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γ-COP, a coat subunit of non-clathrin-coated vesicles with homology to Sec21p

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Constitutive secretory transport in eukaryotes is likely to be mediated by non-clathrin-coated vesicles, which have been isolated and characterized [(1989) Cell 58, 329–336; (1991) Nature 349, 215–220]. They contain a set of coat proteins (COPs) which are also likely to exist in a preformed cytosolic complex named coatomer [(1991) Nature 349, 248–250]. From peptide sequence and cDNA structure comparisons evidence is presented that one of the subunits of coatomer, γ-COP, is a true constituent of non-clathrin-coated vesicles, and that γ-COP is related to sec 21, a secretory mutant of the yeast *Saccharomyces cerevisiae*.

Endoplasmic reticulum, Golgi, Vesicular transport, Coatomer; Sec21, Coat protein γ-COP

1. INTRODUCTION

In eukaryotic cells, constitutive secretory protein transport occurs from the endoplasmic reticulum (ER) via the various stations of the Golgi apparatus to the plasma membrane. Individual steps of this transport have been reconstituted in vitro (see for example [4–6]), and a variety of its biochemical parameters have been elucidated (for a review see [7]). Newly synthesized proteins appear to be transported through the Golgi stack in non-clathrin-coated vesicles, which have been isolated and characterized [2]. They contain a set of coat proteins (COPs) (α-, β-, γ-, δ-, ε- and ζ-COP with molecular weights of 160, 107, 98, 61, 36, and 20 kDa, respectively). These proteins show similarity in molecular weight but are immunologically unrelated to the subunits of the clathrin coat involved in endocytotic membrane traffic. β-COP has been characterized at a molecular level and shows some homology to β-adaptin, a protein involved in the clathrin system. By peptide sequence comparison [2,8], β-COP was proven to represent a component of both the non-clathrin-coated transport vesicles and a cytosolic complex, the coatomer. Coatomer consists of subunits of molecular weights identical to α-, β-, γ-, δ-, ε- and ζ-COPs, indicating that it is a preorganized assembly of the coat of non-clathrin-coated vesicles. We have isolated the individual COPs from Golgi-derived non-clathrin-coated vesicles, as well as their counter parts from coatomer. Here we report that γ-COP is a constituent of both the coatomer and the vesicles, and that this Golgi vesicle-derived coat subunit is related to Sec21p, a protein encoded by a gene required for vesicle budding in ER to Golgi transport [9,10]. This finding provides strong evidence that the coatomer is required for vesicular transport in vivo, and in addition that COP-coated vesicles mediate transport both from the ER to the Golgi, as well as within the Golgi stack.

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2. MATERIALS AND METHODS

Preparation of non-clathrin-coated Golgi-derived transport vesicles, of coatomer, isolation of γ-COP, preparation of tryptic peptides and sequencing was as described [2,3]. For cloning of γ-COP cDNAs a degenerated oligonucleotide probe was designed TG(T,C)TC(C,T)-TG(A,G)AA(T,G,A)AT(T,C)TC(T,C)TGGCGGGTGGCAGC, corresponding to the γ-COP-peptide, AATRQEIFQEQ. This probe was used to screen a λgt10 library from bovine brain (random primed, Clontech). Three independent clones were sequenced in the M13 mp18 system with Sequenase (USB).

For the production of anti-γ-COP peptide antibodies, the dodecapeptide, VAATRQEIFQEQ, according to amino acid positions 254–265 of the partial sequence was synthesized (kindly performed by Dr. R. Frank, Heidelberg), and coupled to persuccinylated bovine serum albumin as a carrier [11], which was activated with isobutyl chloroformate. About 20 mol of peptide was covalently linked to 1 mol of carrier. This product was injected into rabbits, and the antiserum obtained was depleted of antibodies directed against the carrier protein by affinity adsorption to persuccinylated bovine serum albumin bound to nitrocellulose. The resulting supernatant was affinity purified by adsorption to nitrocellulose-bound antigen and subsequent elution with 50 mM citrate buffer, pH 2.3 [12]. The eluant was quickly neutralized by the addition of 1 M Tris, pH 8.3

