data

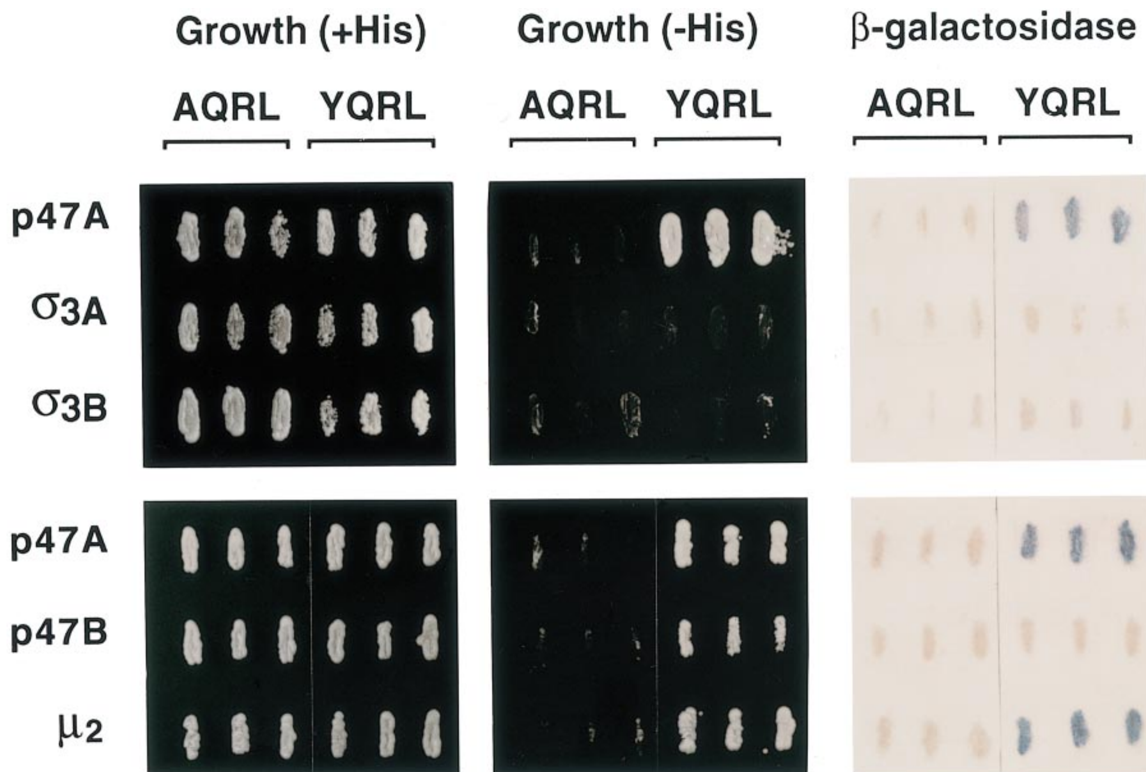


Fig. 8. Two-hybrid analysis demonstrating interaction of p47A and p47B but not σ_{3A} and σ_{3B} with a tyrosine-based sorting signal. Yeast cells were co-transformed with plasmids encoding p47A, p47B, μ_2, σ_{3A} or σ_{3B} fused to the GAL4 DNA binding domain and plasmids encoding the sequences AQRL or YQRL after residues 329–352 of the TGN38 tail fused to the GAL4 transcription activation domain. Three independent clones derived from each co-transformation were tested for their ability to grow on plates lacking histidine (–His) and for expression of β-galactosidase activity (blue color), as shown in the figure.

not shown). In contrast to the transferrin receptor, the lysosomal membrane protein Lamp-1 was not observed in the peripheral structures that stained for σ_3 (Figure 9k and i). Instead, most of the Lamp-1 staining was confined to juxtanuclear vesicles that did not appear to coincide with σ_3-positive structures. These results suggest that a substantial amount of σ_3 is either associated with or adjacent to peripheral endosomal structures containing the transferrin receptor. In addition, a portion of the σ_3 population exists within a juxtanuclear location, in an area of the cell that contains the TGN (Figure 9f), lysosomes (Figure 9i), and both recycling and late endosomes (Yamashiro *et al.*, 1984; Geuze *et al.*, 1988; Griffiths *et al.*, 1988).

