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A new class of carriers that transport selective cargo from the trans Golgi network to the cell surface

Yuichi Wakana, Josse van Galen, Felix Meissner, Margherita Scarpa, Roman S. Polishchuk, Matthias Mann and Vivek Malhotra

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 31 May 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while two referees are very supportive and would support publication after appropriate revision, one referee raises major concerns with respect to the conclusiveness of the dataset that in his/her view preclude publication of the study.

In the meantime, and in the light of the concerns raised by referee 2, I have had a chance to consult with the referees once more. We have now come to the conclusion that we will be happy to consider a revised version of the manuscript in which the points raised by the referees are addressed in an adequate manner. Based on the cross-referee commenting process, I would like to specify the points that need further attention. There is a concern about the specificity of the TGN46 antibody used, and it would be important to provide control data on its specificity (such as a complete SDS gel) and indicate whether it was affinity purified. It would definitely strengthen the mass spectrometry dataset if you could perform the control IP experiment after TGN46 siRNA, and we would encourage you to include this additional control. Yet, given that you have validated relevant candidate proteins by further functional experiments, we would not necessarily insist on these experiments. Furthermore, the PAUF carrier preparation should be characterised more thoroughly, in particular, given that you did not detect TGN46 in the mass spectrometry dataset of the fraction pulled-down with the TGN46 antibody. In his/her cross-referee comments, referee 3 suggests staining PAUF carriers for TGN46 at different time points to show at what stage of their lifecycle

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they actually contain TGN46, and I would like to ask you to follow this suggestion. At the same time, it would be helpful to explain the rationale for TGN46 as a choice for your pull-down experiments more clearly. Finally, further verification that the other cargos tested are indeed in another carrier than PAUF should be provided to address a concern essentially raised by all three reviewers. Please do not hesitate to get back to me at any time in case you would like to consult on any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This is an interesting an important study that describes the isolation of transport carriers en route between the trans Golgi network (TGN) and cell surface. The authors have names these, CARTS for carriers of the TGN to the surface. The vesicles are formed at 32ºC with ATP and cytosol and their formation requires protein kinase D activity; they lack markers of lysosomes, endosomes and Golgi. The authors identify PAUF as a new secretory cargo in HeLa cells, and they confirm the intracellular-equivalent carriers include Synaptotagmin II, Rab6A and Rab8A but not Rab11A, and myosin IIA. They show that myosin is a cargo rather than a functional vesicle-forming factor. They also try to show that collagen and VSV-G are excluded from the carriers. Overall, the work is of high quality and will be of broad interest to the readers of EMBO J.; publication is strongly recommended after the following issues are addressed.

1. An important part of this study seeks to conclude that VSV-G and collagen are not in CARTS. This would be a very important finding, if true. To test this, the authors have used a temperature block followed by release for 15 minutes at 32ºC. They need to show that the VSV-G and collagen are not at ER exit sites under these conditions and are truly localized to the TGN. This is a very important control. Another way around this would be to carry out a longer chase. Without this, they cannot yet conclude that G protein and collagen exit the TGN in a distinct carrier type.

2. The authors conclude that p115 is not required for secretion of PAUF. They only achieved 70% depletion so their conclusion is premature and should be softened. This is not an important aspect of the present study and could be shown in supplemental materials since the conclusion is not yet strongly supported.

3. The mass spec data helped the authors identify new cargoes that were verified by other means, but peptide coverage is very low for most of the proteins detected. The authors should consider analyzing the "coverage" (number of peptides/total number of residues in the protein). Higher coverage indicates higher significance of detection of the protein in question. Otherwise, the Excel file is not very compelling. Programs such as Scaffold by Proteome Software could help rank the hits according to significance. The paper does not need this table but the Table I in the manuscript should try to report coverage.

4. In figure 2, can the authors state what fraction of starting material HRP is precipitated with the anti-TGN46 antibody?

