Caspases and nitric oxide broadly regulate dendritic cell maturation and surface expression of class II MHC proteins

Siew Heng Wong*†‡§, Laura Santambrogio*‡¶, and Jack L. Strominger***

*Department of Cancer Immunology and AIDS, Dana–Farber Cancer Institute, Boston, MA 02115; and †Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138

Contributed by Jack L. Strominger, November 5, 2004

The passage of dendritic cells (DC) from immature to terminally differentiated antigen-presenting cells is accompanied by numerous morphological, phenotypic, and functional changes. These changes include, for example, expression of "empty" class II MHC proteins (MHCIId) at the surface in immature DC, whereas a much larger amount of peptide-loaded MHCIId is expressed at the surface in mature DC. Here we show that, in cultured immature DC derived from murine bone-marrow precursors, a number of molecules involved in intracellular trafficking were present in a cleaved form, degraded by caspase-like proteases. Cleavage was either inhibited or reduced significantly during maturation of DC induced by either LPS and TNF-α or by peptides that inhibit caspase activities. Inducible nitric oxide (NO) synthetase up-regulated by LPS was essential for inhibiting the caspase-like activity during the maturation of DC. Moreover, treatment with LPS or caspase inhibitor resulted in expression of MHCIId/peptide complexes at the cell surface. Thus, the alteration of the endosomal trafficking pathways during the development of DC that parallels the changes in surface expression of MHCIId is regulated at least in part by the activities of caspases, inducible NO synthetase, and its product NO.

antigen presentation | endosomes | LPS | nitric oxide synthetase | protease inhibitors

Terminal differentiation of dendritic cells (DC) into the most powerful antigen-presenting cell is accompanied by numerous morphological and functional changes. Immature DC have a highly organized cytoskeleton and exhibit slow motility, whereas, upon maturation, they acquire a high cellular motility resulting from the depolymerization of F-actin and the loss of vinculin (1). Developmental regulation of endocytosis has also been observed, e.g., immature DC are highly endocytic and very efficient in capturing antigens, a feature that is lost during maturation (2–4). Differences in the level of phagocytosis are in part related to the higher expression in immature DC of activated Cdc42, a member of the Rho family of GTPases that mediates actin-dependent phagocytosis (5). Another intriguing difference between immature and mature DC is the cell-surface expression of class II MHC proteins (MHCIId). Immature granulocyte/macrophage colony-stimulating factor (GM-CSF)-derived DC display at the surface an empty, peptide-receptive form of MHCIId (as well as surface-invariant chain and HLA-DM) but only a few MHCIId/peptide complexes (6, 7), whereas mature immunogenic DC express only high levels of long-lived MHCIId/peptide complexes (8–10). It was proposed that most of the MHCIId complexes that reach the endosomal pathway in immature DC are retained intracellularly because cystatin C (CysC), a natural protease inhibitor that also resides in the late endosomes/lysosomes, inhibits the activity of cathepsin S (11). The latter protease is crucial for the cleavage of the invariant chain fragment lip10 to class II-associated invariant chain peptides during the maturation of DC and the down-regulation of CysC during maturation would enable the complete degradation of lip10 to class II-associated invariant chain peptides. However, this interpretation is not tenable because CysC is not expressed in all subsets of DC and CysC-deficient mice showed no aberration in maturation of or antigen presentation by DC (12). Finally, antigen processing is enhanced in DC upon maturation because of a more efficient recruitment/assembly of the V-ATPase subunits required for lysosome acidification and efficient cathepsin function (13). In general, a profound rearrangement of subcellular functions involving trafficking, protease assembly, and cytoskeleton organization is observed when immature DC are exposed to LPS or other maturing agents.

Membrane dynamics and protein trafficking in the endosomal pathway is tightly regulated by molecular complexes involving several families of proteins and lipids (14). Morphological changes in endolysosomal compartments have been observed in DC after LPS stimulation. In particular, DC lysosomes form tubular extensions that mediate transport between the lysosome-related MHCIId compartments and the cell membrane (15–18). This form of transport, uniquely described in maturing DC, has been proposed to be the sorting mechanism through which peptide-loaded MHCIId reaches the cell surface, and it partially explains the quantitatively different amounts of MHCIId/peptide complexes present on the surface of immature and mature DC. Also a different rate of endocytosis and subsequent degradation of MHCIId/peptide complexes between immature and mature DC has been described as an additional mechanism that regulates the surface stability of MHCIId during DC maturation (10). Additionally, the presence of empty MHCIId only on immature DC further has confirmed differential regulation of endosomal trafficking during DC maturation. Thus, in general, both qualitative (empty versus peptide-loaded) and quantitative (number of molecules) differences in MHCIId at the cell surface have been observed at different stages of DC maturation. However, the mechanisms, i.e., the endosomal targeting molecules differentially involved in the trafficking processes, are still unknown.