Discussion

Cytoplasmic protein coats play major roles in the formation of transport vesicles and in the selection of integral membrane proteins for inclusion into those vesicles (Rothman and Wieland, 1996; Schekman and Orci, 1996). Some coats, such as COPI, COPII, clathrin-AP-1 and clathrin-AP-2, are present in all cells, where they participate in general transport and sorting processes. Other coats, such as the recently described neuron-specific, β-NAP-containing coat, have a more restricted pattern of tissue expression, and are probably involved in specialized sorting functions. In the present study, we demonstrate the existence of another ubiquitously expressed adaptor-like complex (AP-3) which may be a component of a novel membrane coat.

The key to the identification of this complex was the cloning and sequencing of two cDNAs encoding closely related proteins (σ_{3A} and σ_{3B}) that are homologous to the σ_1 subunit of AP-1 and to the σ_2 subunit of AP-2. The σ_{3A} and σ_{3B} gene products were expressed in all tissues and cell lines examined, both at the mRNA and protein levels. In addition, close homologs of σ_{3A} and σ_{3B} exist in *S.cerevisiae* and *S.pombe*, further suggesting that these proteins must play a role in some basic transport or sorting process common to all cells.

The σ_3 proteins were found to be components of a complex with a subunit composition reminiscent of AP-1 and AP-2 and very similar to that of the neuronal β-NAP-containing complex (Simpson *et al.*, 1996). In all cells examined in this study, the complex consisted of a ~23 kDa protein (σ_{3A} or σ_{3B}), a ~47 kDa protein recognized by antibodies to p47A and p47B (Pevsner *et al.*, 1994), a ~140 kDa protein cross-reactive with the neuron-specific β-NAP (Newman *et al.*, 1995) and an unidentified ~160 kDa protein. In non-neuronal cells, the ~47 kDa protein probably corresponds to p47A, while the ~140 kDa protein is likely to be a homolog of β-NAP. Based on information found in expressed sequence tag databases, we have recently identified a non-neuronal protein that displays significant homology to β-NAP over the region used to raise our polyclonal antibodies to β-NAP. Northern blot analyses show that the mRNA for this novel protein is expressed in both neuronal and non-neuronal cell lines

