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Review

Reticulocyte maturation: mitoptosis and exosome release

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ABSTRACT: During the differentiation of erythroid cells, a vast program of maturation takes place, leading to decay or elimination of organelles, including the nucleus, mitochondria, ribosomes, lysosomes, endoplasmic reticulum and Golgi apparatus. During the last step of red cell maturation, remaining organelles, primarily mitochondria and ribosomes but also vestiges of others are finally cleared from the cell. This cleaning session also affects specific proteins that are partially or entirely removed from the cell surface. The interplay of the various events and their causal relationships are approached here.

I. Introduction

In this review, we have focused on two major cellular changes that occur during reticulocyte maturation: (i) mitochondrial disappearance and (ii) membrane remodeling, and have attempted to determine how these two events could be related. Before going into further detail, we present a short description of the main cellular and molecular events in erythropoiesis.

II. General background

In adult mammals, circulating erythrocytes represent the end product of proliferation and differentiation from precursors in bone marrow. Production of committed erythroid precursors from the progeny of pluripotent stem cells is influenced by various factors such

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as interleukines (*e.g.* IL-1, IL-3, IL-6) and growth factors (*e.g.* granulocyte/macrophage colony stimulating factor, GM-CSF). When committed for differentiation along the erythroid lineage, pluripotent stem cells become blast forming unit-erythroids (BFU-E). BFU-E are highly proliferative cells, particularly in the presence of high concentrations of erythropoietin (EPO). They develop into less proliferative colony forming unit-erythroids (CFU-E). CFU-E are late progenitors whose survival and differentiation into erythroblasts is highly dependent on EPO. Cytological and ultrastructural methods have been used to identify different stages in cell differentiation from the earliest cell engaged in the erythroid lineage. Hemoglobin production and accumulation is the main feature of this maturation process. Cell division simultaneously leads to the production of mature cells.

Proerythroblasts are large cells with a large nucleus containing diffuse chromatin and basophilic cytoplasm. Their differentiation into erythrocytes occurs through four successive mitoses within 3-4 days, leading to the production of 16 mature cells from one cell engaged within the lineage. Rapid and important changes inducing biochemical and morphological modifications (*e.g.* decrease in cell size, condensation of nucleus) allow the characterization of basophilic I and II, polychromatic I and II erythroblast stages. At this stage, polychromatic II erythroblasts expulse their nucleus and become reticulocytes. Then reticulocytes cross the blood barrier to gain access to the bloodstream where they mature into erythrocytes. This reticulocyte maturation step, achieved within 1-2 days in the blood stream, is the focus of the present review. During this period, the reticulocyte gets rid of all of its intracellular compartments to take on the appearance of a typical erythrocytic "hemoglobin bag". Meanwhile, hemoglobin synthesis goes on (about 30% of the total Hb produced) and the mean cell volume decreases, leading to Hb concentration and an increase in cell density.

The name "reticulocyte" comes from the reticulum-like pattern that was observed in certain red blood cells after staining blood with methylene blue. These "net-like" structures were demonstrated to be a sign of the "juvenile" nature of these red cells. It is now known that this reticulum is a staining artifact due to the precipitation of diffusely distributed RNA by supravital stains (*e.g.* methylene blue, brilliant cresyl blue). Reticulocyte staining varies with the RNA content, which decreases as the reticulocyte matures. This was used to determine subtypes for reticulocyte maturation. This disappearance of RNA is concomitant to the loss of subcellular structures such as mitochondria, ribosomes, and endosomal vesicles.

III. Mitochondrial degradation

Mitochondria are the last organelles lost by reticulocytes since they are still needed at this stage for ATP production and heme synthesis. The endoplasmic reticulum and Golgi apparatus are mostly lost when the nucleus is expelled. Three mechanisms leading to mitochondrial removal have been observed: organelle degeneration, mitochondrial autophagy, and expulsion in the extracellular medium. We will discuss the latter possibility in § IV.2.