3. RESULTS AND DISCUSSION

In our attempt to characterize the individual non-clathrin-coated vesicle coat subunits, we isolated γ-COP from non-clathrin-coated Golgi-derived vesicles [2], as well as from coatomer [3], by SDS-gel electrophoresis. The protein was submitted to tryptic digestion and the resulting peptides were purified by reverse-phase HPLC on RP 18 columns, and sequenced. A degenerated oligonucleotide probe was synthesized corresponding to one of the peptides (see Materials and Methods) and used to screen a random-primed bovine brain cDNA library (Clontech). The largest insert found was cloned into M13 mp18 and sequenced. This cDNA comprises an open reading frame of 1,569 bp, with a stop codon at position 1,570 (Fig. 1). Four peptides known from microsequencing were found in the derived amino acid sequence (underlined in Fig. 1.). The dodecapeptide corresponding to the segment from amino acid 254–265 in the derived protein sequence was synthesized, linked to a carrier, and used to raise antibodies in a rabbit. Immunological analysis of isolated coatomer with this antiserum is shown in Fig. 2. In the gel system used the migration of β- and γ-COP is reversed as compared to the system used in [2]. In lane 2, coatomer was stained with Coomassie brilliant blue after separation. The positions of the COPs are indicated. As a control, Western blotting was performed with the monoclonal antibody, M3A5, directed against β-COP (and kindly provided by Thomas Kreis) (lane 3 in Fig. 2). The antiserum against the dodecapeptide, VAATRQEIFQEQ, reacted clearly and specifically with γ-COP, as shown in lane 4 in Fig.

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   1 acgggcagcgagggcagcatcgaccgcctcatgaagcagatctcctccttcatgtccgag
     ThrGlySerGluGlySerIleAspArgLeuMetLysGlnIleSerSerPheMetSerGlu

  61 atctcggacgagttcaaggtcgtggtggtgcaggccatcagcgcgctgtgtcagaagtat
  21 IleSerAspGluPheLysValValValValGlnAlaIleSerAlaLeuCysGlnLysTyr

 121 cccgcaagcacgccgtgctcatgaacttcctgttctccatgctgcgggaagagggcggc
  41 ProArgLysHisAlaValLeuMetAsnPheLeuPheSerMetLeuArgGluGluGlyGly

 181 ttcgagtacaagcgggccatcgtggactgcatcatcagcatcatcgaggagaacgcggag
  61 PheGluTyrLysArgAlaIleValAspCysIleIleSerIleIleGluGluAsnAlaGlu

 241 agcaaggagacggggctgtcccacctgtgcgagttcatcgaggactgcgagttcaacgtg
  81 SerLysGluThrGlyLeuSerHisLeuCysGluPheIleGluAspCysGluPheThrVal

 301 ctggccacgcgcatcctgcacctgctgggccaggagggccaggacgacgcaacccttcca
 101 LeuAlaThrArgIleLeuHisLeuLeuGlyGlnGluGlyProGlyProAlaThrLeuPro

 361 agtacatccgcttcatctacaaccgcgtggtgctggagaccgccgaggtcgcgcaggcct
 121 SerThrSerAlaSerSerThrThrAlaTrpCysTrpArgProProArgSerArgArgPro

 421 gtgagtgctctggccaagtttggggcgcagaacgaagagatgctgcccagtatcctggtg
 141 ValSerAlaLeuAlaLysPheGlyAlaGlnAsnGluGluMetLeuProSerIleLeuVal

 481 ctgctgaagaggtgtgtgctggatgacgacaacgaggtcagggaccgggccacctttctac
 161 LeuLeuLysArgCysValMetAspAspAspAsnGluValArgAspArgAlaThrPheTyr

 541 atcaacgtgctggagcagaagcagaaggcgctcaatgcaggctacatcctgaatggtctg
 181 IleAsnValLeuGluGlnLysGlnLysAlaLeuAsnAlaGlyTyrIleLeuAsnGlyLeu

 601 gccgtgtccatccccgggtctggagccgggcgctgcagcagtacacctctggagccgtcggag
 201 AlaValSerIleProGlyLeuGluArgAlaLeuGlnGlnTyrThrLeuGluProSerGlu

 661 aagccccttcgacctcaagtccgtgccccctggccaccgcgccccctggcggagcagaggaca
 221 LysProPheAspLeuLysSerValProLeuAlaThrAlaProLeuAlaGluGlnArgThr