5. The procedure to isolate CARTS includes carbonate washing of membranes post-isolation to remove peripherally associated proteins. Why are lumenal proteins still present after this harsh treatment? Please explain this for the reader.

Other comments.

1. Page 3. The authors discuss extensive characterization of transport carriers and should include these more up to date references:

Borner GH, Antrobus R, Hirst J, Bhumbra GS, Kozik P, Jackson LP, Sahlender DA, Robinson MS. Multivariate proteomic profiling identifies novel accessory proteins of coated vesicles. J Cell Biol. 2012 Apr 2;197(1):141-60.

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Referee #2

This manuscript seeks to characterize the carriers that transport cargo from the trans-Golgi network (TGN) to the plasma membrane. There is a lot of interest in these carriers, and surprisingly little known about them. However, the present study is extremely disappointing. The authors claim to have isolated "CARriers of the TGN to the cell Surface", or "CARTS", using an antibody that recognizes the cytoplasmic domain of the TGN resident protein TGN46. Although they identified a number of proteins in their pulldowns, essential controls are lacking, so the physiological relevance of the identified proteins is unclear.

The first problem is that TGN46 is an odd choice for a secreted cargo protein, because at steady state most of it is in the TGN. There is a small amount at the cell surface, from where it is rapidly endocytosed, and it has also been shown to traffic from the TGN to endosomes and back again. Thus, there is no reason to suppose that vesicles containing TGN46 are en route from the TGN to the plasma membrane; they could be embarked on any one of a number of different trafficking itineraries. The authors would probably argue that the lack of detectable transferrin receptor and cathepsin D in their pulldowns, and the presence of secretory peroxidase (ssHRP), are sufficient proof that the vesicles are TGN-to-plasma membrane transport intermediates. However - and this is

the most serious flaw with the paper - there are no controls for antibody specificity. They are using a commercial polyclonal antibody; it does not even appear to have been affinity purified. How do they know that the proteins they pull down are associated with TGN46-containing carriers? At the very least, they should have carried out an IP using cells treated with an siRNA to deplete TGN46, and then compared the protein composition of pulldowns from control and siRNA-treated cells.

Instead, they present a list of ~100 identified proteins, with no way of determining which are specific and which are not. Indeed, the authors point out (page 9) that many proteins are likely to be present as a result of non-specific binding to the beads, so they have "selected proteins that are likely to be secreted or inserted into the plasma membrane..., and accessory proteins that likely aid in the trafficking of these carriers from the TGN to the cell surface". In other words, they have decided arbitrarily which proteins they like, without knowing whether their presence in the pulldowns is specifically due to their presence in TGN46-containing carriers (aka CARTS) or not. And very oddly, TGN46 itself was apparently not detected in the pulldown by mass spectrometry, only by Western blotting.

Some of these proteins, like PAUF, are known to be secreted, and they found, not surprisingly, that when tagged versions are expressed in HeLa cells, they get secreted. They then decided to look at Rabs (for reasons that are not clear, because no Rabs could be detected in their pulldowns by mass spectrometry), and found some colocalization between MycHis-tagged PAUF and GFP-tagged Rabs 6a and 8a, but not 11a. Again, this is hardly surprising, because Rabs 6 and 8, but not Rab11, have been reported to function on the secretory pathway. But what does this have to do with the initial isolation of TGN46-containing vesicles? In fact, TGN46 is barely mentioned after the first two pages of the Results section; the focus is almost exclusively on PAUF. Two proteins that were identified by mass spectrometry are myosin II and p115, but the authors report that neither appears to be important for PAUF secretion.