1. Mitochondria degeneration: contribution of the 15-lipoxygenase (15-LOX)

The reticulocyte 15-lipoxygenase is a major actor of red cell maturation. Its expression peaks in reticulocytes just before mitochondrial degradation. In fact, it has been shown that synthesis of 15-LOX mRNA takes place in the early stages of erythropoiesis, but its translation is inhibited by interaction with ribonucleoproteins K and E1 until activation in peripheral reticulocytes (Ostareck *et al*., 1997; Ostareck-Lederer *et al*., 1994). Unlike 5- and platelet 12-lipoxygenase, erythroid 15-LOX catalyzes oxygenation of esterified polyenoic fatty acids, including those that are part of complex substrates such as biomembranes (Kühn *et al*., 1990). It has been suggested that oxygenation of fatty acid residues of phospholipids by 15-LOX induces formation of the reactive oxygen species $OH^{\cdot}$ by reaction of fatty acyl hydroperoxides with $CoQH^{\cdot}$ in a Fe^{2+}-dependent way (Schnurr *et al*., 1996). The ROS formed could cause opening of the permeability-transition pore (PTP), inducing mitochondrial depolarization, chemical equilibration of solutes across the membranes and thus matrix swelling and rupture of the outer membrane (Skulachev, 2001). The enzymatic activity of 15-LOX terminates by self-inactivation after approximately 600 oxygenations (Schewe *et al*., 1986) due to the hydroperoxide intermediate (Hartel *et al*., 1982).

It has been demonstrated that 15-LOX associates with mitochondrial membrane in a calcium-dependent manner (Watson and Doherty, 1994). More recently, 15-LOX has been shown to bind *in vitro* to different intracellular organelles such as mitochondria, rough and smooth ER, Golgi apparatus and peroxisomes, but not the plasma membrane (van Leyen *et al*., 1998). This

selectivity contributes to the specific destruction of organelles in an ordered biological process. Indeed, it was shown that 15-LOX binding renders the membranes prone to proteolytic breakdown by an ATP- and ubiquitin-dependent system (cf. next §). Moreover, it has been recently shown that 15-LOX integrates into the organelle membrane in an oligomeric form and permeabilizes the organelle, releasing lumenal proteins and allowing access of proteases to both lumenal and integral membrane proteins (van Leyen *et al*., 1998). *In vitro* maturation of reticulocytes in the presence of the lipoxygenase inhibitor eicosatetraynoic acid (ETYA) retards mitochondrial degradation, as shown by the persistence of marker proteins and by direct EM visualization of mitochondria (Grüllich *et al*., 2001).

2. Ubiquitination and ATP-dependent proteolysis

Mitochondria are the main physiological substrates of proteolysis during reticulocyte maturation. They constitute about 10% of the total protein of reticulocytes (Dubiel and Rapoport, 1989). Amino acids derived from their degradation are used for the synthesis of hemoglobin. This proteolysis is ATP-dependent (Müller *et al*., 1980) and preceeded by the attack of lipoxygenase (Dubiel *et al*., 1981). Protein turnover via the ATP ubiquitin-dependent pathway proceeds through degradative intermediates generated by the ATP-coupled covalent conjugation of ubiquitin to susceptible target proteins (Hershko, 1988). Although ubiquitination appears to be relatively non-selective (Ciechanover *et al*., 1980), certain features of specific target proteins have been found to favor their degradation. An abnormal protein conformation has been proposed to induce the degradation of target proteins through ubiquitination, in some cases requiring the involvement of Hsc70 (Bercovich *et al*., 1997). Attachment of ubiquitin to proteins is carried out by the sequential action of three classes of enzymes (E1, E2, E3). E1 enzyme forms a thiolester bond with the C-terminus of ubiquitin, which is then transferred to a thiol of an E2 enzyme. E2 enzyme can transfer ubiquitin to protein lysine residues, but E3 supports the recognition of specific proteolytic substrates (Ciechanover, 1994). During erythroid differentiation, the ubiquitin pathway does not seem to be activated in a general manner, but rather through the specific induction of particular conjugating enzymes. Indeed, genes encoding the ubiquitin-conjugating enzymes E2-20K and E2-230K are induced in erythroid cells (Wefes *et al*., 1995). Moreover, the high levels of both proteins noted in reticulocytes decrease in mature red cells, suggesting a function in the differentiation process. Induction of these enzymes could be a way to selectively degrade some mitochondrial proteins during reticulocyte maturation.

The protein substrates marked for degradation by ubiquitination are then rapidly degraded in the cytosol by the 26S proteasome (Hershko and Ciechanover, 1998; Vogues *et al*., 1999). The 26S proteasome has a 20S proteolytic core, complexed with a 19S cap at either end of its barrel structure. ATP hydrolysis provides the chemical energy necessary to unwind the otherwise stable structure of the substrate protein and facilitate its translocation into the proteolytic compartment (Coux *et al*., 1996). The proteasome contains at least five identifiable proteolytic activities and was thus first named the multicatalytic proteinase complex (Dahlmann *et al*., 1988). Degradation occurs in a highly progressive fashion into small peptides of 3-20 residues that are further hydrolyzed to amino acids by other peptidases (Kisselev *et al*., 1998).

