Journal of Cell Science 110, 1169-1178 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS4351

Plasma membrane targetting, vesicular budding and release of galectin 3 from the cytoplasm of mammalian cells during secretion

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SUMMARY

Galectin 3, a 30 kDa galactoside-binding protein distributed widely in epithelial and immune cells, contains no signal sequence and is externalized by a mechanism independent of the endoplasmic reticulum (ER)-Golgi complex. We show here that hamster galectin 3 overexpressed in transfected cos-7 cells is secreted at a very low rate. A chimaera of galectin 3 fused to the N-terminal acylation sequence of protein tyrosine kinase p56^{lck}, Nt-p56^{lck}-galectin 3, which is myristoylated and palmitoylated and rapidly transported to plasma membrane domains, is efficiently released from transfected cells indicating that movement of cytoplasmic galectin 3 to plasma membrane domains is a rate limiting step in lectin secretion. N-terminal acylation is not sufficient for protein secretion since p56^{lck} and the chimaera Nt-p56^{lck}-CAT are not secreted from transfected cells. The amino-terminal half of galectin 3 is sufficient to direct export of a chimaeric CAT protein indicating that part of the signal for plasma membrane translocation lies in the N-terminal domains of the lectin. Immunofluorescence studies show that Nt-p56^{lck}-galectin 3 aggregates underneath the plasma membrane and is released by membrane blebbing. Vesicles of low buoyant density isolated from conditioned medium are enriched in galectin 3. The lectin is initially protected from exogenous collagenase but is later released in soluble protease-sensitive form from the lectin-loaded vesicles. Using murine macrophages, which secrete their endogenous galectin 3 at a moderate rate especially in the presence of Ca^{2+}-ionophores, we were also able to trap a galectin 3-loaded vesicular fraction which was released into the culture supernatant.

Key words: Galectin, Macrophage, Secretion, Transfected cell

INTRODUCTION

Galectins are a family of proteins that bind to galactose-containing ligands (Barondes et al., 1994). To date, eight family members have been described which differ in tissue localization and embryonic expression. Members of this family have been implicated in a variety of functions that include neoplastic transformation and growth regulation (Raz et al., 1990; Yamaoka et al., 1991) and immune responses (Liu, 1993).

Evidence for extracellular roles of galectin 3 is available. The lectin binds to carbohydrate determinants such as polylactosamine and ABO(H) blood group structures that are prominent on epithelial cell surfaces and matrix glycoproteins (Sato and Hughes, 1992). Galectin 3 added exogenously can modulate cell adhesion to laminin and fibronectin glycoforms carrying appropriate sugar receptors and promotes migration of cells though a laminin-rich gel (Sato and Hughes, 1992; Le Marer and Hughes, 1996). Galectin 3 also modulates cyst formation and tubulogenesis in kidney cell cultures in vitro, probably by effects on integrin mediated interactions (Bao and Hughes, 1995). In monocytes and neutrophils galectin 3 ligation of surface glycoprotein receptors activates an oxidative burst and secretion of cytokines (Liu, 1993; Zuberi et al., 1994). Similarly diverse effects have been reported for other galectins and it appears that the galectins are a family of regulatory molecules which perform a variety of biological functions depending on their extracellular location and their specific cell surface receptors. Further understanding of these roles requires some detailed ideas of how the proteins are secreted and the regulation of their secretion.

Galectins, as well as several mammalian proteins such as interleukins 1 α/β, the acidic and basic fibroblast growth factors and thioredoxin that are known to be synthesized on cytoplasmic ribosomes, are subsequently secreted from cells by non-classical mechanisms (Muesch et al., 1990). Since these proteins lack a signal sequence, secretion does not involve passage of nascent proteins through the endoplasmic reticulum and various drugs commonly used to block the classical secretory pathway, such as brefeldin A and monensin, do not block galectin 3 secretion (Lindstedt et al., 1993; Sato et al., 1993). Interestingly, secretion is strongly stimulated by heat shock (42°C) and by calcium ionophores (Sato et al., 1993; Sato and Hughes, 1994).

Normally galectin 3 secretion is slow and incomplete, for example 20-30 percent of newly synthesized lectin over 24 hours in BHK cells (Sato et al., 1993), making studies of the secretory mechanism difficult. Recent studies (Zlatkine et al., 1997) showed that chimaeras (Fig. 1) of the first ten amino acid residues of p56^{lck} protein tyrosine kinase, containing one glycine site for myristoylation and two cysteine palmitoylation

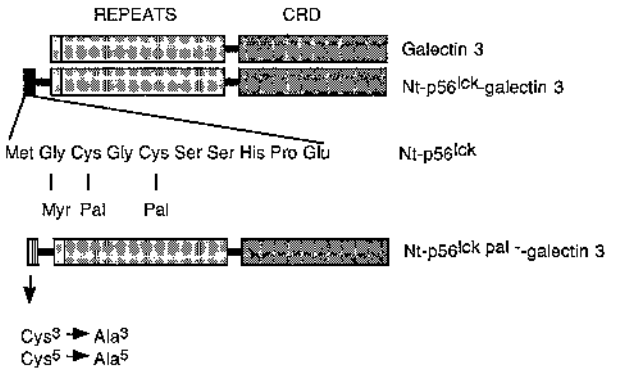

Fig. 1. Schematic representation of the structures of galectin 3 and chimaeras. The carbohydrate recognition domain (CRD) and the glycine-proline rich repeat sequence of galectin 3 are shown flanked by short linker sequences. N-terminal extension with the first ten amino acid residues of $p56^{lck}$ provides one and two sites, respectively, for acylation by myristate and palmitate. Site-directed mutagenesis deletes both palmitoylation sites in the Nt-$p56^{lck\ pal-}$-galectin 3 mutant.