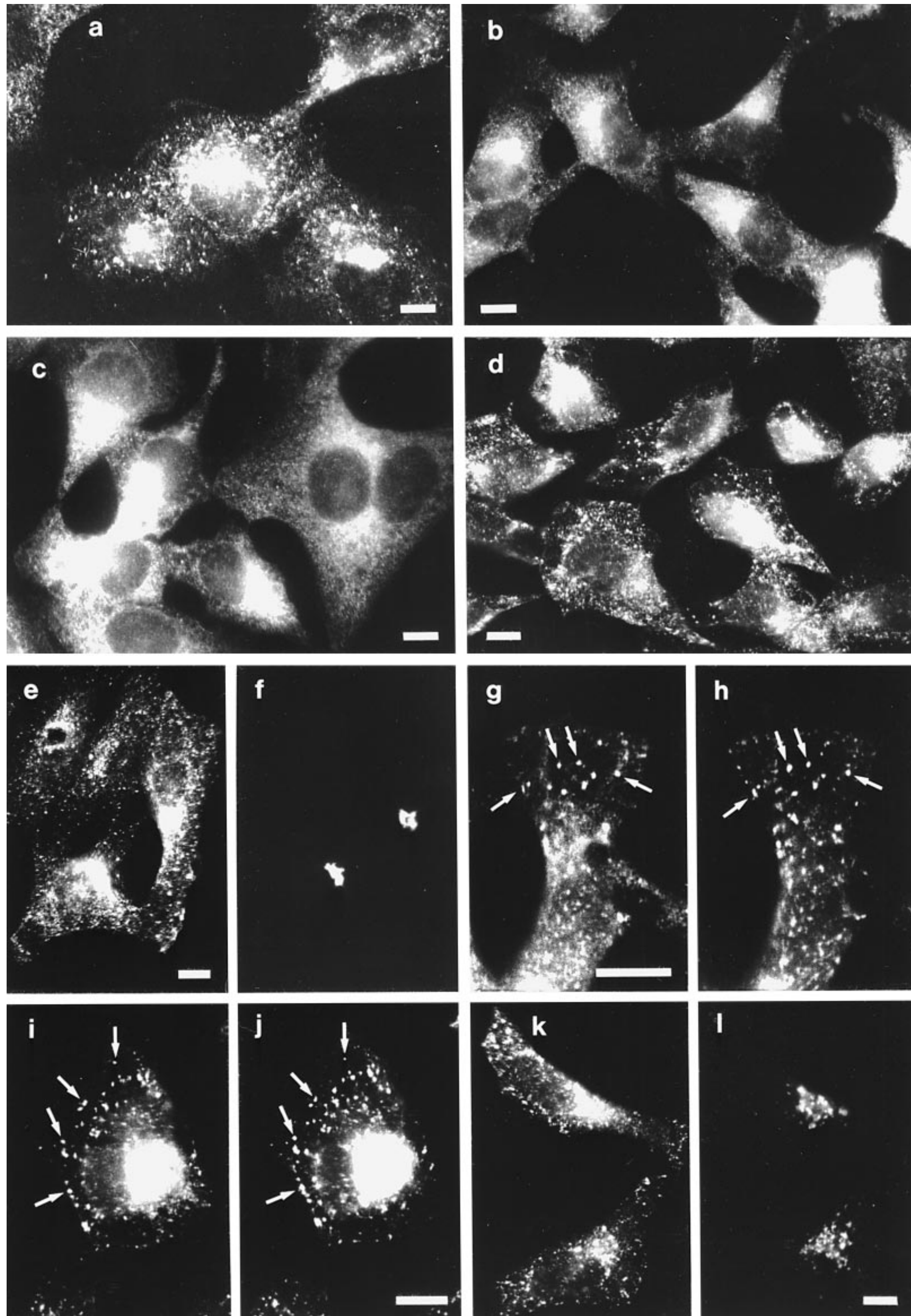


Fig. 9. Subcellular localization of σ_3 examined by immunofluorescence microscopy. (a–d) Effect of BFA on the distribution of σ_3 in HeLa cells. (**a**) Untreated cells; (**b**) cells treated for 5 min with 2 µg/ml BFA; (**c**) cells treated for 15 min with 2 µg/ml BFA; (**d**) cells treated for 15 min with 2 µg/ml BFA followed by 40 min in the absence of BFA. After each treatment, cells were fixed with formaldehyde, permeabilized with saponin, and stained with rabbit antibodies to σ_3 and Cy3-conjugated antibodies to rabbit IgG. (e–l) Comparison of the co-localization of σ_3 with various organellar markers in HeLa cells. (e, f) Normal cells stained for (**e**) σ_3 and (**f**) an epitope-tagged form of TGN38. (g and h) Normal cells stained for (**g**) σ_3 and (**h**) transferrin receptor. (i and j) Cells treated for 15 min with BFA and 40 min in the absence of BFA stained for (**i**) σ_3 and (**j**) transferrin receptor. (k, l) Normal cells stained for (**k**) σ_3 and (**l**) Lamp-1. Cells were fixed with formaldehyde, permeabilized with saponin, and stained with rabbit antibodies to σ_3 and mouse antibodies to the other markers, followed by Cy3-conjugated antibodies to rabbit IgG and fluorescein-conjugated antibodies to mouse IgG. Arrows point to structures in which σ_3 and the transferrin receptor co-localize. Bars, 10 µm.

(E.Dell'Angelica and J.Bonifacino, unpublished observations). Thus, we suggest that this β-NAP homolog is a likely candidate for the ~140 kDa component of the non-neuronal complex. In neuronal cells, σ_{3A} or σ_{3B} could be components of both a neuron-specific complex (containing p47B and β-NAP) and a ubiquitous complex (containing p47A and a β-NAP homolog). The subunits of both complexes could be interchangeable, as is the case for the closely related β_1- and β_2-adaptin (Page and Robinson, 1995).

Essential to the function of organellar coats is the ability to undergo reversible association with membranes. The σ_3-containing complex was found to share this property with all other known coats, as it was found to be partially associated to membranes and to be susceptible to dissociation from membranes by treatment with moderate concentrations of salt. Moreover, the σ_3-containing complex was reversibly dissociated from membranes upon treatment of intact cells with BFA. In this respect, the σ_3-containing complex behaves like COPI and AP-1, both of which are sensitive to BFA (Donaldson *et al.*, 1990; Robinson and Kreis, 1992; Wong and Brodsky, 1992), and unlike AP-2, which is not affected by BFA (Robinson and Kreis, 1992; Wong and Brodsky, 1992). The sensitivity of the σ_3-containing complex to BFA suggests that the regulation of its recruitment to membranes is similar to that of COPI and AP-1 and may involve small GTP binding proteins belonging to the ARF family (Klausner *et al.*, 1992).