3. Autophagy

Autophagic structures containing mitochondria observed during erythroid differentiation have been reported by several authors (Gronowicz *et al*., 1984; Heynen *et al*., 1985; Kent *et al*., 1966). However, it is not clear if these structures are destined for degradation by lysosomal proteases or fusion with the plasma membrane. Both may occur depending on the differentiation stage of the erythroid lineage. Digestion in autolysosomes may be the major destination of mitochondria in the early stages (Takano-Ohmuro *et al*., 2000), but the autophagic structures may be rerouted for organelle expulsion into the extracellular medium (Simpson and Kling, 1968) while the lysosomal compartment vanishes.

IV. Membrane remodeling

During the maturation process, the membrane surface area and cell volume are considerably reduced, contributing to an increase in cell density. It has been shown that rat reticulocytes lose approximately one-third of their membrane lipids during *in vivo* maturation (Shattil and Cooper, 1972). The decrease in surface area is attributed to "remodeling" of the plasma membrane. One of the main consequences of this remodeling is a loss of endocytic capacity of the mature red cells. Indeed, mature erythrocytes are no longer able to take up molecules from the extracellular medium by receptor-

mediated endocytosis. Various receptors such as the transferrin receptors (TfR) (Jandl and Katz, 1963), β_2-adrenergic receptors (β_2AR) (Bilezikian, 1978) and insulin receptors (IR) (Thomopoulos *et al*., 1978) are lost during the final stage of the differentiation process. Moreover, clathrin-coated pits, *i.e.* the structures in charge of surface receptors sorting and internalization, are never found on the erythrocyte surface, suggesting that not only cargo molecules, but also constituents of the endocytic machinery are lost during maturation. At the same time, there are other alterations in membrane structure and function, such as changes in the transport of amino acids (Tucker and Young, 1980), sugars (Zeidler *et al*., 1976), nucleosides (Jarvis and Young, 1982), calcium (Wiley and Shaller, 1977) and sodium and potassium (Ellory, 1977).

Different processes, such as autophagy and/or membrane shedding (Seelig, 1972; Simpson and Kling, 1968) were reported to induce this membrane remodeling. More recently, vesicle secretion has been demonstrated to be a major process involved in the control of membrane function during red cell differentiation (Johnstone *et al*., 1991; Pan and Johnstone, 1983). We will review this latter phenomenon and propose some ideas concerning protein sorting in the secreted vesicles, called exosomes. The molecular bases of the process have been mainly studied using the transferrin receptor since this protein, which is highly abundant on the reticulocyte surface (around 100,000 copies), is completely lost by the cells through exosome secretion (Fig. 1).

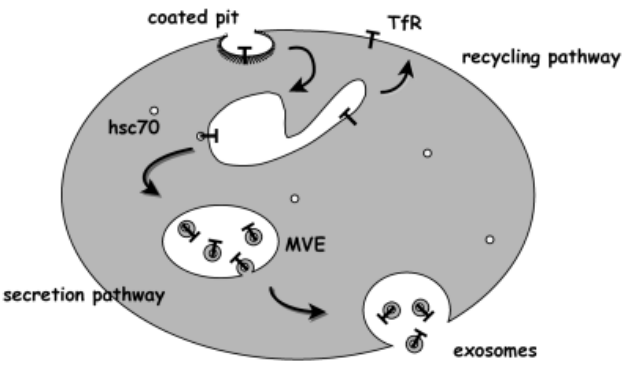

FIGURE 1. TfR pathways during reticulocyte maturation. TfR (**T**) are internalized at the cell surface and then, either recycled back to the plasma membrane (recycling pathway), or segregated in small vesicles constituting multivesicular endosomes (MVE). The receptor is expelled into the extracellular medium, as part of an exosome, when MVE fuse with the plasma membrane (secretion pathway).