sites at positions 3 and 5 (Koegl et al., 1994), with hamster galectin 3 and another normally cytoplasmic protein chloramphenicol acetyl transferase (CAT) were efficiently targetted in transiently transfected Cos 7 cells to plasma membranes and also perinuclear Golgi compartments to some extent. In contrast Nt-$p56^{lck}$-galectin 3 chimaeras lacking palmitoylation sites after site-directed Cys→Ala mutagenesis showed no or minimal transport to plasma membranes. These proteins were distributed throughout the cytoplasm as was the lectin lacking the Lck motif when expressed in Cos 7 cells. The conclusion of these results was that the addition of a myristoylated and palmitoylated sequence to galectin 3 or CAT is a powerful signal for plasma membrane targetting. We have now used this system to follow the transport of galectin 3 out of the cell. We show that trafficking of galectin 3 from biosynthetic sites to the plasma membrane is a rate-limiting step in secretion and that plasma membrane association alone is not sufficient to direct non-classical export of a cytosolic protein since other chimaeric proteins containing the $p56^{lck}$ acylation sequence are not secreted. We provide evidence that the chimaeric galectin 3 protein is secreted from cells in vesicles from which it is released into the external medium. Finally, we show that vesicles of similar properties containing endogenous galectin 3 are released from murine macrophages.

MATERIALS AND METHODS

Antibodies and reagents

Trans ^{35}S-label (>70% L-methionine, specific activity 1,057 Ci/nmole) was obtained from ICN Biomedicals, High Wycombe, UK. Palmitate (9,10-^{3}H labelled, specific activity 51 Ci/mmol) and myristate (9,10-^{3}H labelled, specific activity 40-60 Ci/mmol) were from Amersham International and NEN, UK. *Achromobacter iophagus* collagenase was from Boehringer Mannheim. Rabbit polyclonal antisera raised against hamster galectin 3, its carbohydrate-recognition domain (CRD) and N-terminal domain epitopes were as described (Mehul et al., 1994, 1995). Rat monoclonal antibody M3/38 (anti-Mac-2) against mouse galectin 3 was from Boehringer Mannheim, East Sussex, UK. Rabbit polyclonal IgG against chloramphenicol acetyl transferase (CAT) was purchased from 5 prime-3 prime, Inc., Colorado. Rabbit polyclonal anti-serum raised against $p56^{lck}$ was a generous gift from A. I. Magee. Goat anti-rat and anti-rabbit IgG peroxidase, fluorescein or Texas red conjugate were from Sigma.

Expression plasmids

CAT cDNA was generated by PCR and inserted into the pSG5 plasmid. Hamster galectin 3 cDNA was isolated from clone Q5 (Mehul et al., 1994) by *Xba*I and *Bam*HI digestion and subcloned into the pSVL plasmid (Pharmacia). Nt-$p56^{lck}$-galectin 3 and Nt-$p56^{lck}$-CAT constructs were subcloned into the pSG5 plasmid using *Eco*RI-*Bam*HI restriction sites and N-terminal $p56^{lck}$ or mutated N-terminal $p56^{lck}$ adaptors. Full details of these constructs are described elsewhere (Zlatkine et al., 1997). Briefly, galectin 3 cDNA was isolated and purified from the Q5 clone (Mehul et al., 1994) by *Sal*I-*Bam*HI site restriction. Adaptors were generated by annealing of two primers: 5′ AATTCATGGGCTGTGGCTGCAGCTCACACCCGGAAGATG 3′ and 5′ TCGACATCTTCCGGGTGTGAGCTGCAGCCACAGCCCATG 3′ (for wild-type N-terminal $p56^{lck}$), or 5′ AATTCATGGGCGCAGGCGCAAGCTCACACCCGGAAGATG 3′ and 5′ TCGACATCTTCCGGGTGTGAGCTTGCGCCTGCGCCCATG 3′ for mutated N-terminal $p56^{lck}$ adaptors (underlines correspond to alanine codons substituted for Cys^3 and Cys^5 codons of the wild-type sequence). CRD of galectin 3 (starting at T110) fused to the Nt-$p56^{lck}$ sequence was generated by PCR using as template DNA the plasmid pSG5 containing the Nt-$p56^{lck}$-galectin 3 construct and the following primers; 5′(phosphorylated) TTCCGGGTGTGAGCTGCAGCC 3′ and 5′ ACAGTGCCCTATAAGCTGCCCTTG 3′. PCR was performed using Amplitaq DNA polymerase and Taq-extender (Stratagene) as follows: one cycle at 94°C for 3 minutes, 30 cycles at 94°C for 30 seconds, 56°C for 1 minute, 72°C for 2 minutes and 1 cycle for 5 minutes at 72°C. PCR products were incubated with *Dpn*I and pfu DNA polymerase to digest template DNA plasmid and blunt end the PCR products before agarose-gel purification. To make chimaeria Nt-$p56^{lck}$-Nt-galectin 3-CAT, Nt-$p56^{lck}$-galectin 3 was cut with *Eco*RI and *Bam*HI. The gel-purified product was digested with *Hin*fI and ligated together with a restriction fragment obtained by *Sal*I and *Bam*HI treatment of Nt-$p56^{lck}$-CAT plasmid into pSG5. The gel-purified product was blunt-ended using Mung bean nuclease and ligated and contains the Nt-$p56^{lck}$ acylation sequence, residues 1-120 of hamster galectin 3 (Mehul et al., 1994) followed by the complete CAT coding sequence. Ligation and transformation of *Escherichia coli* DH5 was performed as described previously (Mehul et al., 1994). Large scale purification of plasmid DNAs was carried out using a Qiagen Kit. Nucleotide sequences were verified by the dideoxy-chain termination method using a Sequenase Kit, version 2.0 (U.S. Biochemical Corp.).