As a first step in understanding why empty MHCIId molecules are transported to the cell surface and why so little MHCIId/peptide complex appears at the surface in immature DC, the expression of protein molecules that play vital roles in the endosomal trafficking pathways during the maturation of DC was investigated.

Abbreviations: DC, dendritic cell; BMDC, bone-marrow-derived DC; GM-CSF, granulocyte/macrophage colony-stimulating factor; i-NMMA, Nω-monomethyl-l-arginine; iNOS, inducible nitric oxide synthetase; MHCIId, class II MHC protein.

1S.H.W. and L.S. contributed equally to this work.

2On leave of absence from: Institute of Molecular and Cell Biology, Singapore 117609.

3Present address: Department of Microbiology, National University of Singapore, Singapore 117597.

4Present address: Department of Pathology, Albert Einstein College of Medicine, New York, NY 10461.

5To whom correspondence should be addressed. E-mail: jstrom@fas.harvard.edu.

© 2004 by The National Academy of Sciences of the USA
Materials and Methods

DC Preparations. Bone-marrow-derived DC (BMDC) from B10.BR mice (I-A^b, I-E^b) (The Jackson Laboratory) were established by a the bulk-culture method as described in refs. 6 and 7. Briefly, bone-marrow cells were harvested from the long bones and plated on six-well plates (Millipore) at a cell density of 2.5 × 10^6 per ml in complete DMEM with 5% FCS containing 15 ng/ml of mouse recombinant GM-CSF (R & D Systems). Cells were fed every 2 days with complete DMEM containing 15 ng/ml GM-CSF. For immature DC, cells were harvested after day 10, and the surface phenotype was confirmed by FACS analysis for the expression of cell-surface MHCII (empty, KL304; peptide-loaded, Y3P), CD11b, and CD11c and by immunoprecipitation (Fig. 7, which is published as supporting information on the PNAS web site). For mature DC, cells were further subcultured in media with 1 µg/ml LPS (Sigma) for 40–48 h.

Western Blot and FACScan Analyses. Western blot analysis was performed as described in ref. 19. All mAb used in the Western blot analysis were purchased from BD Transduction Laboratories. Rabbit polyclonal Ab against syntaxin 7 was a generous gift from Wanjun Hong. FACScan analysis were performed as described in refs. 6 and 7. All mAbs used in this experiments were purchased from Pharmingen.

Treatment of DC with Nω-Monomethyl-L-Arginine (L-NMMA) and Caspase Activities. DC grown in the presence of GM-CSF for 10–12 days were incubated with 50 µM L-NMMA for 12 h before incubation with 1 µg/ml LPS and L-NMMA for an additional 40 h, i.e., for a total of 52 h. Treated cells were collected and processed for Western blot analysis. L-NMMA was purchased from Calbiochem.

Preparation of cell lysates for assay of caspase activity and cleavage of fluorogenic substrates were performed as described in ref. 20. Briefly, DC (in six-well plates with 10 × 10^6 cells per well), either untreated or LPS-treated, were washed twice with Hank’s balanced salt solution before the addition of 200 µl of chilled resuspension buffer (25 mM Hepes, pH 7.5/5 mM EDTA/5 mM MgCl2/5 mM DTT/1 mM PMSF). On ice, cells were scraped and pooled into a 1.5-ml Eppendorf tube and left on ice for 5–10 min. Lysates were then frozen on dry iced and subsequently passed through 10 cycles of freeze–thaw processes before centrifugation at 13,000 × g for 30 min. Supernatants were transferred to new tubes and stored at −80°C until they were used.