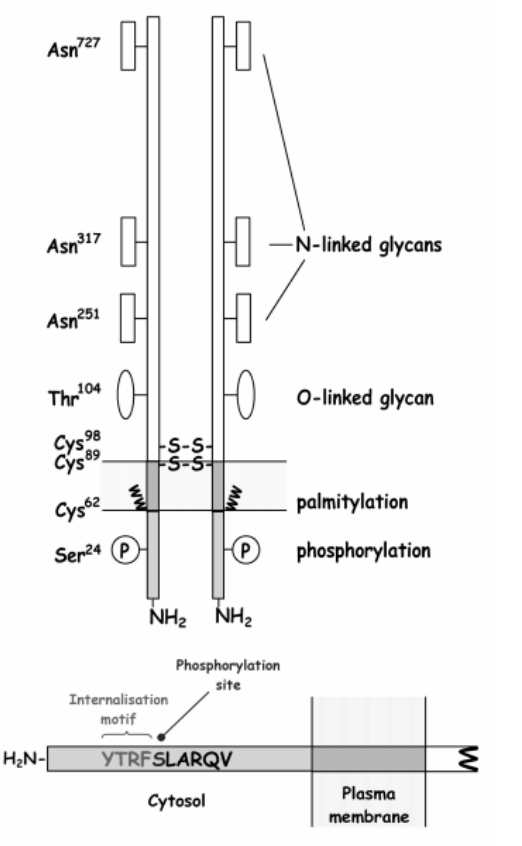

FIGURE 2. Diagramatic representation of the TfR and of the putative domain of interaction with Hsc70.

1. Structure and function of the TfR

The transferrin receptor is a type II transmembrane receptor, with two identical monomers of 90-95 kDa linked via two disulfide bonds (Cys^{89} and Cys^{98}) (Jing and Trowbridge, 1987; Schneider *et al*., 1982). Each subunit consists of (i) an extracellular ligand-binding domain, (ii) a single hydrophobic transmembrane-spanning domain, and (iii) a short N-terminal cytoplasmic tail (Fig. 2). The extracellular part of TfR is post-transcriptionally modified by one complex chain and two mannose-rich oligosaccharides at three N-Asp glycosylation sites (Asn^{251}, Asn^{317}, Asn^{727}) (Omary and Trowbridge, 1981) and also contains O-linked oligosaccharides (Thr^{104}) (Do *et al*., 1990). Glycosylation appears to be necessary for normal function of the receptor (Rutledge *et al*., 1994; Williams and Enns, 1991). However, since it was recently shown that the N-glycans of the mature receptor has no impact on ligand binding (Orberger *et al*., 2001), glycosylation may control receptor folding or sorting

during endocytosis and recycling. The cytoplasmic domain is palmitylated on Cys^{62} adjacent to the transmembrane-spanning region (Jing and Trowbridge, 1987) and phosphorylated on Ser^{24} by PKC (Davis *et al*., 1986b). Neither the acylation nor the phosphorylation of the receptor seems to be critical for its internalization and recycling (Jing and Trowbridge, 1990; Rothenberger *et al*., 1987).

The TfR mediates cellular iron accumulation by binding and internalization of the iron transport protein transferrin (Dautry-Varsat *et al*., 1983). At neutral pH, TfR binds iron-loaded transferrin on the cell surface at high affinity (Kd around 10^{-9}M). This binding step is energy and temperature independent. The receptor-ligand complex is then clustered and internalized through clathrin-coated pits that pinch off from the plasma membrane to form coated vesicles. After clathrin uncoating, the vesicles fuse with endosomes. Iron is released from transferrin in the acidic environment of endosomes, and apo-transferrin still bound to its receptor returns to the cell surface. The receptor is freed from apo-transferrin when encountering the neutral extracellular pH, thus allowing a new round of binding and endocytosis to begin.

Clustering of different receptors in clathrin-coated pits has been shown to depend on a short amino acid sequence present in the cytoplasmic domain of the various receptors and recognized by cytosolic proteins named adaptor proteins (AP1 and AP2). These adaptor proteins simultaneously interact with clathrin and other accessory components of the endocytic machinery and contribute to receptor segregation and vesicle formation. The adaptor protein involved in clathrin-coated pits on the cell surface is the AP2 complex. It is a heterotetramer, with two large subunits (α and β2), a medium sized or μ subunit and a small or σ subunit. The β2 subunit contains the clathrin-binding domain (Shih *et al*., 1995), while the μ subunit is implicated in the interaction with the receptors (Bonifacino and Dell'Angelica, 1999). Distinct sorting signals for selection within clathrin-coated vesicles have been identified. The best characterized is the YXXØ signal (where Ø is a bulky hydrophobic residue and X can be any amino acid, although Y+2 is frequently an R). The corresponding sequence in the TfR cytoplasmic tail is $YTRF^{23}$ (Collawn *et al*., 1993) (Fig. 2). Receptor recycling to the plasma membrane does not seem to require the presence of a specific signal on the receptor. Both the fluorescently tagged lipid N-(N-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]) sphingomyelin (C6-NBD-SM) and the TfR were recycled according to the same kinetics and with equal efficiency after internalization by reticulocytes (Vidal *et al*., 1997). Conversely, interactions with another adaptor protein (AP3) could be involved for protein targeting to lysosomes (Robinson and Bonifacino, 2001). In all cells, TfR thus cycles very efficiently between the plasma membrane and the endosomal compartment, avoiding lysosomal degradation.