Cell culture, transfection and metabolic labeling

Cos-7 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum and antibiotics. Transfection was performed by electroporation (250 μF, 0.3 kV) using 10 to 20 μg of plasmid DNA. Transfected cells were grown for 24 to 48 hours at 37°C with 5% CO_2 in monolayer culture usually in 3.5 cm or 6 cm diameter Nunc dishes in Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics. For amino-acid labeling, the cells were incubated for 16 hours at 37°C with methionine and cysteine-free Eagle's medium (1 ml) containing 10% dialyzed FCS and 150 μCi of Trans ^{35}S-label. For fatty acid labelling, cells were incubated for 16 hours in Eagle's medium supplemented with dialyzed FCS and [9,10-^{3}H] palmitate (250 μCi/ml) or [9,10-^{3}H] myristate (150 μCi/ml). In pulse chase experiments cells were incubated with methionine and cysteine-free Eagle's medium containing 10% dialyzed FCS for 20 minutes, then in the same medium (1 ml) containing 150 to 200 μCi/ml of Tran ^{35}S-label for 30-45 minutes at 37°C.

After incubation, cells were washed twice with complete Eagle's medium containing 10% FCS and incubated in the same medium (1 ml) for various periods of chase. Culture supernatants were collected and kept on ice after the addition of 0.5 ml of RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Na deoxycholate, 0.1% SDS, 1% NP40, 1 mM EDTA) containing 1 mM PMSF. Cell monolayers were gently washed with cold PBS, lysed with 1 ml of RIPA buffer at 2°C, passed through a narrow gauge needle and kept on ice. Alternatively, cells before lysis were incubated in cold PBS containing 100 mM thiodigalactoside (TdG) on ice for 20 minutes (TdG fraction). Medium and TdG fractions were centrifuged at 6,500 ***g*** at 4°C for 10 minutes to remove cell debris.

Immunoprecipitation and western blotting

Labelled fractions were precleared with 40-100 μl of a 50% (w/w) suspension of Protein A-Sepharose in RIPA buffer for 2 hours at 4°C followed by centrifugation at 4°C for 5 minutes at 6,500 ***g***. The cleared supernatants were incubated with an appropriate polyclonal antibody (1:100) overnight at 4°C in the presence of 40 μl of Protein A-Sepharose. The beads were washed five times with RIPA buffer for 10 minutes at 4°C, once with 100 mM Tris-HCl, pH 8, and then heated at 90°C for 5 minutes in Laemmli sample buffer or at 60°C for fatty acid labelling. Samples were separated by SDS-PAGE using 10%, 12.5% or 15% gels. Fluorography of dried gels was done using Kodak X-Omat Film. In some experiments, proteins were transferred after SDS-PAGE to nitrocellulose and blotted with rabbit polyclonal antibodies directed against galectin 3 CRD or N-terminal domain epitopes (1:2,000 dilution) for 2 hours at room temperature followed by peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution). Bands were located by the SuperSignal CL-HRP Substrate System (Pierce, Rockville Ind.).

Immunofluorescence microscopy

Transfected or non-transfected cells were plated on glass coverslips, grown for 24-48 hours, washed three times with phosphate buffered saline (PBS) and fixed with methanol at −20°C for 8 minutes or directly incubated at 4°C for 20 minutes with a primary antibody diluted in PBS containing 1% BSA before fixation. In some experiments cells were fixed with 3.7% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilization was performed by incubation of the fixed cells with 0.2% of Triton X-100 at room temperature for 10 minutes After fixation cells were washed twice with PBS, once with PBS-3% BSA and then incubated for 2 hours at 4°C with primary antibodies diluted 1:50 in PBS-1% BSA. Cells were washed five times with PBS and once with PBS-1% BSA and incubated for 1 hour with affinity-purified fluorochrome-conjugated goat anti-rabbit or anti-rat IgG (1:50) in PBS-1% BSA. Cells were extensively washed with PBS and slides were mounted in Hydromount (National Diagnostics, UK) and examined with a Bio-Rad Lasersharp MRC600 imaging system attached to an Olympus BH2 microscope using Zeiss ×40 or ×63 Planapochromat lenses. Untransfected cells were observed as negative controls.

Vesicle isolation

Cos-7 cells transfected with Nt-p56^{lck}-galectin 3-pSG5 plasmid were grown for 48 hours and then labelled with 50 μC/ml of Trans ^{35}S-label for 30 minutes at 37°C as described above. Chase was performed in non-radioactive medium for different periods and culture supernatants were collected. In some experiments transfected cells were labelled to equilibrium over 24 hours. Supernatants were centrifuged at 2,000 ***g*** for 30 minutes at 4°C. Hepes buffer (10 mM Hepes, pH 7.2, 150 mM NaCl, 0.02% NaN_3) was added (1:1, v/v) and the mixtures were centrifuged at 90,000 ***g*** for 2 hours at 4°C. Pellets were harvested and resuspended in Hepes buffer. Pellet fractions were laid on continuous sucrose gradients (10-50% w/v) in Hepes buffer and centrifuged for 16 hours at 4°C and 170,000 ***g***. Fractions were collected from the bottom of the tube and assayed for radioactivity (30 μl samples). Other samples were taken for SDS-PAGE (30 μl) or immunoprecipitation (300 μl) with galectin 3 specific antibodies. Appropriate gradient fractions were combined, centrifuged at 90,000 ***g*** for 2 hours at 4°C and the pellets were resuspended in PBS. In other experiments, the pellets and supernatant fractions from high-speed (90,000 ***g***) centrifugation were diluted in RIPA buffer and immunoprecipitated with galectin 3-specific antibodies.