To assay for caspase activities, 20 µg of protein was incubated at 37°C in a buffer containing 25 mM Hepes, pH 7.5, 10% sucrose, 0.1% CHAPS, and 10 mM DTT, with the respective fluorogenic substrates [caspase-1 substrate VI (Z-YVDAD-afc), caspase-2 substrate I (Z-VDVAD-AFC), caspase-3 substrate IV (Ac-DEVD-AFC), caspase-4 substrate II (Ac-LEVD-AFC)] (all from Calbiochem), in a 96-well, flat-bottom microtitre plate. Fluorescence resulting from cleavage of the substrates was quantified after 2, 4, and 6 h by using a Millipore fluorocytometer at a maximum excitation of ~400 nm and a maximum emission of ~505 nm. The caspases substrates were not completely specific for the caspases indicated.

Results

Biochemical Changes in Essential Protein Molecules of the Endosomal Trafficking Pathways During the Maturation of DC. Molecular changes in the dynamics of the endosomal compartments and at the cell surface have been suggested to be the cause of alterations in structure and trafficking of MHCII during DC maturation as well as the level of endocytosis (5, 8, 10, 11). However, the precise molecular mechanisms that regulate these changes in DC during development have not been completely elucidated. To approach this question, changes of subcellular expression and processing of some protein molecules that play pivotal roles in the endosomal pathways were investigated. For example, AP-1 and AP-2 adaptor protein complexes are components of clathrin-coated vesicles associated with the transGolgi network/ endosomes and plasma membrane, respectively. AP-1 and AP-2 have important functions in the attachment of clathrin to the membrane, in cargo selection, and in the recruitment of accessory protein components for vesicle formation and sorting (21–23). The AP-1 adaptor complex has been thought to function in trafficking between the transGolgi network and the late endosomes, whereas the AP-2 adaptor complex is essential for the endocytic pathways.

As a first step, the expression of α- and γ-adaptin, principal components of AP-2 and AP-1 complexes, respectively, was examined biochemically during maturation of DC. Immature BMDC from B10.BR (I-A^b) or SJL/J (I-A^b) mice prepared by the bulk-culture method in the presence of GM-CSF for 8–10 days were used in all of the experiments and were thoroughly characterized by FACS analysis and immunoprecipitation (see Materials and Methods and Supporting Text, which is published as supporting information on the PNAS web site). Protein extracts from either untreated immature DC or LPS or TNF-α-treated, mature DC were separated by SDS/PAGE and probed with specific mAb against α- and γ-adaptin. Untreated immature DC, mAb against α-adaptin detected a major polypeptide band of ~67 kDa and, surprisingly, only a very faint band of ~110 kDa full-length α-adaptin in a Western blot (Fig. 1). Interestingly, when cells were treated with LPS, the 110-kDa polypeptide band increased greatly and the intensity of the 67-kDa polypeptide band decreased. Thus, the 67-kDa polypeptide appeared to be a cleavage product of the full-length α-adaptin. The same phenomenon was also observed in TNF-α-treated DC, although to a lesser extent. Similarly, the specific mAb against γ-adaptin detected only a faint 80- to 85-kDa band in the control. However, a slightly larger major protein band of ~90 kDa, full-length γ-adaptin, appeared after treatment with LPS and TNF-α, suggesting that the maturation process also inhibited cleavage of full-length γ-adaptin and its smaller forms that had been further proteolyzed in the control. Although the relative intensities of these bands varied in different experiments (Fig. 2a; see also Fig. 4), the overall result was always the same. A similar cleavage has been shown for β-adaptin (see Fig. 6), although the mAb used does not distinguish between β1 (AP-1 complex) and β2 (AP-2 complex) adaptins.

Several other proteins of the endosomal membrane trafficking pathways involved in budding and fusion events were examined similarly. mAb specific for syntaxin 7 [soluble N-ethylmaleimidesensitive factor attachment protein receptor (SNARE) molecule of the early/late endosomes], syntaxin 8 (SNARE molecule of the early/late endosomes), dynamin (function in formation of endocytic vesicles from the cell surface), Vti1A (Golgi, transGolgi network, and late endosomal SNARE), Vti1B (transGolgi network and late endosomal SNARE), lysosomal-associated membrane protein 1, and clathrin heavy chain were used as probes in Western blots. No changes in lysosomal-associated membrane protein 1 and clathrin heavy chain were seen (data not shown). LPS treatment increased the total protein levels of all of these molecules. In addition, cleavage products of ~16 kDa were observed for syntaxin 8 and Vti1A in both the immature and mature DC. Again, LPS treatment dramatically increased the amount of the longer full-length band and decreased relatively that of the smaller band (Fig. 1). Cleavage products for syntaxin 7, Vti1B, and dynamin were not resolved in this experiment but were clearly seen in subsequent experiments (Fig. 2a; see also Fig. 4). One or more of the following interpretations could apply to the different proteins: (i) LPS treatment inhibited degradation of the mature proteins, (ii) LPS
inhibited specific cleavage of the mature protein by a protease, although the cleavage band(s) could not be detected because of the loss of the mAb epitope, and/or (iii) LPS increased the synthesis of the protein. However, the changes observed could not be correlated with mRNA levels measured by RT-PCR (data not shown), suggesting that these results could not be explained at the transcription regulation level. The possibility of alternative splicing regulated by LPS was not eliminated by these experiments.