What could be the function of the AP-3 complex? By analogy with AP-1 and AP-2, we speculate that one function might be the recognition of sorting signals at some site of transport vesicle formation. In support of this idea, our results show that both p47A and p47B, the medium chains of the ubiquitous and the neuron-specific forms of the complex, respectively, bind the tyrosine-based signal YQRL from TGN38 in a tyrosine-dependent fashion (Figure 8). The fact that μ_1 and μ_2 also interact specifically with tyrosine-based sorting signals (Ohno *et al.*, 1995) suggests that this property is characteristic of members of the medium-chain family of adaptor subunits. This conservation of function is remarkable since p47A, p47B, μ_1 and μ_2 are only 30–40% identical to one another. Experiments are now under way to determine whether the complete complex also binds tyrosine-based sorting signals and to establish the structural features of the signals that interact preferentially with p47A. Despite the homology of σ_{3A} and σ_{3B} to the amino-terminal half of p47A and p47B (Figure 1b), the two small chains do not bind tyrosine-based signals, suggesting that they must play another role within the complex.

In addition to the ability of the σ_3-containing complex to recognize signals, another characteristic that is critical for establishing the function of the complex is its subcellular localization. Our immunofluorescence microscopy analyses show that the endogenous σ_3-containing complex seems to be more concentrated in a juxtanuclear area, suggesting that the protein might be associated with some central organelle such as the TGN. However, the staining extends far beyond the TGN into the periphery of the cells. The partial but significant co-localization of the peripheral σ_3-containing structures with the transferrin receptor observed in our studies suggests that the complex may be associated with some compartment of the endosomal system. The exact nature of this compartment will have to be established by immunoelectron microscopy; our attempts to perform immunoelectron microscopy with the antibodies to σ_3 have so far been unsuccessful, probably because of the lower sensitivity of the techniques employed with respect to immunofluorescence microscopy. Nevertheless, the association of the σ_3-containing complex with endosome-like structures observed by immunofluorescence microscopy suggests that the complex may play a role in protein sorting in peripheral regions of the cell.

Two sorting processes that depend at least in part on tyrosine-based sorting signals deserve consideration as processes that may be mediated by the σ_3-containing complex: basolateral targeting (reviewed by Matter and Mellman, 1994) and transport from early to late endosomes (Rohrer *et al.*, 1996). Targeting of some proteins to the basolateral plasma membrane of polarized epithelial cells has been shown to be dependent on tyrosine-based sorting signals; some of these signals are indistinguishable from endocytic signals, whereas others are not active in endocytosis (Matter and Mellman, 1994). The sorting events that direct transport to the basolateral plasma membrane are thought to occur at both the TGN and endosomes (Matter *et al.*, 1993), locations that are compatible with the apparent distribution of the σ_3-containing complex. Transport of some proteins from early to late endosomes may also be dependent in part on tyrosine-based signals. This includes lysosomal proteins such as Lamp-1 (Harter and Mellman, 1992; Rohrer *et al.*, 1996) and TGN proteins such as TGN38 (Bos *et al.*, 1993; Humphrey *et al.*, 1993) and furin (Schäfer *et al.*, 1995; Voorhees *et al.*, 1995). All of these proteins undergo rapid internalization from the plasma membrane together with recycling receptors such as the transferrin receptor. At some early endosomal location, however, those intracellular proteins must be segregated from recycling receptors, so that they can travel to their respective destinations deeper into the endocytic pathway. We speculate that such a sorting event might take place in some early endosomal compartment. Both the potential of the σ_3-containing complex for recognizing tyrosine-based sorting signals and its apparent localization to endosomes would be compatible with such a role.