Murine macrophage J774.2 cells from the European Collection of Animal Cell Cultures were labelled in suspension culture (1 ml) for 3 hours with 400 μCi/ml Trans ^{35}S-label and chased in cold medium containing 5 μM calcium ionophore A23187 (Sato and Hughes, 1994). The cells were centrifuged down at 6,500 ***g*** for 5 minutes and resuspended in fresh chase medium every 30 minutes for a total of 6 hours of chase. The combined clarified supernatants, kept on ice, were applied to continuous sucrose gradients. In other experiments, 0.5 ml packed J774.2 cells were labelled in methionine-free Eagle's medium (1 ml) with 2 mCi TransLabel for 45 minutes at 37°C. The cells were washed three times by centrifugation at 6,500 ***g***, suspended in complete Eagle's medium (3 ml) and chased at 37°C. Samples (1 ml) were removed at intervals and centrifuged at 2,000 ***g*** and 6,500 ***g*** for 5 minutes each at 2°C to remove cells. The cell-free supernatants were then centrifuged at 90,000 ***g*** for 2 hours at 2°C. The high speed pellets and supernatants were diluted into RIPA (1 ml) at 2°C and used for immunoprecipitation of galectin 3 as before.

Collagenase treatment and other procedures

Particulate fractions in 10 mM Hepes, pH 7.2, 150 mM NaCl buffer (0.5 ml) were adjusted to 5 mM $CaCl_2$ and treated with *Achromobacter iophagus* collagenase (25 mUnits) at 37°C. In some cases the collagenase was omitted and the mixtures were adjusted to 5 mM EDTA final concentration before incubation. Other particulate samples were adjusted to 0.1% Triton X-100 and sonicated for 10 minutes at 4°C before addition either of EDTA (5 mM final concentration) or collagenase (25 mUnits). After incubation, each mixture (0.5 ml) was mixed with RIPA buffer (0.5 ml) containing 5 mM EDTA and used for immunoprecipitation of galectin 3. Lactate dehydrogenase (LDH) activity was determined using the Cytotox 96 non-radioactive cytotoxicity assay kit obtained from Promega (Madison, WI). Sugar binding activity of galectin 3 or chimaeric proteins was determined by affinity chromatography using lactose-Agarose (Sigma) as described previously (Mehul et al., 1994).

RESULTS

Galectin3 expression in Cos-7 cells

Cos 7 cells express very little galectin 3 normally and expression is greatly increased in cells transfected transiently with either hamster full length cDNA (Fig. 2) or with the Nt-p56^{lck}-galectin 3 cDNA (result not shown). The lectin expressed in transfected cells retained full carbohydrate binding activity as shown by lactose-agarose affinity chromatography (Fig. 2).

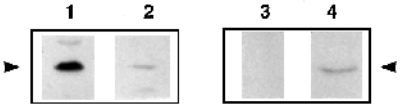

Fig. 2. Galectin 3 expression in [^{35}S]methionine-labelled Cos-7 cells transfected with full length hamster galectin 3 cDNA. 1, Transiently transfected cells; 2, non-transfected cells; 3,4, asialofetuin agarose chromatography of transfected cell lysates: unbound (3) and bound (4) fractions. Equal proportions of cell lysates or column fractions were immunoprecipitated with galectin 3-specific antibodies and immune complexes were separated by SDS-PAGE followed by fluorography. The arrowheads show migration of galectin 3.