 721 gaaagcaccccggtcaccgccgccaagcagcccgagaaggtggccgccaccccggcaggag
 241 GluSerThrProValThrAlaAlaLysGlnProGluLysValAlaAlaThrArgGlnGlu

 781 atctttccaggagcagctggcggctgtgcccgagttccaggggctggggccctcttccaag
 261 IlePheGlnGluGlnLeuAlaAlaValProGluPheGlnGlyLeuGlyProLeuPheLys

 841 tcctgcctgagcccgtggccctcactgagtcggagacggagtacgtcatccgctgcacc
 281 SerSerProGluProValAlaLeuThrGluSerGluThrGluTyrValIleArgCysThr

 901 aagcacaccttcactgaccacatggtcttccagtttgactgccacgaacacgctcaacgac
 301 LysHisThrPheThrAspHisMetValPheGlnPheAspCysThrAsnThrLeuAsnAsp

 961 cagaccctggagaacgtcacggtgcagatggagccctcggagggcctacgaggtgctgtgt
 321 GlnThrLeuGluAsnValThrValGlnMetGluProSerGluAlaTyrGluValLeuCys

1021 tacgtgcccgcccgagcctgccctacaaccagccccggaaccctgctacacgctggtggcc
 341 TyrValProAlaArgSerLeuProTyrAsnGlnProGlyThrCysTyrThrLeuValAla

1081 gtgcccaaggaagacccccacggcggtggctgcacgttccagctgcgtgatgaagttcacc
 361 LeuProLysGluAspProThrAlaValAlaCysThrPheSerCysValMetLysPheThr

1141 gtcaaggactgcgacccccacgaccgggaggcggacgacgagggttacgaggacgagtat
 381 ValLysAspCysAspProThrThrGlyGluAlaAspAspGluGlyTyrGluAspGluTyr

1201 gtgctggaggatctggaagtcacgatagcggatccacatccagaaggtcatgaagctgaac
 401 ValLeuGluAspLeuGluValThrIleAlaAspHisIleGlnLysValMetLysLeuAsn

1261 ttcgaggcagcctgggacgaggtgggggatgagttccagaaggaggagacgttcaccttg
 421 PheGluAlaAlaTrpAspGluValGlyAspGluPheGlnLysGluGluThrPheThrLeu

1321 tccaccatcaagcacctcgaggaggctgtgggcaatatcgtcaaagttcttaggaatgcac
 441 SerThrIleLysThrLeuGluGluAlaValGlyAsnIleValLysPheLeuGlyMetHis

1381 cctttgtgagcggtctgacaaagtgccggacaacaagaacacgcacacgctgctcctggcc
 461 ProCysGluArgSerAspLysValProAspAsnLysAsnThrHisThrLeuLeuLeuAla

1441 ggcgtgttccggggaggcatgacatcctggtgcgctccccggctgctgcttttggacaca
 481 GlyValPheArgGlyGlyMetAspIleLeuValArgSerProLeuLeuLeuLeuAspThr

1501 gtcactatgcaggtgacagccagaagttcggaggagctgccgggtggacatcgtcttggcg
 501 ValThrMetGlnValThrAlaArgSerSerGluGluLeuProValAspIleValLeuAla

1561 tctgttggg
 521 SerValGly
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Fig. 1. Partial cDNA sequence of γ-COP. A random-primed λgt10 cDNA library from bovine brain (Clontech) was screened with an oligonucleotide probe as described in Materials and Methods, and three independent clones were sequenced. Tryptic peptides from γ- COP, isolated as described in [2,3], were microsequenced and compared with the γ-DNA-derived protein sequence. The four peptides found in the derived sequence are underlined. Amino acids are given in the three letter code.