In the final section, they test whether transport intermediates containing MycHis-tagged PAUF also contain GFP-tagged collagen or VSV-G protein. By this time, they have shifted their definition of CARTS to mean any punctate element that is positive for PAUF, when it started off as a definition of TGN46-containing transport intermediates isolated in their pulldown assay. They find that less than 20% of these PAUF-positive puncta are also positive for collagen or VSV-G. Their control is to cotransfect with MycHis-tagged PAUF and mRFP-tagged PAUF, which results in >98% colocalization. However, this is not a fair comparison. It is hardly surprising that two different tagged versions of PAUF behave in the same way, but different proteins traffic through different compartments with different kinetics, especially if one is transmembrane and the other is lumenal. Indeed, synaptotagmin II, which was also found in the pulldown, and which they claim is present in the same transport intermediates as PAUF, in fact shows only limited colocalization with PAUF (Fig. 4B). One wonders how much TGN46 would be present in the PAUF-positive puncta. I suspect that there would be little or none, even though CARTS were initially defined by the presence of TGN46. Their warning to the reader on page 19, "we caution the use of VSV-G protein as a general marker for TGN to cell surface transport", is about 20 years out of date. Kai Simons's lab showed in the early-to-mid 1990s that VSV-G travels to the plasma membrane in different carriers from hemagglutinin, not only in epithelial cells but in fibroblasts as well.

In summary, although TGN-to-plasma membrane trafficking is a fascinating topic, the present study lacks objectivity and is the kind of work that gives proteomics a bad name.

Referee #3

The goal of this study is to purify and characterize transport carriers operating between the TGN and the PM. To this end, the authors immune-isolate TGN46-containing membranous objects from digitonin-permeabilized cells incubated in the presence of cytosol and ATP. TN46 is known to associate with transport carriers and to cycle between the TGN and the PM. Thus, the choice of this protein as a bait for immune-precipitation is probably valid (the PM is probably disrupted by digitonin in their experiments). The authors also show that the isolated TGN46 membranes contain HRP, as would be expected of sealed transport carriers.

Mass spectrometry of the immuno-isolated TGN46 membrane reveals the presence of over hundred

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proteins. Among these the authors select for further examination a few secretory cargoes including PAUF, lysozyme and others, as well as 10 other proteins including synaptotagmin II and myosin II. They then select PAUF to characterize PAUF carriers in live cells. They show that these carriers contain (ie, colocalize with), and require for function, two of the proteins identified by mass spectrometry, ie, synaptotagmin II and myosin II. The PAUF carriers also contain other previously characterized relevant proteins, such as PKD (which is necessary for carrier fission) and Rab 8 and 6. Altoghether, these data indicate that the isolated TGN membranes contain PAUF carriers.

My comments:

1) It would be reassuring for readers to have at least some level of morphological characterization of the isolated TGN46 membranes and of the digitonin-treated cell from which they derive. It is possible that HRP is contained only in a minor fraction of the TGN46 membranes. The rest might be just membrane fragments of uncertain significance.

2) It remains unclear whether the authors have isolated one or more types of carriers. It would be important to examine whether the PAUF carriers contain also other cargo proteins that co-immune-precipitate with TGN46 (eg, MHC, desmoglein, lysozyme etc). If they do not, do these other carriers show the same features?
In general, a membrane preparation such as that described in this study should be characterized more thoroughly. I think that adding the information indicated above would strengthen the manuscript.

3) A limit of this study is the paucity of new information it provides on the molecular mechanism of carrier formation. As noted by the authors, the previously isolation of clathrin, and COP carriers led to the identification of the molecular machinery of budding. This is not the case here.

Minor comments

1) H89 is not a specific PKD inhibitor. The evidence based on the use of DN PKD is more convincing.

2) The authors claim that PAUF carriers do not contain PC and VSVG. The PCI experiments are not convincing. PCI is a large cargo that is not normally expressed in Hela cells. Is PCI transported and secreted normally in these cells? We do not know whether the spots that contain PCI derive from the TGN. The TGN localization of PCI is not shown.
The comparison between VSVG and PAUF is more convincing, but it would be better to carry it out using identical conditions for the two cargoes, ie, without VSVG synchronization.

3) The colocalization between synaptotagmin II and PKD is so complete that one suspects fluorescence overspill across the two channels.