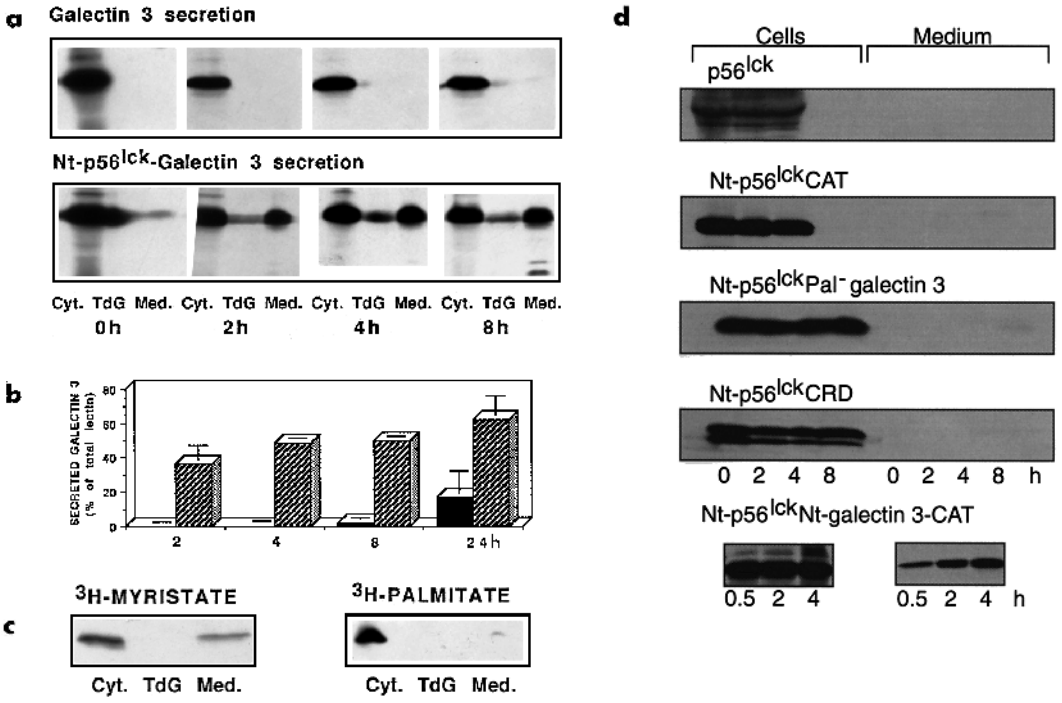

Fig. 3. Secretion of proteins from transiently transfected Cos-7 cells. (a) Cos-7 cells 48 hours post-transfection with either galectin 3 or Nt-p56^{lck}-galectin 3 constructs (see Fig. 1) were labelled for 30 minutes at 37°C with [^{35}S]methionine and chased for the times indicated. Culture supernatants were collected. The cells were washed with thiodigalactoside (TdG) to elute cell surface-bound lectin and then lysed. Equal proportions of culture supernatants and cell lysates were immunoprecipitated with galectin 3 antibodies and immune complexes were separated by SDS-PAGE followed by fluorography. (b) Gel fluorograms (see a) were scanned and the proportion of secreted (the sum of lectin in culture supernatants and TdG extracts) galectin 3 (black) and Nt-p56^{lck}-galectin 3 (cross hatched) calculated as a percentage of total lectin. Means of several separate experiments are shown. (c) Cos-7 cells transiently transfected with Nt-p56^{lck}-galectin 3 were labelled for 2 hours at 37°C with either ^{3}H-myristate or ^{3}H-palmitate and chased for 2 hours at 37°C. Galectin 3 was immunoprecipitated from cell lysates, TdG eluted fractions and culture supernatants as described in a. (d) Cos-7 cells transiently transfected with the constructs indicated were labeled as in a. CRD is the carbohydrate recognition domain of galectin 3. Nt-galectin 3 is the N-terminal sequence (residues 1-120) of the hamster lectin. Expressed proteins were immunoprecipitated from lysed cells and culture supernatants with antibodies against galectin 3 or CAT as appropriate and analysed by SDS-PAGE and fluorography. The 8 hour chase lysates of p56^{lck} and N-p56^{lck}-CAT transfected cells were not examined.

Plasma membrane targetting of galectin 3 by acylation increases its secretion in transfected Cos 7 cells

Pulse-chase analysis showed that secretion of galectin 3 itself from transiently transfected Cos 7 cells is very slow (Fig. 3a,b). By contrast one half of the newly synthesised Nt-p56^{lck}-galectin 3 chimaera (Fig. 1) was secreted within 4 hours (Fig. 3a,b). As expected deletion of the palmitoylation sites which blocks translocation of cytosolic chimaeras to plasma membrane domains (Zlatkine et al., 1997) resulted in negligible secretion of the chimaera Nt-p56^{lck}-pal^{-} galectin 3 (Fig. 1) over 8 hours of chase (Fig. 3d). Most of the external Nt-p56^{lck}-galectin 3 protein was recovered from the culture supernatant of transfected cells but a small amount was eluted (Fig. 3a) from the cell surface with a sugar hapten, thiodigalactoside (TdG). Cell lysis monitored by lactate dehydrogenase assays was not detected in either transfection over 8 hours of chase and was still less than 10% after 24 hours (results not shown), indicating that rapid secretion of Nt-p56^{lck}-galectin 3 was not due to cell death. The chimaeric protein recovered from secretions was labelled with both ^{3}H-myristate and ^{3}H-palmitate (Fig. 3c) showing that the acyl substituents on the Lck-tail had survived export from the cell over the period of pulse-chase. However, palmitoylation can be a reversible process, including palmitoylation of p56^{lck} (Paige et al., 1993), and some deacylation of chimaeric protein seems also to have occurred.

Acylation is not a sufficient signal for protein secretion

Recent studies have shown that p56^{lck} protein tyrosine kinase is rapidly targetted to glycolipid enriched plasma membrane domains following myristoylation and palmitoylation of the N-terminal sequence (Shenoy-Scaria et al., 1994; Rodgers et al., 1994). However, the acylated Lck protein is not secreted from transiently transfected Cos-7 cells (Fig. 3d). The chimaera Nt-p56^{lck}-CAT is similarly transferred to the plasma membrane in transfected Cos 7 cells (Zlatkine et al., 1997) but is not secreted (Fig. 3d). These results show that acylation alone is insufficient for release of proteins from cells and suggest that the signal for the efficient secretion of Nt-p56^{lck}-galectin 3 is contained within the lectin sequence. At least part of this signal is contained within the N-terminal domains of galectin 3. First, pulse chase experiments show that a chimaera of the N-terminal Lck sequence, containing the sites for myristylation and palmitoylation, fused to the galectin 3 CRD was secreted from transiently transfected Cos-7 cells at a very low rate over 8 hours of chase (Fig. 3d). Trivial reasons for this result are unlikely from control experiments showing that the CRD chimaera was readily labelled with myristate and palmitate (results not shown). Second, the chimaera Nt-p56^{lck}-Nt-galectin 3-CAT containing the acylation sequence fused to the N-terminal domains of galectin 3 followed by CAT was secreted (Fig. 3d) with kinetics very similar to chimaera Nt-p56^{lck}-galectin 3 (Fig. 3a).