Cleavage of Endosomal Trafficking Molecules Is Blocked by Caspase Inhibitors. These observations initiated a search for protease candidates by examining the amino acid sequences of the proteins. Interestingly, analysis of the amino acid sequences of α-adaptin, β-adaptin, γ-adaptin, syntaxin 7, syntaxin 8, Vti1A, Vti1B, and dynamin revealed many potential caspase recognition/cleavage sites (data not shown). To examine the relevance of these sites, immature DC were incubated with inhibitors of both the group I (caspases 1, 3, 4, and 7) and group III (caspases 6, 8, 9, and 10) caspases for 4 h before Western blot analysis (Fig. 2a). As controls, cells were incubated with DMSO (the solvent used to solubilize the inhibitors) for a similar period. The cleavages of α-adaptin, β-adaptin, Vti1A, Vti1B, syntaxin 7, syntaxin 8, and dynamin were inhibited by caspase inhibitors of both group I and group III. Notably, the effect of group I and group III caspase inhibitors on the expression and cleavage patterns were similar to that of LPS treatment (compare Figs. 1 and 2a), i.e., the intensity of the smaller cleaved polypeptide declined as the amount of the full-length polypeptide increased, but the effect of the inhibitors was always greater than that of LPS. Note that, in these experiments, small cleavage products for syntaxin 7 (25 kDa) and dynamin (80 kDa) (not seen in Fig. 1) were evident. Interestingly, in the case of γ-adaptin, no smaller form was evident in the control (as in Fig. 1 as well), which is likely due to loss of the epitope detected by the Ab used in the Western blot. γ-Adaptin contains two canonical caspase cleavage sites at amino acids 627–630 (DDLID) and 743–746 (DMDTD). The polypeptide used in the immunoprecipitation to prepare the mAb encompassed amino acids 642–821. Thus, the cleavage at residue 746 would reduce the size of γ-adaptin (822 residues,
Caspase Activity Is Inhibited by LPS Treatment as the Result of Induction of Inducible Nitric Oxide Synthetase (iNOS). Next, the question of whether caspase activity was reduced (inhibited) during physiological maturation of DC induced by LPS was examined. Four different fluorescent caspase peptide substrates (see Materials and Methods) were incubated with a fixed amount of protein extracts derived from LPS-treated and untreated DC. The fluorescence accumulated as a result of substrate cleavage after 2, 4, and 6 h was measured, and the extent of inhibition at each time point was assessed. At all three time points used, the activities using caspase substrates 1, 2, 3, and 4 were reduced 30–60% during the maturation of DC (Fig. 2b). Thus, the inhibition of cleavage in LPS-treated mature DC appeared to be caused by a reduction of caspase activities.

LPS has previously been shown to up-regulate the expression of the iNOS in many immune cells, including macrophages and DC (24, 25). Furthermore, nitric oxide (NO) has been reported to inhibit caspasies by S-nitrosylation of cysteine residues at the active sites (26, 27). Therefore, inhibition of caspases by NO (through S-nitrosylation) could be an essential mechanism controlling the activity of caspases during DC maturation. LPS-induced up-regulation of the 120-kDa iNOS in DC was detected by Western blot analysis and immunofluorescence microscopy (Fig. 3a Upper and Lower).

91,350 Da) by 76 residues (82,815 Da), which is compatible with the gel sizes observed. A further cleavage at residue 630 would eliminate all Ab recognition, as observed. On the other hand, group I and group III caspase inhibitors had either very little or no effect on the expression levels or cleavage patterns of MHCII, syntaxin 11, lysosomal-associated membrane protein 1, or H2-DM (data not shown).