2. MVE formation and exosome secretion

During its maturation, the capacity of reticulocytes to bind transferrin decreases to null at the final erythrocyte stage. This loss of Tf binding was first thought to be due to inactivation of TfR by de-glycosylation or proteolysis (Leibman and Aisin, 1977), but it was then demonstrated that TfR are in fact expelled in the extracellular medium, associated with membrane vesicles. These vesicles (about 60 nm in diameter), formed from the endocytic compartment are found in plasma of phlebotomized or anemic animals of different species (Johnstone *et al*., 1989) when the multivesicular endosomes (MVE) fuse with the plasma membrane (Fig. 1). They are also found in human plasma and their concentration was demonstrated to reflect the rate of erythropoiesis. Patients with erythroid hypoplasia presented lower amounts of vesicles compared to patients with erythroid hyperplasia (Huebers *et al*., 1990). Accordingly, plasma of patients treated with EPO showed the presence of high amounts of vesicles (Brugnara *et al*., 1993). This could be used as a novel method for the detection of recombinant EPO abuse in athletes (Parisotto *et al*., 2000). Using an *in vitro* culture technique, it was demonstrated that reticulocytes, and not lymphocytes or mature erythrocytes, were the cells secreting these vesicles (Johnstone *et al*., 1987). The contribution of circulating reticulocytes to TfR secretion has been confirmed *in vivo* by hyper-transfusing reticulocyte-rich or reticulocyte-poor blood to rats (R'zik *et al*., 2001). Erythroblasts, the precursors of reticulocytes, have also been demonstrated both *in vitro* and *in vivo* to produce vesicle-associated TfR (R'zik *et al*., 2001; Shintani *et al*., 1994). Moreover, a truncated soluble form of the TfR (around 85 kDa) has also been found in the plasma (Baynes *et al*., 1994). Biochemical studies have shown that this soluble material consists of the extracellular domain of the intact receptor with a truncation site between Arg^{100} and Leu^{101}, induced by a membrane-associated serine protease at a site distinct from that produced by trypsin (Shih *et al*., 1990). Depending on the cell system and/or the species used for the studies,

different amounts of cleaved receptors were found in the culture supernatants or plasma. TfR cleavage can occur directly from vesicles released by reticulocytes by the action of a granulocyte-associated protease (Johnstone, 1996). The amount of cleaved TfR released by cells could also vary depending on the stage of erythropoiesis. Indeed, contrary to reticulocyte MVE that does not contain any late endosomal or lysosomal markers (Dardalhon *et al*., 2002), it is possible that in early erythroid cells the protease content of MVE is not null due to the presence of an active lysosomal pathway.

Although blebbing from the plasma membrane has been described in maturing red cells (Gasko and Danon, 1974), electron microscopic (EM) studies using colloidal gold-transferrin (AuTf) have shown that these vesicles originate from the endosomal compartment (Harding *et al*., 1983). Gold beads were mainly localized within coated pits and vesicles after short (about 5 min) cell labeling at 37°C. When reticulocytes were incubated for longer times (> 20 min), AuTf was found to decorate vesicular inclusions (about 60 nm) of multivesicular structures (Fig. 3). With longer chase-time, gold-transferrin was found to be associated with vesicles released in the extracellular medium by the fusion of multivesicular structures with the plasma membrane. These multivesicular endosomes were shown to be negative for lysosomal enzymes (*e.g*. acid phosphatase, arylsulfatase) by EM staining (Harding *et al*., 1983). Expulsion through exocytosis was also reported to be an event leading to mitochondria removal during reticulocyte maturation (Gronowicz *et al*., 1984). As shown in Figure 3, autophagic structures can be localized in continuity with, or at least close to, colloidal gold-Tf labeled MVE. The addition of 3-methyladenine (3-MA), a well-known inhibitor of autophagy, during *in vitro* reticulocyte maturation, decreased TfR secretion in the extracellular medium, suggesting that there are at least some similarities or links between autophagy and exosomal processing (Dardalhon *et al*., 2002). However, the effect of 3-MA could also be related to its PI_3-kinase inhibitor characteristics (Petiot *et al*., 2000). Indeed, PI_3-kinase is a very likely participant in the formation of MVE, since wortmannin was demonstrated to inhibit morphogenesis of the MIIC compartment in antigen presenting cells (Fernandez-Borja *et al*., 1999), a multivesicular compartment that induces exosome release in B lymphocytes (Raposo *et al*., 1996) and dendritic cells (Théry *et al*., 1999). Lipids may be important participants during the budding step. We found that the fluorescent lipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) was sorted in exosomes after endocytosis by reticulocytes, while C6-NBD-SM was very efficiently recycled to the plasma membrane (Vidal *et al*., 1997). Recent studies demonstrated the existence of lysobisphosphatidic acid (LBPA)-rich membrane domains in the internal mem-