In conclusion, I note that other excellent groups have attempted the purification of post-Golgi carriers before, and failed. Despite its limitations, this is the first study that succeeds in providing a valid purification method and in identifying some carrier components. As such, it is worthy of publication in the EMBO J.

1st Revision - authors' response 04 July 2012

Referee #1

This is an interesting an important study that describes the isolation of transport carriers en route between the trans Golgi network (TGN) and cell surface. The authors have names these, CARTS for carriers of the TGN to the surface. The vesicles are formed at 32ºC with ATP and cytosol and their formation requires protein kinase D activity; they lack markers of lysosomes, endosomes and Golgi.

The authors identify PAUF as a new secretory cargo in HeLa cells, and they confirm the intracellular-equivalent carriers include Synaptotagmin II, Rab6A and Rab8A but not Rab11A, and myosin IIA. They show that myosin is a cargo rather than a functional vesicle-forming factor. They also try to show that collagen and VSV-G are excluded from the carriers. Overall, the work is of high quality and will be of broad interest to the readers of EMBO J.; publication is strongly recommended after the following issues are addressed.

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Done (Supplementary Figure 7).

2. The authors conclude that p115 is not required for secretion of PAUF. They only achieved 70% depletion so their conclusion is premature and should be softened. This is not an important aspect of the present study and could be shown in supplemental materials since the conclusion is not yet strongly supported.

OK. Moved to the supplemental materials.

3. The mass spec data helped the authors identify new cargoes that were verified by other means, but peptide coverage is very low for most of the proteins detected. The authors should consider analyzing the "coverage" (number of peptides/total number of residues in the protein). Higher coverage indicates higher significance of detection of the protein in question. Otherwise, the Excel file is not very compelling. Programs such as Scaffold by Proteome Software could help rank the hits according to significance. The paper does not need this table but the Table I in the manuscript should try to report coverage.

Mass spectrometry was just to get a collection of proteins with the intent of then selecting a small number for further analysis. To us, it is like a genome wide screen that is used to select a few for further analysis.

4. In figure 2, can the authors state what fraction of starting material HRP is precipitated with the anti-TGN46 antibody?

0.01% of the total HRP activity was detected in the immunoprecipitated membranes. This is probably relevant considering 1-5% budding efficiency, 20% immunoprecipitation efficiency, and the presence of some HRP in the ER even after a 20°C block.

5. The procedure to isolate CARTS includes carbonate washing of membranes post-isolation to remove peripherally associated proteins. Why are lumenal proteins still present after this harsh treatment? Please explain this for the reader.

Proteins that remain tightly bound might not be released. This is now explained in the paper.

Other comments.

1. Page 3. The authors discuss extensive characterization of transport carriers and should include these more up to date references:

Borner GH, Antrobus R, Hirst J, Bhumbra GS, Kozik P, Jackson LP, Sahlender DA, Robinson MS. Multivariate proteomic profiling identifies novel accessory proteins of coated vesicles. J Cell Biol. 2012 Apr 2;197(1):141-60.

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Stenmark H. Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol. 2009 Aug;10(8):513-25.

Done.

We thank this reviewer for constructive criticism and valuable suggestions.

Referee #2

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The first problem is that TGN46 is an odd choice for a secreted cargo protein, because at steady state most of it is in the TGN. There is a small amount at the cell surface, from where it is rapidly endocytosed, and it has also been shown to traffic from the TGN to endosomes and back again. Thus, there is no reason to suppose that vesicles containing TGN46 are en route from the TGN to the plasma membrane; they could be embarked on any one of a number of different trafficking itineraries. The authors would probably argue that the lack of detectable transferrin receptor and cathepsin D in their pulldowns, and the presence of secretory peroxidase (ssHRP), are sufficient proof that the vesicles are TGN-to-plasma membrane transport intermediates. However - and this is the most serious flaw with the paper - there are no controls for antibody specificity. They are using a commercial polyclonal antibody; it does not even appear to have been affinity purified. How do they know that theproteins they pull down are associated with TGN46-containing carriers?At the very least, they should have carried out an IP using cells treated with an siRNA to deplete TGN46, and then compared the protein composition of pulldowns from control and siRNA-treated cells.