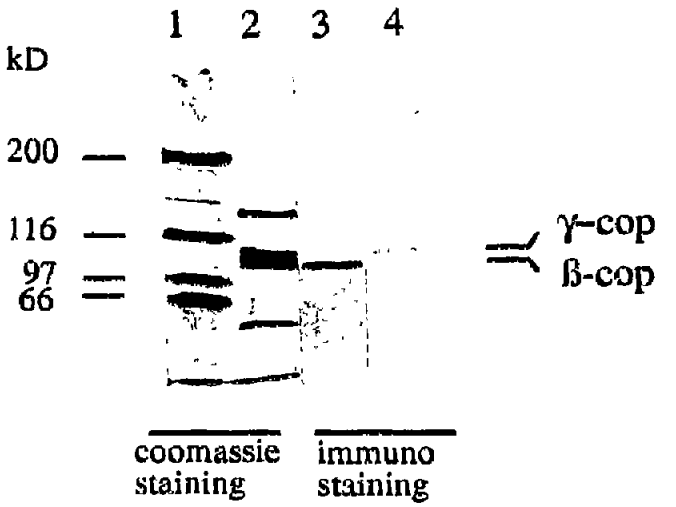

Fig. 2. Antibodies against a cDNA-derived synthetic peptide react with γ-COP. Antibodies against the peptide, VAATRQEIFQEQ, were prepared as described in Materials and Methods. Coatomer isolated according to [3] was separated by SDS-gel electrophoresis on a 6% acrylamide gel with a ratio of monomer to *N,N'*-methylenebisacrylamide of 100.1, with 6 M urea in the separating gel. Lane 1, molecular weight standard proteins; lane 2, coatomer; lane 3, immunostaining after electrotransfer to Immobilon support of coatomer with the monoclonal antibody, M3A5, directed against β-COP (kindly provided by Thomas Kreis), lane 4, sample as in lane 3 immunostained with affinity-purified (see Materials and Methods) antibodies directed against the cDNA-derived dodecapeptide.

2. We take this as confirmation that the cDNA shown in Fig. 1 encodes a 524 amino acid region comprising part of this coat protein. The missing portion of the full-length cDNA is not present in the cDNA library used, as re-screening of a λgt11 cDNA library made from denatured RNA, random plus oligo-dT-primed (Clontech), did not yield inserts that would have completed the full cDNA of γ-COP. Nor did application of the RACE procedure [13] with bovine mamarian gland epithelial cell RNA lead to additional cDNA information.

Computer-assisted structure comparison revealed a striking homology to Sec21p, as shown in Fig. 3. The Bestfit program was used that introduces gaps for the optimal alignment of the sequences. In a stretch of 569 positions 28.3% of the amino acids are identical, and a similarity of 52.3% is obtained if conserved amino acids are considered. Individual peptide stretches of between 40 and 60 amino acids are found with identities of more than 50% at either side of the two major gaps.

In the Blast P program for protein comparison [14] a high score for the relation of γ-COP to Sec21p of 204 is obtained, with a smallest Poisson probability of 2.5×10^{-25}. For comparison, the next best score was 56 for a hypothetical 119.5 kDa protein [15] with a smallest Poisson probability of 2.7×10^{-6}. These results, together with the identities and similarities shown in Fig. 3, provide a statistically firm evidence that Sec21p and γ-COP are related proteins.

Sec21p [16] is a protein of 935 amino acids with a molecular weight of about 103 kDa. This is in close agreement to the apparent molecular weight of about 100 kDa for γ-COP, as estimated by SDS-gel electrophoresis. The alignment of our partial protein sequence with Sec21p is optimal in the C-terminal part of Sec21p. This is in good agreement with a stop codon found at position 1,570 of the cDNA sequence, and indicates that our cDNA codes for a C-terminal part of γ-COP, comprising almost two thirds of the entire protein.

What implications do our findings have on our knowledge on the mechanism of vesicular biosynthetic protein transport? First, γ-COP is the second subunit of the cytosolic coatomer complex that is now shown to be present in transport vesicles as well. Thus, the remaining subunits of coatomer are also highly likely to represent the coat of non-clathrin-coated transport vesicles. Second, and more importantly, the similarity of a coatomer subunit from animal transport vesicles to a yeast secretory mutant protein offers strong confirmation that coatomer plays a functional role in vesicular

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γ-cop   1 TGSEGSIDRLMKQISSFMSEISDEFKVVVVQAISALCQKYPRKHAVLMNF 50
sec21 369 TGFSKNISSLISTITNFIHDVSDDFKIIIIDAVRTLSLNFPQEWKSILNF 418