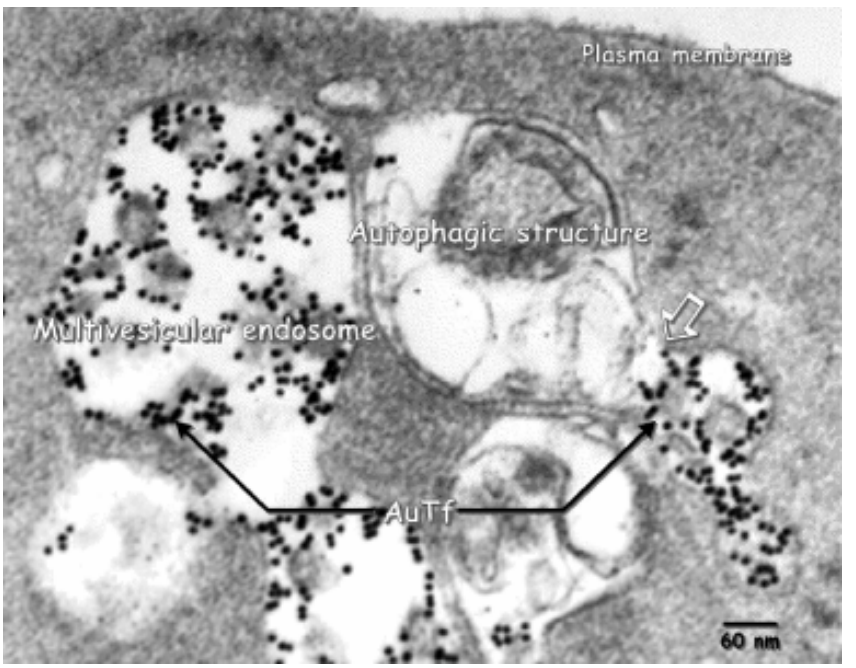

FIGURE 3. Electron microscopy of reticulocyte internal vesicles labelled with colloidal gold-transferrin. Reticulocytes incubated with colloidal-gold transferrin for 30 min at 37°C. The arrow shows continuity between autophagic structures and typical colloidal gold-Tf (AuTf) labelled MVE.

branes of late endosomes and suggested a role of such domains in lipid and protein sorting from these organelles (Kobayashi *et al*., 1998). Moreover, we recently observed, by EM, that GM1 is sorted in the internal vesicles of MVE of rat reticulocytes (unpublished). This, together with the sorting of GPI-anchored proteins (Rabesandratana *et al*., 1998) and the Src kinase Lyn (unpublished) in reticulocyte exosomes, suggests that lipid rafts, *i.e.* the glycosphingolipid (GSL)-enriched domains, may be involved in reticulocyte exosome formation. Accordingly, perfringolysin O, which binds to cholesterol enriched with lipid rafts, has been shown to label internal vesicles in multivesicular bodies of antigen presenting cells. Moreover, exosomes secreted from these cells were also labeled by the toxin (Mobius *et al*., 2002).

3. TfR sorting in exosomes

Exosome release leads to the complete loss of TfR from maturing reticulocytes whereas no band 3, a major membrane protein in red cells, can be detected in exosomes. Other membrane proteins such as glucose transporters, acetylcholinesterase, nucleoside transporters, and amino acid transporters are found in released vesicles (Johnstone *et al*., 1987). The molecular basis of the process of protein sorting into exosomes is still not known. These proteins clearly have very different structures since for example TfR is a single spanning membrane receptor, acetylcholinesterase is a glycosylphosphatidylinositol (GPI)-anchored protein, and glucose transporter is a 12-membrane-spanning helice protein. Moreover, depending on the species (*e.g*. sheep *versus* pig), proteins (*e.g.* nucleoside transporter) are lost to various proportions.