We used an affinity purified anti-TGN46 antibody. The amount of material required for this experiment is beyond our capacity and therefore it was not performed. The idea we had was simple; isolate membranes that are enriched in TGN46 but devoid of Golgi membrane resident proteins, proteins contained in the other secretory compartments and the endocytic compartments. We achieved our goal to the best of our abilities. This was then followed by mass spectrometry, see below.

Instead, they present a list of ~100 identified proteins, with no way of determining which are specific and which are not. Indeed, the authors point out (page 9) that many proteins are likely to be present as a result of non-specific binding to the beads, so they have "selected proteins that are likely to be secreted or inserted into the plasma membrane..., and accessory proteins that likely aid in the trafficking of these carriers from the TGN to the cell surface". In other words, they have decided

arbitrarily which proteins they like, without knowing whether their presence in the pulldowns is specifically due to their presence in TGN46-containing carriers (aka CARTS) or not. And very oddly, TGN46 itself was apparently not detected in the pulldown by mass spectrometry, only by Western blotting.

The mass spectrometry or genome wide analysis generates a lot of data and not all is specific. The data has to be confirmed by other procedures and we did that by testing a few potentially likely candidates. There is nothing wrong with this approach.

TGN46 is present in extremely small amounts in the transport carriers and therefore not visible by IF and mass spectrometry. TGN46 is a heavily glycosylated protein of about 45 kDa, which migrates at around 110 kDa by western blotting. Any number of reasons, including heavy glycosylation, could prevent detection by IF and mass spectrometry. We do not see this as a big concern. Although we know a lot about the mechanism of COPI assembly on the membranes, the exact cargo contained in these vesicles remains controversial. We expect to learn more about the CARTS now that we know the cargoes they transport and the potential role of accessory proteins such as myosin II, Syt II, Rab6a and 8a, and the fact that they are distinct from the VSV-G containing carriers.

Some of these proteins, like PAUF are known to be secreted, and they found, not surprisingly, that when tagged versions are expressed in HeLa cells, they get secreted.

Yes, but we still had to confirm it by formally testing the secretion of PAUF by HeLa cells. There are no specific antibodies and we therefore used a tagged construct instead.

They then decided to look at Rabs (for reasons that are not clear, because no Rabs could be detected in their pulldowns by mass spectrometry), and found some colocalization between MycHis-tagged PAUF and GFP-tagged Rabs 6a and 8a, but not 11a.

It has been reported that VSV-G containing presumed Golgi to cell surface carriers contain Rab6 and Rab8 (Miserey-Lenkei et al, 2010; Lukas et al, 1993). It was therefore important for us to test their association with the CARTS.

Again, this is hardly surprising, because Rabs 6 and 8, but not Rab11, have been reported to function on the secretory pathway.

Rab11 is associated with carriers that form at the endosomes. It was therefore important to test its association with the CARTS.

But what does this have to do with the initial isolation of TGN46-containing vesicles?

It has to do with the characterization of TGN46 containing carriers. We plan to test more known proteins of the Golgi to cell surface pathway to better understand their roles in protein secretion.

In fact, TGN46 is barely mentioned after the first two pages of the Results section; the focus is almost exclusively on PAUF.

It is a protein of unknown function and we used it simply to isolate the carriers. That was the sole purpose of the description of TGN46. I (Vivek) used albumin as a marker to isolate the now known COPI vesicles (1989). There was no description of albumin other than its use a marker to tag the potential transport carriers. The significance of TGN46 is similar to albumin that was used to isolate intra-Golgi transport carriers.

Two proteins that were identified by mass spectrometry are myosin II and p115, but the authors report that neither appears to be important for PAUF secretion.