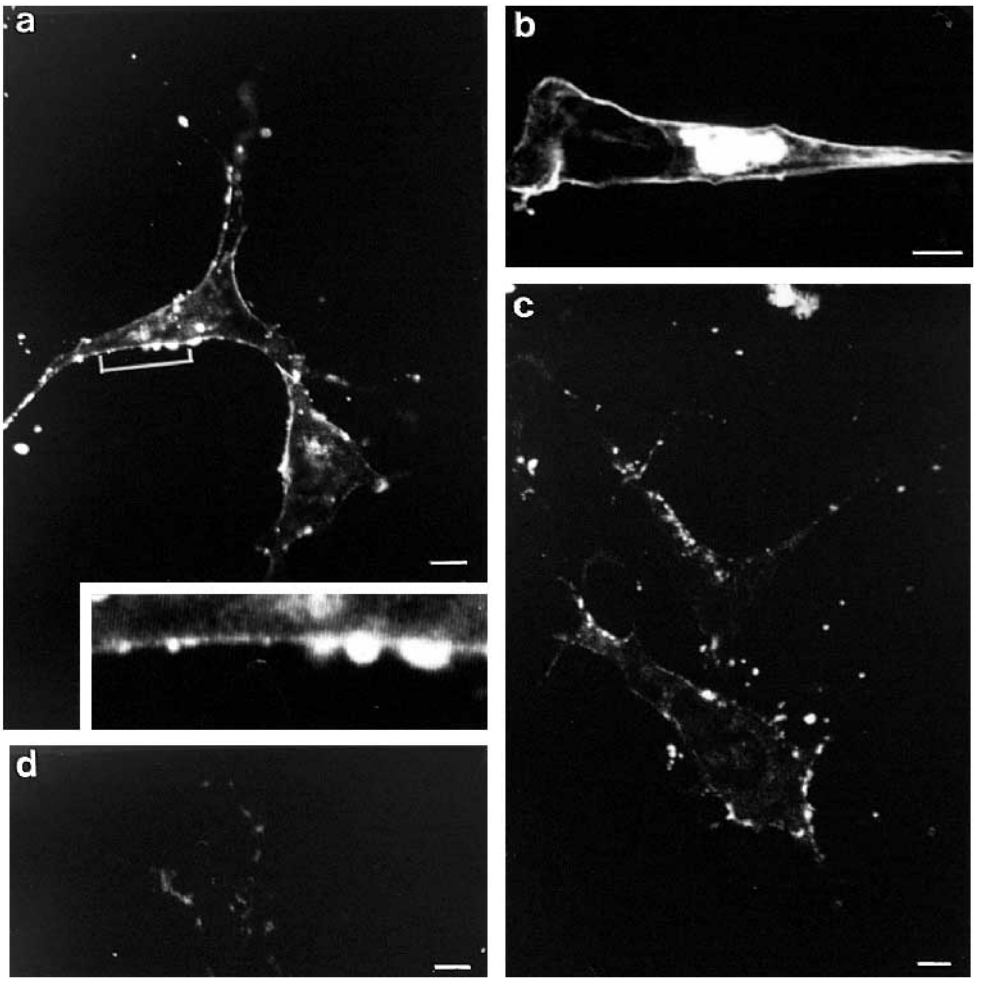

Fig. 4. Immunofluorescence staining of Cos-7 cells transfected with Nt-p56^{lck}-galectin 3 (a,c,d) or Nt-p56^{lck}-CAT (b). (a) Fixed and permeabilized cells stained for galectin 3. Note dominant and punctate localization of Nt-p56^{lck}-galectin 3 at the plasma membrane, significant perinuclear Golgi staining and (white bar and inset) pronounced blebbing of lectin from the plasma membrane. (b) Methanol fixed cells stained with CAT-specific antibody. Note uniform localization along the plasma membrane and significant perinuclear Golgi staining. (c,d) Galectin 3 antibody staining of unpermeabilized cells (d) or of intact cells (c) treated with bacterial collagenase before fixation and permeabilization. Bars, 10 μm.

Membrane association of chimaeric proteins in transfected cells

Fig. 4 shows the distribution of Nt-p56^{lck} chimaeras in transfected cells. We used for galectin 3 detection the M3/38 monoclonal antibody which recognizes an epitope in the N-terminal half of the lectin. In contrast to the chimaeric CAT protein, which was distributed uniformly along the cytoplasmic side of the plasma membrane as well as prominently in perinuclear presumably Golgi structures in transfected cells (Fig. 4b), the galectin 3 chimaeric protein was located mainly at the cell periphery showing a markedly discontinuous appearance with numerous aggregates of lectin underlining the plasma membrane (Fig. 4a). At higher magnification many of these aggregates appeared to be blebbing from the cell surface (Fig. 4a). In addition we observed antibody-positive aggregates deposited on the growth substratum which were absent in the chimaeric CAT-transfected cells. The cell-associated galectin 3-rich structures were inaccessible to antibody in non-permeabilized cells (Fig. 4d) and were resistant to digestion of intact cells by bacterial collagenase (Fig. 4c), an enzyme that degrades the N-terminal domains of galectin 3 with loss of M3/38 reactivity.

Secretion of Nt-p56^{lck}-galectin 3 in enclosed vesicles

The pronounced blebbing of galectin 3-containing structures from transfected cells (Fig. 4) suggested that the lectin was released from cells within a limiting membrane. In order to identify a secreted vesicular fraction containing galectin 3, Cos-7 cells transiently transfected with Nt-p56^{lck}-galectin 3 cDNA were pulse labelled with [^{35}S]methionine and chased for 2 hours. The collected culture supernatants were cleared by a low speed centrifugation step and a particulate fraction was obtained by centrifugation for 2 hours at 90,000 ***g***. Isopycnic centrifugation of the particulate fraction on a continuous 10-50% sucrose gradient gave a radioactive peak floating at approximately 25% sucrose (Fig. 5d). The peak contained a 30 kDa protein as the major labelled component, shown to be galectin 3 by immunoprecipitation (Fig. 5e,f). Only trace amounts of galectin 3 were recovered in other fractions from the gradient, including the soluble protein fraction at the top of the gradient (Fig. 5d,f). Fig. 5a-c shows that galectin 3 is also a major protein of vesicles isolated after long term labelling. Similar results were obtained by silver staining after SDS-PAGE of purified vesicles (results not shown). We conclude that the vesicles are enriched in Nt-p56^{lck} galectin 3 relative to other proteins suggesting that evagination occurs at membrane domains from which pre-existing membrane proteins have been in part segregated.