γ-cop  51 LFSMLRE.EGGFEYKRAIVDCIISIIEENAESKETGLSHLCEFIEDCEFT 99
sec21 419 LIDVLKNSEGGFKFKNSIVEALIDIVSFVPQSKELALENLCDFIEDCEFN 468

γ-cop 100 VLATRILHLLGQEGPGPATLPSTSASSTTAWCWRPPRSRR.PVSALAKFG 148
sec21 469 EILVRILHLLGKEGPSAPNPSLYVRHIYNRVYLENSIIRSAAVVALSKFA 518

γ-cop 149 AQNEE..MLPSILVLLKRCVMDDDNEVRDRATFYLNVLE ..QKQKALNA 193
sec21 519 LTKNDPTLYESIISLLKRIANDKDDEVRDRATIALEFIDSARNKDDVIAQ 568

γ-cop 194 GYILNGLAVSIPGLERALQQYT.. ..... .LCPSEKPFD 224
sec21 569 NLIESKYFYDIPSLESKLSSYISSNTDSFATAFDVNQVRKFTEDEMKAIN 618

γ-cop 225 LKSVPLATAPLAEQRTESTPVTAAKQPEKVAATR..... ....Q 259
sec21 619 LKRKQEQIFNQKSETTLDTTPEAESVPEKRADANSFAGPNLDDHQEDLLA 668

γ-cop 260 EIFQEQLAAVPEFQGLGPLFKSSPEPVALTESETEYVIRCTKHTFTDHMV 309
sec21 669 TKYADELLSIEQIKPFGQLVNSSR.AISLTEPEAEFVVRGVKHLFKDNVV 717

γ-cop 310 FQFDCTNTLNDQTLENVTV..QMEPSEAYEVLCYVPARSLPYNQPGTCYT 357
sec21 718 LQFNITNTLTDIALDNVSVVCTPEISDEAELEELFTLQVDRLLPSEEAAC 767

γ-cop 358 LVALPKEDPTAVACTFSCVMKFTVKDCDPTTGEA. DDEGYEDEYVLEDL 405
sec21 768 YVAFKKLDEIVMEGFLN NLTFTTKEINPDTNEPFDGDEGFQDEYEIIDSI 816

γ-cop 406 EVTIADHIQKVMKLNFEAAWDEVGDEFQKEETFTLSTIKTLECAVGNIVK 455
sec21 817 FLNAGDYVKSSFTGNFSAIFDELPCE..EVAVFNIQEDLSIQEYVDKIIL 864

γ-cop 456 FLGMHPCERSDKVPDNKNTHTLLLAG..VFRGGHDILVRSRLLLLDTVTM 503
sec21 865 NSSCLPVESTQTAPSDSNSHTLKLFGKSALTGSKVALQIKMIKSSKGLAL 914

γ-cop 504 QVTARSSEELPVDIVLASV 522
sec21 915 KVHCKGEDSLLCSDLVNGL 933
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Fig. 3. Comparison of the partial cDNA-derived amino acid sequence of γ-COP with Sec21p. Identical amino acids are indicated by a vertical dash; conserved amino acids are indicated by a colon.

transport in vivo. Sec21p has been characterized as one of several temperature-sensitive yeast mutants defective in protein transport, and results from synthetic lethality studies indicate a role for Sec21p in budding of transport vesicles from the ER [10]. The close homology of a coat subunit isolated from Golgi-derived transport vesicles, and a protein required for ER-to-Golgi transport would certainly be consistent with the simple idea that transport vesicles from both the ER and the Golgi apparatus recruit their coats from a common pool of cytosolic precursor, the coatomer, and that the coat is required for vesicle budding. We would predict that Sec21p, like γ-COP will exist in a similar coatomer complex in yeast. In summary, the mechanisms underlying vesicle budding from the ER and from the Golgi are likely to be very similar, if not identical, in animals and in yeast, as has been found earlier for the process of membrane fusion [17]. Results from yeast cell-free ER-to-Golgi transport assays have revealed evidence for a vesicular carrier but no coat has been described to date [18,19]. In light of our findings, it appears likely that a transient COP-coated vesicle intermediate has been missed.

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NOTE ADDED IN PROOF

Independent work by R. Schekman and co-workers (Nature, in press) leads to conclusions similar to our own