Fig. 3. iNOS, induced by LPS, reduced the cleavage of α-adaptin and γ-adaptin during DC maturation. (a) Upper Protein extracts (80 μg) from immature (untreated) and mature DC (plus LPS) were analyzed by Western blot by employing mAbs specific for iNOS (NOSII). (a) Lower Confocal microscopic analysis for iNOS (using anti-iNOS phycoerythrin) performed on immature and LPS-matured DC as above. The red dye appears white in this black and white figure. (b) Extracts from DC either untreated (lane 1) or treated with the iNOS inhibitor L-NMMA (lane 2), LPS (lane 3), or L-NMMA plus LPS (lane 4) were analyzed by Western blot by using Abs against α-adaptin.

To test the role of NO as the upstream inhibitor of caspase activity, L-NMMA, which inhibits all three known forms of NOS (iNOS, endothelial NOS, and neuronal NOS) was used. LPS inhibited the degradation of α-adaptin (Fig. 3b, compare control lane 1 with lane 2). L-NMMA alone had no effect (Fig. 3b, lane 3). However, in the presence of both L-NMMA and LPS, the inhibitory effect of LPS on degradation of α-adaptin was greatly reduced (Fig. 3b, lane 4). Similar results were attained for γ-adaptin. Therefore, iNOS played an important role (through NO) in the control of endosomal trafficking, at least in part, by inhibiting the selective cleavage/degradation of vital molecules of the endosomal pathways by caspases. In addition, spermine nononate, which releases NO nonenzymatically at physiological pH (28), mimicked the inhibitory effect of LPS or caspase inhibitors on protein cleavage (data not shown).

Next, the question of whether the loss of the iNOS gene alone might enhance the cleavage in immature DC and/or cause a decline in the cleavage inhibitory effect in LPS-treated DC was investigated. iNOS−/− mice (The Jackson Laboratory) were used to prepare iNOS−/− DC that were compared with the immature DC of wild-type (iNOS+/+) littermate controls. As expected, the loss of iNOS increased the cleavage of α-adaptin, γ-adaptin, and dynamin in the immature DC (Fig. 4, compare lanes 1 and 3). Additional smaller cleaved forms of syntaxin 8 and Vti1A were also seen.

However, in the LPS-induced mature DC, we observed an unexpected enhancement of the full-length forms of these proteins in iNOS−/− and an expected enhancement in iNOS+/+ mice. These data raised the possibility that the inhibitory effect seen in the LPS-treated iNOS−/− DC could be due to NO produced by the activities of other forms of NOS, such as endothelial and neuronal NOS, which were up-regulated by LPS. In addition, gene expression compensation by other genes with similar functions in gene knockout animals is also possible. However, an alternative mechanism for the production of NO is
unknown and would not explain the enhanced cleavage seen in NOS−/− mice. Nevertheless, the possibility that LPS may inhibit cleavage through some other mechanism is not excluded.

Because our data suggested that iNOS activity (through NO) could prevent the cleavage of molecules essential for membrane trafficking between the endosomes and the cell surface, a decline of cell-surface membrane proteins would be expected in the iNOS−/− DC. To demonstrate this point, the distribution of cell-surface membrane proteins, such as TIR, MHCII, B7-1, B7-2, and CD24, in wild-type (iNOS+/+) and iNOS−/− immature DC was determined by FACS analysis. As expected, the expression of these membrane proteins on the cell surface declined in the iNOS−/− DC compared with wild type (data not shown).

LPS and Caspase Inhibitors Redistribute MHCII to the Surface of BMDC.

To fully understand the significance of the caspase-mediated AP-1 cleavage in trafficking, BMDC were treated overnight with LPS or group I caspase and analyzed by flow cytometry for changes in MHCII distribution. Untreated cells displayed at their surface only a small amount of total MHCII, the majority being empty, peptide-receptive MHCII (Fig. 5). Overnight treatment with LPS decreased empty MHCII and greatly increased the surface expression of peptide-loaded MHCII. Similarly, an increase in peptide-loaded MHCII, with a concomitant decrease in the empty form, was evident after overnight treatment with CI.