As Fig. 6 shows the amount of Nt-p56^{lck} galectin 3 loaded vesicles increased with time over a 4 hour chase. Fig. 6 also shows that the lectin was protected within the vesicles from

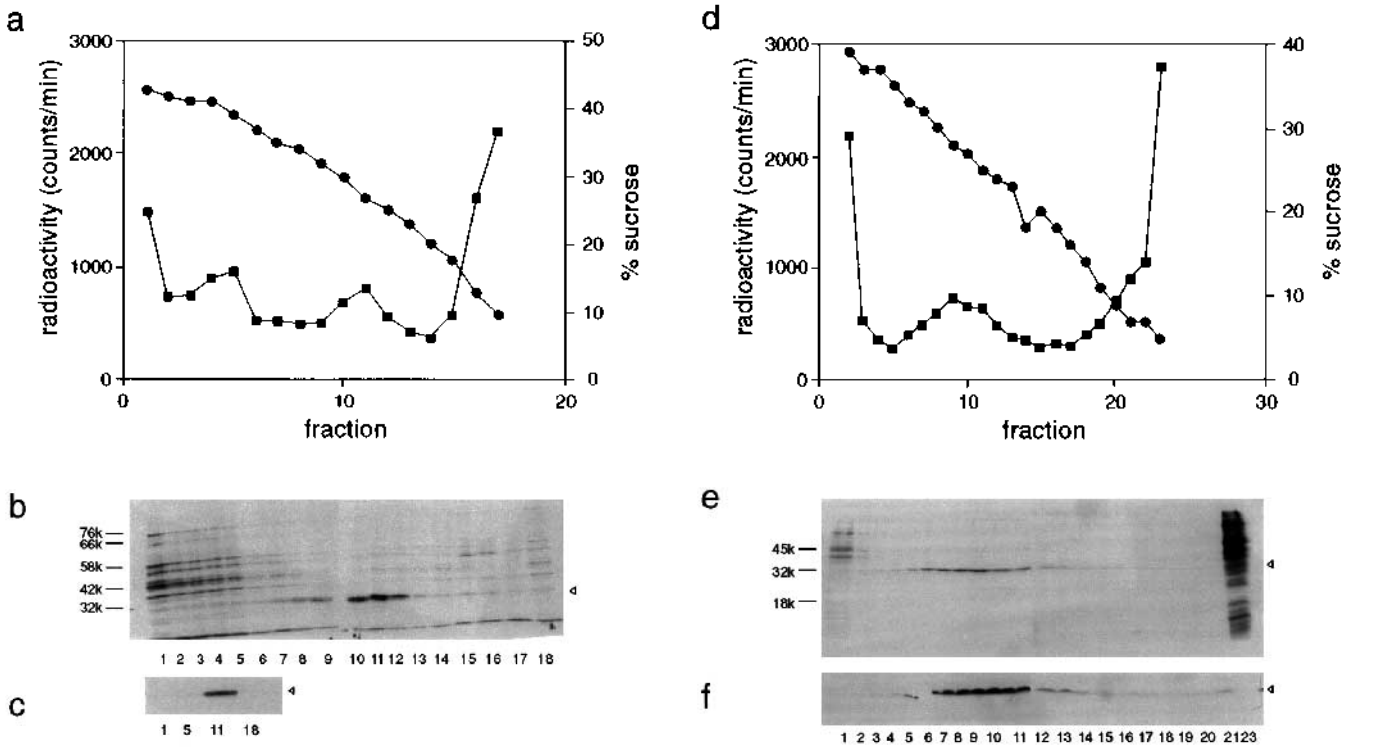

Fig. 5. Isolation of galectin 3-loaded vesicles from Nt-p56^{lck}-galectin 3-transfected Cos-7 cells. Cells were labelled after transient transfection with [^{35}S]methionine for 20 hours (a-c) or 30 minutes followed by a 2 hour chase (d-f). Particulate fractions pelleted from culture supernatants were loaded onto 10-50% continuous sucrose gradients and centrifuged at 170,000 ***g*** overnight. Fractions collected from the gradients were counted (■) and sucrose density (●) was determined by refractometry (a,d). Equal samples of gradient fractions were subjected to SDS-PAGE followed by fluorography (b,e). Equal samples were also immunoprecipitated with galectin 3 specific antibodies and examined by SDS-PAGE (c,f). Migration of galectin 3 is indicated by arrowheads. Molecular mass markers are shown on the left of each panel.

bacterial collagenase digestion. After sonication of the vesicular fraction in the presence of Triton X-100, collagenase treatment degraded galectin 3 to a 18 kDa fragment, presumed to be the collagenase-resistant CRD fragment since it was immunoprecipitated by a polyclonal antibody raised against the CRD. We have shown previously that the collagenase-resistant fragment of hamster galectin 3 is approximately 18 kDa in size and retains lactose-binding activity (Mehul et al., 1994).

Nt-p56^{lck}-Galectin 3 is released from externalized vesicles

The presence of Nt-p56^{lck}-galectin 3 within vesicles isolated from culture supernatants raised the question of whether the lectin is eventually released in soluble form into the culture medium. Cos-7 cells transiently transfected with Nt-p56^{lck}-galectin 3 cDNA (Fig. 1) were pulse labelled for 30 minutes with [^{35}S]methionine. The low amount of galectin 3 secreted during this period, detected by immunoprecipitation, was

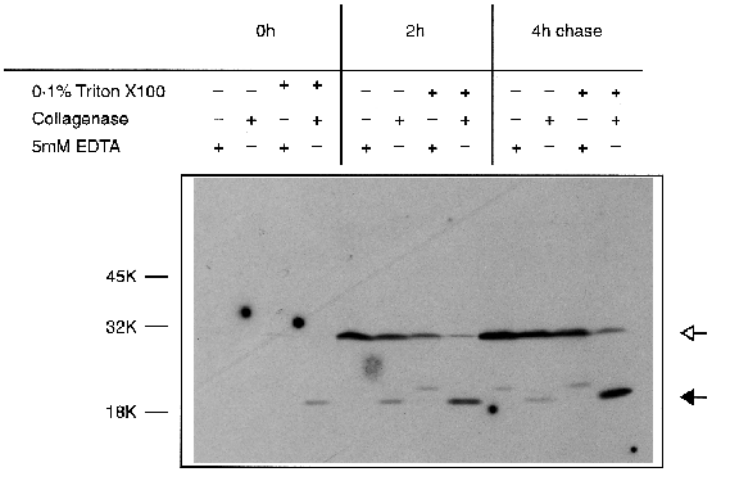

Fig. 6. Galectin 3 in vesicles recovered from the conditioned medium of transfected Cos-7 cells is protected from bacterial collagenase digestion. Cells transiently transfected over 48 hours with Nt-p56^{lck}-galectin 3 cDNA were labelled with [^{35}S]methionine for 30 minutes and chased for 0, 2 or 4 hours. Particulate fractions pelleted at 90,000 ***g*** from the conditioned culture supernatants were suspended in 10 mM Hepes, pH 7.2, 150 mM NaCl with or without 5 mM EDTA. In some cases, particulate fractions were sonicated in the presence of 0.1% Triton X-100, then incubated with or without bacterial collagenase and 5 mM $CaCl_2$ as indicated. Following incubation at 37°C overnight (10-12 hours) the mixtures were diluted into SDS-buffer, immunoprecipitated with antibodies directed against the CRD of galectin 3 and the immune complexes were examined by SDS-PAGE and fluorography. Intact galectin (open arrow) and the CRD fragment (closed arrow) and molecular mass markers are indicated.