However, there must be some common features between all of these proteins. For example, the sorting signal must "appear" during the lifespan of the protein. Acylation does not seem to be involved since the secreted TfR is still palmitylated. Concerning phosphorylation, it has been shown that the exosomal TfR cannot be phosphorylated *in vitro* by exogenously added PKC, suggesting that the phosphorylation site is no longer accessible (Adam and Johnstone, 1987). So far, this is the only difference described between exosomal TfR and cell surface receptors.

In reticulocyte exosomes, another protein is enriched with TfR in a stoichiometric ratio. This protein has been identified as clathrin-uncoating ATPase (also known as the heat shock cognate 70 kDa protein: Hsc70) (Davis *et al*., 1986a). This protein has been shown to interact with the TfR cytoplasmic domain (Mathew *et al*., 1995), and it was suggested that this interaction induces sorting of the receptors into exosomes. This hypothesis is strengthened by the fact that Hsc70 is also present in exosomes secreted by dendritic cells (Théry *et al*., 1999), and thus may be a general marker of exosome formation. Interestingly however, clustering of TfRs on the reticulocyte cell surface induced by antibodies or lectins was shown to induce receptor sorting into exosomes (Vidal *et al*., 1997). Similarly, clustering of acetylcholinesterase by antibodies resulted in an increase in the exosomal release of the enzyme. These observations demonstrated that protein sorting into exosomes is not necessarily induced by cytosolic sorting machinery. The fact that GPI-anchored proteins such as acetylcholinesterase (AChE), CD55, CD58, CD59 are enriched in reticulocyte exosomes (Johnstone *et al*., 1987; Rabesandratana *et al*., 1998), whereas they do not cross the plasma membrane, supports the concept that protein sorting into exosomes may occur in the absence of a cytoplasmic domain.

The possibility that protein aggregation may be the signal triggering molecules towards the exosome pathway could account for these observations. For example, Hsc70 could interact with proteins following partial unfolding of the cytoplasmic domain. If TfR were to unfold, Hsc70 would become associated with the TfR without participating further in the sorting process. It is known that, besides its role in uncoating clathrin from coated vesicles, Hsc70 has general chaperone properties and is involved in protein folding and unfolding (Strickland *et al*., 1997).

Previous studies have described the general characteristics of the HSP70 class of molecular chaperones in binding to peptides (Fourie *et al*., 1994). Hsc70 binds preferentially to hydrophobic sequences containing basic amino acids. The ADP-bound form of HSP70 has a high affinity for peptides whereas the ATP-form has a lower affinity, thus resulting in dissociation (Palleros *et al*., 1994). Using an *in vitro* binding assay to study characteristics of the interaction between Hsc70 and TfR, we demonstrated that Hsc70 binds to exosomal TfR with the characteristics expected of a chaperone/peptide interaction. We used deoxyspergualin (DSG) and LF15-0195, two immunosuppressive agents that interact with Hsc70 (Komesli *et al*., 1999; Nadeau *et al*., 1994). Both compounds diminished the interaction between Hsc70 and TfR in our *in vitro* binding assay. Both induced an increase in TfR release in exosomes when added during *in vitro* maturation of reticulocytes, demonstrating that TfR aggregation instead of Hsc70 binding may be the

signal targeting TfR molecules towards the exosome pathway.

V. Mitoptosis in maturing reticulocytes

Mitochondrial degradation in maturing reticulocytes is really a programmed death phenomenon (mitoptosis) (Skulachev, 1999), since nucleus expulsion coincides with activation of 15-LOX translation and induction of genes coding for ubiquitin conjugating enzymes. Moreover, these enzymes are no longer active in mature red cells, *i.e.* after the disappearance of mitochondria. The fact that the nucleus is expulsed before activation of mitochondrial breakdown is probably not fortuitous. Indeed, since mitochondria are now recognized as key elements in the control of the cell survival and death (Kroemer and Reed, 2000), this may be a way for maturing reticulocytes to avoid apoptosis.