Materials and methods

Cells

All cells used in this study were of human origin. HeLa, H4, SK-N-MC and SK-N-SH cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). M1 fibroblasts were kindly provided by E.Long [National Institutes of Health (NIH)]. RD4 cells were a gift from J.Harford (NIH). NTera-2 cells were kindly provided by K.Ozato (NIH). All of the above cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin (complete DMEM). MOLT-4 and Jurkat cells were obtained from the ATCC and cultured in RPMI 1640 medium supplemented with 9% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cloning of σ_{3A} and σ_{3B} cDNAs

The human cDNA clone 131033 (Washington University-Merck EST Project) was obtained from the IMAGE Consortium. The clone consisted of a fragment of the full-length σ_{3A} cDNA spanning nucleotide 268 to the start of the poly(A) tail. The missing portion of the cDNA was

isolated from both human brain and human pancreas Marathon-Ready™ cDNA libraries (Clontech, Palo Alto, CA) by two rounds of 5′ RACE PCR amplification. The specific primers used in the first and second (nested) 5′ RACE reactions were complementary to nucleotides 849–873 and 624–648, respectively. The clone c-2rd10 from the Genexpress cDNA Program, comprising nucleotides 190–1829 of the full-length σ_{3B} cDNA, was obtained from Genethon (Cedex, France). The missing 5′ segment was isolated from a human brain Marathon-Ready™ cDNA library by using a single 5′ RACE reaction. The specific primer was complementary to nucleotides 665–690. Each 5′ RACE product was cloned into the pNoTA/T7 shuttle vector (5 Prime → 3 Prime, Boulder, CO). In each case, at least three independent clones were selected and sequenced. Identical sequences were obtained for the 5′ fragments of the σ_{3A} cDNA which had been isolated from either the brain or pancreas libraries.

Northern blot analysis

Human multiple tissue Northern blots were purchased from Clontech. For the analysis of σ_{3A} and σ_{3B} mRNAs in human cell lines, total RNA was isolated using the TRIzol™ reagent (Gibco-BRL, Gaithersburg, MD), fractionated by denaturing agarose electrophoresis and subsequently blotted onto nylon membranes. A fragment of the σ_{3A} cDNA spanning nucleotide 518 to the start of the poly(A) tail was obtained by digestion of clone 131033 with *Pvu*II and *Not*I. For σ_{3B}, the fragment comprising nucleotides 641–1102 was obtained by digestion of clone c-2rd10 with *Afl*II and *Bgl*II. A second probe corresponding to nucleotides 60–640 of the σ_{3B} cDNA was prepared by digestion of the 5′ RACE product (see above) with *Nco*I and *Afl*II. Probes were ^{32}P-labeled by using the Megaprime™ DNA labeling system (Amersham, Arlington Heights, IL) and diluted in Hybrisol™ I (Oncor, Gaithersburg, MD) before hybridization for 16–24 h at 42°C. Blots were washed four times with 2× SSC, 0.05% (w/v) SDS for 5 min at room temperature, and then twice with 0.1× SSC, 0.1% (w/v) SDS for 30 min at 55°C. For the probes derived from the σ_{3B} cDNA, an additional 30-min incubation at 60°C in 0.1× SSC, 0.5% (w/v) SDS was performed.

Production of fusion proteins

In order to prepare glutathione *S*-transferase (GST) fusion proteins containing amino acid residues 113–193 of σ_{3A} and σ_{3B}, the corresponding segments of the cDNAs (including the stop codon) were engineered by PCR to be cloned into the *Bam*HI–*Eco*RI sites of the pGEX-5X-1 vector (Pharmacia Biotech, Uppsala, Sweden). A GST-fusion protein containing residues 647–796 of human β-NAP (Newman *et al.*, 1995) was generated by PCR amplification of the corresponding fragment from the cDNA clone 165789 (Washington University-Merck EST Project, obtained from the ATCC) and subsequent directional cloning of the PCR product into the *Bam*HI–*Eco*RI sites of the pGEX-5X-1 vector. The cDNAs encoding rat p47A and p47B [kindly provided by J.Pevsner and R.Scheller, Stanford University (Pevsner *et al.*, 1994)], were engineered by PCR for directional cloning into the *Eco*RI–*Xho*I and *Bam*HI–*Sal*I sites of pGEX-5X-1 vector, respectively. The sequence of all the constructs was verified by manual sequencing. For protein expression and purification, competent *Escherichia coli* strain DH5α cells (Gibco-BRL) were transformed with the above constructs. Cell culture, induction of fusion protein expression and batch purification using glutathione–Sepharose 4B beads (Pharmacia Biotech) were performed according to the manufacturer's instructions.