This is incorrect. Myosin II is required for PAUF secretion but not the biogenesis of CARTS. This is an important distinction. Please see for details the paper of Echard and colleagues in NCB on the proposed role of myosin II in membrane fission. We can't explain the significance of p115 in the trafficking of CARTS, but because it is a tether, it was important to test its involvement.

In the final section, they test whether transport intermediates containing MycHis-tagged PAUF also contain GFP-tagged collagen or VSV-G protein. By this time, they have shifted their definition of CARTS to mean any punctate element that is positive for PAUF, when it started off as a definition of TGN46-containing transport intermediates isolated in their pulldown assay.

It is difficult to monitor the transport of integral membrane proteins such as TGN46 because they are always present on the plasma membrane, whereas PAUF secretion can be easily quantitated. PAUF is also more concentrated in the carriers than TGN46 and therefore a better marker for the CARTS.

They find that less than 20% of these PAUF-positive puncta are also positive for collagen or VSV-G. Their control is to cotransfect with MycHis-tagged PAUF and mRFP-tagged PAUF, which results in >98% colocalization. However, this is not a fair comparison. It is hardly surprising that two different tagged versions of PAUF behave in the same way, but different proteins traffic through different compartments with different kinetics, especially if one is transmembrane and the other is lumenal. Indeed, synaptotagmin II, which was also found in the pulldown, and which they claim is present in the same transport intermediates as PAUF, in fact shows only limited colocalization with PAUF (Fig. 4B).

There is a lot in the literature on whether some proteins traffic via the endosomes to the cell surface. This is probably another sorting step but it has hardly revealed any novel insights. We were surprised that VSV-G and PAUF are transported in separate carriers. VSV-G is used extensively to monitor basolateral protein traffic. Our studies reveal that not all proteins transported to the basolateral surface go along with the VSV-G.

Please see figure 5B. They show perfect co-localization. Quantification revealed that more than 80% of PAUF containing CARTS co-localize with Syt II.

One wonders how much TGN46 would be present in the PAUF-positive puncta. I suspect that there would be little or none, even though CARTS were initially defined by the presence of TGN46.

How can the reviewer claim that there would be little to none of TGN46? TGN46 is a heavily glycosylated protein that migrates at almost twice the predicted size. It is possible that this affect its detection by fluorescence microscopy and mass spectrometry. We repeat, TGN46 was used to isolate the potential carriers and that is it. We made no attempt to dwell more on the fate or the significance of TGN46 further with respect to the CARTS. We do not see the relevance of this criticism.

Their warning to the reader on page 19, "we caution the use of VSV-G protein as a general marker for TGN to cell surface transport", is about 20 years out of date. Kai Simons's lab showed in the early-to-mid 1990s that VSV-G travels to the plasma membrane in different carriers from hemagglutinin, not only in epithelial cells but in fibroblasts as well.

Kai made the distinction between basolateral (VSV-G) and apical (HA) carriers. We are discussing the carriers that transport cargo to the basolateral surface only. Hence the cautionary note. Regardless, we have removed this statement from the paper.

In summary, although TGN-to-plasma membrane trafficking is a fascinating topic, the present study lacks objectivity and is the kind of work that gives proteomics a bad name.

We disagree. The mass spectrometry data helped us in characterizing the carriers with respect to the cargo and accessory proteins such as myosin II and Syt II.

Referee #3

The goal of this study is to purify and characterize transport carriers operating between the TGN and the PM. To this end, the authors immune-isolate TGN46-containing membranous objects from digitonin-permeabilized cells incubated in the presence of cytosol and ATP. TN46 is known to

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associate with transport carriers and to cycle between the TGN and the PM. Thus, the choice of this protein as a bait for immune-precipitation is probably valid (the PM is probably disrupted by digitonin in their experiments). The authors also show that the isolated TGN46 membranes contain HRP, as would be expected of sealed transport carriers.