Adaptin Cleavage During Apoptosis Is Different from That Observed in Immature DC. Because caspses are importantly involved in protein cleavage and degradation during apoptosis, one concern was that the adaptin cleavage observed in immature DC was related to apoptosis (29, 30). For this reason, CD11c-positive purified BMDC were stained with annexin V directly after purification or, as a positive control for apoptosis, after UV irradiation. A microglial cell line, N9, and a macrophage cell line, RAW, which do not express the cleaved form of adaptin fragment (52–54) and an additional γ-adaptin fragment (52–54) observed in the apoptotic BMDC (and splenic dendritic cells; data not shown). Furthermore, in N9 and RAW cells, which normally only express full-length adaptins, the apoptosis-associated cleaved fragments were also found (Fig. 6b). Thus, the adaptin cleavage observed in immature DC is distinct from that occurring during apoptosis.

Discussion

Understanding the molecular mechanism(s) that regulate endosomal trafficking remains one of the fundamental gaps in the knowledge of antigen presentation. As the protein complexes responsible for vesicle budding, sorting, docking, and fusion have become more fully elucidated, studies have been focused on the post translational modification of these proteins as possible targets for the modulation of the efficiency of antigen presentation (31). In the present study, a caspase activity in immature DC is demonstrated to be responsible for cleavage of essential proteins of the endosomal trafficking pathways and has a profound effect on DC maturation. These caspase activities were in turn controlled at least partially by the activity of iNOS, which is up-regulated during the maturation of DC and its product NO.

The unexpected initial observation that several important molecules of the endosomal pathways were cleaved in immature DC and that their cleavages were inhibited in mature DC led to the finding that caspses have novel roles in the control of the endosomal pathway during DC development. Caspses are a large family of cysteine proteases and use cysteine as the nucleophilic group to cleave substrate at the C terminus of aspartic acid (32, 33). They were initially extensively characterized in the context of their function in apoptosis. Notably however, mammalian caspses have also evolved additional roles in inflammatory response (33). More recently, roles of caspses...
in T cell activation or, more broadly, activation of T, B and NK cells, erythroid differentiation, and some aspects of cardiac function have been demonstrated (34–41). Additionally, a caspase-mediated endoproteolysis of the NF-κB factor Relish has an important role in the innate immune response in Drosoφila (42).

Here, still another role of caspases is demonstrated, a role in the regulation of the endosomal trafficking pathways that appears to include MHCII distribution during maturation of DC. In immature DC, the trafficking pathway through the endosomal pathways appears to be inhibited by the caspase-dependent degradation of several proteins essential for endosomal trafficking (Fig. 1). These studies of course do not demonstrate that any of the cleaved proteins is directly involved in the trafficking of MHCII. Although AP-1 and AP-2 adaptor protein complexes are known to be components of clathrin-coated vesicles that are involved in cargo selection for intracellular transport, other factors might be more directly involved in MHCII transport (14).

The cleavage of many proteins involved in intracellular transport in immature DC is certain to have consequences for many processes. Interestingly, the knockout of either γ-adaptin itself or μ1A, both components of the AP-1 complex, is lethal in embryos (43, 44). The small amount of full-length AP-1 complex that might remain in immature DC, together with cleaved AP-1 complex, may be sufficient for cell survival functions.

Because the majority of γ-adaptin was also cleaved in the immature DC, clathrin-mediated endocytosis would be expected to increase during maturation of DC when cleavage is inhibited. Consistent with this observation, although macropinocytosis was drastically diminished in mature DC, the numbers of clathrin-coated pits and vesicles were either unaltered or slightly increased in mature DC, suggesting that uptake of extracellular material by receptor-mediated endocytosis may be enhanced by at least maintained during DC maturation (5, 45). In addition, recent findings that mature DC internalized more structurally intact viruses compared with immature DC (46) further support this model. The cleavages of several other endosomal transport proteins indicated that intracellular transport is drastically affected in immature DC.

Thus, caspase activity and its control by NO play an important role in regulating maturation of DC by altering the structure of proteins involved in important intracellular transport processes. Many functions in addition to surface expression of MHCII are likely to be affected. The control of the endosomal trafficking machinery in this way may open up important areas of study in other fields as well.

We thank Wanjun Hong (Institute of Molecular and Cell Biology, Singapore) for the gift of the syntenin 7 rabbit polyclonal Ab, Gaα Raposo for review of the manuscript, and M. L. Wong for excellent technical assistance. S.H.W. is grateful to Professor Wanjun Hong, Professor Y. H. Tan, and Hong Lan Png for support. This work was supported by an Institute of Molecular and Cell Biology, Singapore, Fellowship (to S.H.W.) and by National Institutes of Health Research Grants CA47554 and AI-49524 (to J.L.S.) and AI-48832 (to L.S.).