Antibodies

Monoclonal anti-α-adaptin antibody (clone 100/2) was purchased from Sigma Chemical Co. (St Louis, MO). Anti-transferrin receptor antibody (clone B3/25) was obtained from Boehringer Mannheim (Indianapolis, IN). The anti-HA epitope antibody was from BabCo (Richmond, CA). The anti-Lamp-1 antibody clone H4A3 was obtained from the Developmental Studies Hybridoma Bank (John Hopkins University, Baltimore, MD). The sequence corresponding to residues 166–180 of σ_{3B} was used to raise anti-peptide antibodies in rabbits. The anti-σ_3 antibodies were purified by affinity chromatography (Harlow and Lane, 1988) using as a matrix GST–σ_{3A}(113–193) which had been coupled to Affi-Gel 15 (Bio-Rad, Hercules, CA). Anti-p47 antibodies were obtained by immunizing rabbits with a peptide corresponding to residues 393–404 of rat p47A (Pevsner *et al.*, 1994), and were affinity-purified using as the ligand the peptide immobilized to SulfoLink® coupling gel (Pierce, Rockford, IL). Antibodies against β-NAP were raised in rabbits by immunization with the GST–β-NAP(647–796) fusion protein. The presence of antibodies with specificity for β-NAP was tested by Western blot analysis of bovine brain cytosol and of a peptide comprising residues 647–796 of human β-NAP; the latter was obtained by digestion of the GST–β-NAP(647–796) fusion protein with factor Xa (Sigma) and subsequent purification by SDS–PAGE. The anti-β-NAP antiserum was used without further purification. Preimmune sera and antibodies to either TGN38 (Bosshart *et al.*, 1994) or to bovine serum albumin (BSA; Cappel, Cochranville, PA) were used as irrelevant antibody controls.

Preparation and fractionation of lysates from M1 cells

M1 cells were grown to almost confluence and then washed twice with ice-cold phosphate-buffered saline (PBS). Subsequently, cells were scraped and lysed in ice-cold buffer by 15 passages through a 25-gauge syringe. Two alternative homogenization buffers were used: buffer A consisted of 10 mM HEPES, pH 7.0, 0.15 M KCl, 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM dithiothreitol, while buffer B contained 10 mM HEPES, pH 7.0, 0.25 M sucrose, 1 mM EGTA and 0.5 mM $MgCl_2$. In both cases, the following protease inhibitors were used: 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride. To obtain cytosolic and total membrane fractions, the lysate was centrifuged at 120 000 *g* for 90 min at 4°C in a Beckman TLA 45 rotor (Beckman Instruments, Palo Alto, CA). Alternatively, the lysate was first centrifuged at 800 *g* for 15 min, and the resulting supernatant was further centrifuged at 120 000 *g* for 90 min in order to obtain a fraction enriched in microsomal membranes. For extraction experiments, the fraction enriched in microsomes was resuspended in buffer A with the aid of a Potter homogenizer fitted with a Teflon pestle. Aliquots of the resuspended membranes were incubated for 1 h at room temperature in 0.2× buffer A alone or in 0.2× buffer A containing either 3 M urea, 0.2 M Na_2CO_3 (pH 11.3), NaCl (0.3 or 1 M) or Tris–HCl (0.3, 0.5 or 1 M, pH 7.4). After the incubation period, samples were centrifuged at 120 000 *g* for 90 min, and both pellets and supernatants were collected for further analysis.

For gel filtration experiments, 0.1 ml of either cytosol or the material extracted from membranes with 0.2× buffer A/0.5 M Tris–HCl were loaded onto a Superose 6 column (1×60 cm, Pharmacia Biotech) equilibrated and eluted at 4°C with 0.2× buffer A containing 0.3 M Tris–HCl, pH 7.4. The flow rate was 0.4 ml/min. Fractions (0.4 ml) were collected and analyzed by Western blotting. The following proteins were used as standards (Stokes' radii are given in parentheses): bovine thyroglobulin (85 Å), horse spleen apoferritin (61 Å), BSA (36 Å), carbonic anhydrase (24 Å) and horse heart cytochrome c (17 Å).

In vitro translation of σ_{3A} and σ_{3B}

The complete open reading frames (ORFs) of σ_{3A} and σ_{3B} were subcloned into the *Not*I–*Eco*RI sites of the pBluescript II KS phagemid (Stratagene, La Jolla, CA). Eukaryotic *in vitro* translation was carried out by using the TnT® Coupled Reticulocyte Lysate System (Promega, Madison, WI). The translated material was immunoprecipitated with the anti-σ_3 antibody and then analyzed by SDS–PAGE.