Mass spectrometry of the immuno-isolated TGN46 membrane reveals the presence of over hundred proteins. Among these the authors select for further examination a few secretory cargoes including PAUF, lysozyme and others, as well as 10 other proteins including synaptotagmin II and myosin II. They then select PAUF to characterize PAUF carriers in live cells. They show that these carriers contain (ie, colocalize with), and require for function, two of the proteins identified by mass spectrometry, ie, synaptotagmin II and myosin II. The PAUF carriers also contain other previously characterized relevant proteins, such as PKD (which is necessary for carrier fission) and Rab 8 and 6. Altoghether, these data indicate that the isolated TGN membranes contain PAUF carriers.

My comments:

1) *It would be reassuring for readers to have at least some level of morphological characterization of the isolated TGN46 membranes and of the digitonin-treated cell from which they derive. It is possible that HRP is contained only in a minor fraction of the TGN46 membranes. The rest might be just membrane fragments of uncertain significance.*

We now include immuno-EM's of the PAUF containing carriers in the paper.

2) *It remains unclear whether the authors have isolated one or more types of carriers. It would be important to examine whether the PAUF carriers contain also other cargo proteins that co-immune-precipitate with TGN46 (eg, MHC, desmoglein, lysozyme etc). If they do not, do these other carriers show the same features? In general, a membrane preparation such as that described in this study should be characterized more thoroughly. I think that adding the information indicated above would strengthen the manuscript.*

These carriers contain lysozyme C and the data is included (Figure 3F).

3) *A limit of this study is the paucity of new information it provides on the molecular mechanism of carrier formation. As noted by the authors, the previously isolation of clathrin, and COP carriers led to the identification of the molecular machinery of budding. This is not the case here.*

In the case of COPI carriers the isolation helped in the identification of the coats. Years of research has led to a better understanding of the assembly of the coats and their potential role in events leading to the budding. There are still a number of issues that remain unclear: for example, the cargoes and the fission event per se.

COPII. Genetics had revealed most of the components of these carriers even prior to their isolation.

Give us time, we will get there.

Minor comments

1) H89 is not a specific PKD inhibitor. The evidence based on the use of DN PKD is more convincing.

We agree, but PKD-KD induced tubules might be easily fragmented by centrifugation to collect transport carriers. It is also difficult to generate the amount of material needed for immunoisolation from knockdown cells. The fact that PAUF is included in PKD-KD induced tubules and PKD kinase activity is required for the secretion of PAUF, gives us the confidence to suggest the involvement of PKD in the biogenesis of CARTS. We have made this argument clear in the text.

2) The authors claim that PAUF carriers do not contain PC and VSVG. The PCI experiments are not convincing. PCI is a large cargo that is not normally expressed in Hela cells. Is PCI transported and secreted normally in these cells? We do not know whether the spots that contain PCI derive from the TGN. The TGN localization of PCI is not shown.

Collagens are made and secreted by HeLa cells. In fact this led to the identification of TANGO1 as a collagen exporter. We showed that the PAUF-MycHis co-localized with TGN46 at the perinuclear region where PAUF was also co-localized with PCI, indicating PCI is localized to the TGN in HeLa cells.

The comparison between VSVG and PAUF is more convincing, but it would be better to carry it out using identical conditions for the two cargoes, ie, without VSVG synchronization.

Without synchronization is difficult because the two proteins have very different characteristics. We find that VSV-G is transported rapidly to the cell surface, whereas PAUF containing carriers are retained in the cytoplasm and secreted very slowly. Also, upon transfection, we find that PAUF is predominantly at the TGN and VSV-G is on the plasma membrane which prevents us from seeing transport carriers. To address the reviewer's issue our new data includes more time points which show that VSV-G and PAUF containing carriers do not co-localize.

3) The colocalization between synaptotagmin II and PKD is so complete that one suspects fluorescence overspill across the two channels.