1. ROS production

Mitochondria generate reactive oxygen species (ROS) as by-products of oxidative metabolism. Superoxide ($O_2^{\cdot-}$) formation is dangerous for cells since $O_2^{\cdot-}$ can give rise to a hydroxyl radical ($OH^{\cdot}$), with H_2O_2 as the intermediate. These ROS cause modifications in the amino acids of proteins, which generally results in the loss of their function. The mildly oxidized proteins are rapidly degraded by the 20S proteasome in an ATP- and ubiquitin-independent pathway (Davies, 2001). Oxidative damage induces rearrangement of the secondary and tertiary protein structure with consistent exposure of internal hydrophobic stretches. These hydrophobic stretches can bind directly to 20S proteasomes without requiring ubiquitination. More severe protein oxidation results in chemical fragmentation and covalent cross-linking reactions that can induce protein aggregates and the formation of inclusion bodies.

2. Caspase activation

Pore formation by 15-LOX (van Leyen *et al*., 1998) and/or PTP opening by ROS (Skulachev, 2001) could lead to the loss of outer mitochondrial integrity and subsequent release of intermembrane proteins into the cytosol. Several of these proteins are involved in apoptosis. In particular, cytochrome *c* can form a complex with a cytosolic factor (Apaf-1) and dATP, that is able to hydrolyze inactive procaspase-9 to active caspase-9 (Zou *et al*., 1999), which in turn can activate downstream caspases such as caspase-3, -6, and –7 (Thornberry and Lazebnik, 1998). Accordingly, preliminary experiments showed the presence of caspase-3 activity in reticulocyte cytosol from phenylhydrazine-treated rats (unpublished). In the same line, we have revealed by Western blot a truncated form of Lyn in reticulocyte exosomes, compared to the form detected on the cell surface (unpublished). Such a truncated form (about 3 kDa truncation) of Lyn has already been described in apoptotic hematopoietic cells (Luciano *et al*., 2001).

3. Consequences on exosome release?

Mitochondrial degradation may contribute to exosome formation and /or protein sorting in secreted vesicles. The presence of 14-3-3 protein, an anti-apoptotic molecule, in exosomes and on reticulocyte endocytic vesicles (Bette-Bobillo *et al*., 1998) could be a sign of the involvement of apoptotic signaling in reticulocyte maturation and/or exosome secretion. In agreement, we found that the addition of the 15-LOX inhibitor ETYA during *in vitro* reticulocyte maturation led to a decrease in exosomal TfR secretion (unpublished).

Mitoptosis may be the signaling of a cascade of events leading to an irreversible process. Interestingly, the formation of a multivesicular structure from an early endosomal compartment (TfR^+) is unique to maturing erythroid cells, suggesting that intracellular characteristics are crucial during reticulocyte maturation. In line with this, rabaptin-5 was shown to be one of the 40 or so known substrates of caspases (Swanton *et al*., 1999). Rabaptin-5 is a downstream effector of rab5, which is essential for endosome fusion. Interestingly, rabaptin-5 also interacts with rab4, the GTPase in charge of early endosome recycling (van der Sluijs *et al*., 1992). Cleavage of rabaptin-5 by caspase-3 in maturing reticulocytes may induce changes in membrane dynamics, *i.e.* an inward rate of membrane flow by endocytosis that would exceed the outward flow by recycling, allowing the formation of multivesicular structures. Moreover, as we already underlined, autophagic structures are often observed in continuity or close to MVE (Fig. 3), and exosome secretion is inhibited by 3-MA, an anti-autophagic drug (Dardalhon *et al*., 2002), suggesting a link between both processes. Interestingly, apoptotic signaling in neurons activates autophagy (Xue *et al*., 1999), and both apoptosis and autophagy are induced upon the opening of mitochondrial PTP (Lemasters *et al*., 1998). Mitoptosis in reticulocytes may thus be involved in MVE formation, a process whereby, like autophagic vacuole formation, the cell “digests” itself from within.

Concerning protein sorting in exosomes, mild oxidation of endosomal transmembrane proteins by ROS may induce changes in their conformation, exposing hydrophobic stretches, and leading to their clustering in the endosomal membrane. This is in complete agreement with our characterization of the interaction between hsc70 and TfR in exosomes (Géminard *et al*., 2001). However, we have found that a 10 amino acid synthetic peptide (YTRFSLARQV) contained in the cytosolic domain of the TfR (Fig. 2) was able to compete with interactions between Hsc70 and reduced and carboxymethylated lactalbumin (RCMLA), suggesting that this sequence is involved in the binding to Hsc70 (unpublished). Since the YTRF internalization motif is part of the sequence, another possibility is that this sequence, which is normally engaged with partners such as AP2 and thus inaccessible to Hsc70, would be unmasked after degradation of the adaptor protein. These hypotheses should be checked in further studies.

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VI. References

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