Immunoprecipitation experiments

Cells were metabolically labeled for 14–16 h at 37°C with 2.5 mCi of [^{35}S]methionine (Express Protein Label, Dupont-New England Nuclear, Boston, MA) in a mixture of methionine-free and regular DMEM (91:9, v/v) supplemented with 9% (v/v) bovine fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin. After the labeling period, cells were washed twice with ice-cold PBS and then used immediately for the preparation of cytosol (see above) or total cell lysate. The latter was prepared by incubation for 15 min on ice with buffer C [1% (w/v) Triton X-100, 0.3 M NaCl, 50 mM Tris–HCl, 10 mM iodoacetamide, 5 mM EDTA, 0.1% (w/v) BSA, 0.02% (w/v) NaN_3, pH 7.4] containing 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride and 2 μg/ml leupeptin. The lysate was cleared by centrifugation at 15 000 *g* for 15 min at 4°C followed by filtration through a 0.45 μm filter unit (Millipore, Bedford, MA).

For sucrose gradient fractionation, 350 μl of cytosol from metabolically labeled cells was layered on top of a linear 5–20% (w/v) sucrose gradient (total volume 12 ml) in buffer A. The sample was centrifuged in an SW-41 rotor (Beckman Instruments) at 39 000 r.p.m. for 13 h at 4°C. Sixteen 0.76-ml fractions were collected from the bottom of the tube and diluted with 0.5 ml of buffer C prior to immunoprecipitation.

Immunoprecipitations were carried out by incubating the samples for 1 h at 4°C with antibodies that had been previously bound to 15 μl of protein A–Sepharose beads. Subsequently, the beads were washed four times with 0.1% (w/v) Triton X-100, 0.3 M NaCl, 50 mM Tris–HCl, pH 7.4 and once with PBS. Bound proteins were eluted from the beads by treatment for 5 min at 95°C with either

Laemmli buffer (for SDS–PAGE analysis) or 0.1 M Tris–HCl, pH 7.4, 1% (w/v) SDS and 10 mM dithiothreitol (for recapture experiments). In the latter case, the eluted material was diluted 20-fold with buffer C, centrifuged at 15 000 *g* for 15 min to remove aggregates, and then subjected to a second round of immunoprecipitation before SDS–PAGE analysis.

Electrophoresis and Western blotting

SDS–PAGE analysis was performed by using either the Laemmli system (Laemmli, 1970) or the Tricine system (Schägger and von Jagow, 1987). ^{35}S-labeled samples were detected by fluorography. Electroblotting onto either nitrocellulose or PVDF membranes and incubations of the membranes with primary and secondary antibodies were performed as described by Harlow and Lane (1988). Horseradish peroxidase-labeled antibodies were detected by using the ECL system (Amersham).

Yeast two-hybrid analyses

The constructs GAL4bd–YQRL and GAL4bd–AQRL have been described previously (Ohno *et al.*, 1995). The complete ORFs of rat p47A and p47B were engineered by PCR for cloning into the *Eco*RI–*Xho*I and *Bam*HI–*Sal*I sites, respectively, of the pACTII vector (kindly provided by S.Elledge, Baylor College of Medicine, Houston, TX). The ORFs of σ_{3A} and σ_{3B} were engineered by PCR and cloned into the *Bam*HI–*Xho*I sites of the same vector. Yeast transformation and two-hybrid assays were performed according to the instructions for the MATCHMAKER two-hybrid system (Clontech).

Immunofluorescence microscopy

HeLa cells were grown to 50–80% confluence on glass coverslips. In some experiments, cells were transiently transfected (Graham and van der Eb, 1973) with a construct containing TGN38 tagged with the HA epitope (kindly provided by J.Lee, NIH). In another set of experiments, cells were incubated at 37°C with 2 μg/ml BFA in complete DMEM before fixation. Cells were fixed for 10 min at room temperature with 2% formaldehyde in PBS. Successive incubations with primary and secondary antibodies (diluted in PBS, 0.1% saponin, 0.1% BSA) were carried out for 1 h at room temperature. After each incubation period, unbound antibodies were removed by rinsing with PBS. The coverslips were mounted onto glass slides with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL).

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