We obtained a same result with co-expression of Syt II-3xFLAG and GFP-PKD2-KD (data not shown). Therefore the colocalization is not due to the cross-reaction of antibodies or bleedthrough caused by wrong filters of our microscope.

In conclusion, I note that other excellent groups have attempted the purification of post-Golgi carriers before, and failed. Despite its limitations, this is the first study that succeeds in providing a valid purification method and in identifying some carrier components. As such, it is worthy of publication in the EMBO J.

We thank this reviewer for constructive criticism and valuable suggestions.

2nd Editorial Decision 22 July 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Referees #1 and 3 have now seen your revision and their comments are enclosed below. As you can see both referees appreciate the introduced changes and support publication here. Referee #1 has a few minor text edits, while referees #3 would like to see "more precisely the extent of the colocalization between PAUF carriers and TGN46". If you have meaning full data to address this point then it would be good to include it. If not, then we will go ahead with the paper without it. Please let me know if this issue can be addressed.

Also, we now encourage the publication of source data, particularly for electrophoretic gels and blots. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? This concerns just a few of the figures. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me. You can send the source data files to this email address.

Once we get these last issues fixed then we will proceed with the acceptance here.

Thank you for submitting your interesting study to the EMBO Journal!

Sincerely yours,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The paper is much improved by new data showing kinetics of cargo export to confirm the conclusion that collagen and VSV-G are in distinct carriers that do not represent ER exit sites. The paper should be published with these minor edits:

Abstract line 2 destined FOR the cell surface; line 9 delete the word: predominantly

Intro line 2 delete: the; line 4, delete: extensively; line 5 delete: membranes; line 8, delete: the before early; line 10, delete: the before endosomes; next para line 1, delete: membranes; line 5, delete: membranes and destined FOR the cell surface; Page 4: replace "remain a challenge" with "has not been achieved to date." last para: line 1, delete: specific; line 4, delete: the.

Referee #3

The authors have made a serious effort to address my concerns, with new experiments and textual changes. I think the manuscript now provides good evidence for a new type of post-Golgi carriers, and reveals some of the molecular components of these carriers. This is an interesting and difficult achievement.

I have one remaining request: to define more precisely the extent of the colocalization between PAUF carriers and TGN46. Though this might be technically difficult, as the authors point out, it would add an important detail to the picture.

2nd Revision - authors' response 24 July 2012

Reviewer #1.

The paper is much improved by new data showing kinetics of cargo export to confirm the conclusion that collagen and VSV-G are in distinct carriers that do not represent ER exit sites. The paper should be published with these minor edits:

Abstract line 2 destined FOR the cell surface; line 9 delete the word: predominantly. Intro line 2 delete: the; line 4, delete: extensively; line 5 delete: membranes; line 8, delete: the before early; line 10, delete: the before endosomes; next para line 1, delete: membranes; line 5, delete: membranes and destined FOR the cell surface; Page 4: replace "remain a challenge" with "has not been achieved to date." last para: line 1, delete: specific; line 4, delete: the.

All the changes suggested by the reviewer are included in the final version.

Reviewer #3.

The authors have made a serious effort to address my concerns, with new experiments and textual changes. I think the manuscript now provides good evidence for a new type of post-Golgi carriers, and reveals some of the molecular components of these carriers. This is an interesting and difficult achievement.

We thank the reviewer for highlighting the significance of our findings.

I have one remaining request: to define more precisely the extent of the colocalization between PAUF carriers and TGN46. Though this might be technically difficult, as the authors point out, it would add an important detail to the picture.

We have found that the quantity of cargo transferred by the CARTS is exceedingly small and their visualization by microscopy requires highly sensitive antibodies or overexpression. We have not been able to visualize TGN46 in the CARTS by fluorescence microscopy, which makes it technically difficult to test colocalization with the other markers of the CARTS. We are generating cells stably expressing TGN46 and PAUF to help address this important issue. However, this will take considerable time and we request the reviewer to allow publication of our manuscript without this data.

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