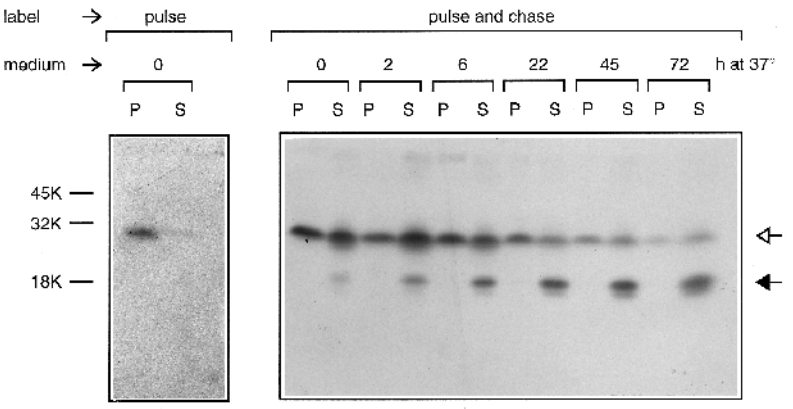

Fig. 7. Galectin 3 is released from the vesicular fraction of conditioned medium of transfected Cos-7 cells and is slowly degraded by endogenous proteases. Cells transiently transfected for 48 hours with Nt-p56^{lck}-galectin 3 cDNA were labelled with [^{35}S]methionine for 30 minutes and culture supernatants were collected after a slow speed 2,000 ***g*** centrifugation either immediately (pulse) or after 2 hours chase (pulse and chase). The conditioned medium supernatants from the pulse chased cells were incubated at 37°C for various times (0-72 hours) as indicated. Cell-free supernatants were centrifuged at 90,000 ***g***, the pellets (P) and high speed centrifugation supernatants (S) were mixed with SDS-buffer and immunoprecipitated with antibodies directed against the CRD of galectin 3. Immune complexes were examined by SDS-PAGE and fluorography. The migration of galectin 3 (open arrow), galectin 3 CRD (closed arrow) and molecular mass markers is indicated. Fluorography was for 14 and 6 days in the left and the right panels, respectively.

almost completely in a particulate fraction (Fig. 7). When the pulse labelled cells were chased for 2 hours at 37°C less than half of the galectin 3 present in the culture supernatant was recovered in the particulate fraction after centrifugation at 90,000 ***g*** at 2°C; the remainder had been released in soluble form (Fig. 7).

Interestingly, the initial rapid rate of lectin release into a soluble form decreased when cell-free culture supernatants were incubated at 37°C (Fig. 7), suggesting lectin release from the externalized vesicles was potentiated in some way by the cells. However, the proportion of lectin recovered in the soluble fraction after high-speed centrifugation steadily increased when the cell-free culture supernatants were incubated at 37°C, and after 72 hours incubation less than 10% of galectin 3 could be pelleted (Fig. 7). During prolonged incubation of conditioned medium at 37°C, there was a slow but eventually almost complete fragmentation of galectin 3 into a 18 kDa. component immunoprecipitated by polyclonal antibody raised against galectin 3 CRD. In addition the signal intensity decreased significantly especially after the more prolonged incubation times. Affinity chromatography of a serum-free culture supernatant incubated at 37°C for 1 to 2 days showed that the 18 kDa fragment retained carbohydrate-binding activity confirming its assignment as the galectin 3 CRD (result not shown). Presumably the conditioned medium contains endogenous proteases secreted from Cos-7 cells with an ability to degrade the N-terminal domains, and more slowly the CRD, of the lectin.

Vesicular secretion of endogenous galectin 3 from murine macrophages

We searched next for evidence of an intermediate vesicle in the export of endogenous galectin 3 from cells. We used murine macrophages that secrete lectin at a moderate rate, especially in the presence of calcium ionophores (Sato and Hughes, 1994), although considerably less than secretion of Nt-p56^{lck}-galectin 3 from transfected Cos-7 cells. Mouse macrophage J774.2 cells were subjected to a 3 hour pulse labelling and chased in medium containing 5 μM A23187 for 6 hours in 30 minute bursts, over which time we reasoned much of the exported lectin would be retained within the vesicles. Sucrose gradient centrifugation of the combined conditioned medium produced a light vesicular fraction (Fig. 8a), similar to that obtained from transfected COS cells (Fig. 5), that contained galectin 3 (Fig. 8b). In addition lectin was recovered in the soluble fraction (fraction 18) from the sucrose gradient, presumably due to release of the lectin from vesicles during collection of the conditioned medium. The galectin 3 associated with the vesicular fraction was resistant to bacterial collagenase during 4 hours at 37°C unless the vesicles were first permeabilized, when it was degraded extensively to the 18 kDa CRD (Fig. 8c). This was shown both by the detection of the 18 kDa fragment by CRD-specific antibodies as well as by the pronounced loss of a signal at 35 kDa, the molecular mass of intact mouse galectin 3, using antibodies directed against N-terminal domain epitopes that are lost after collagenase degradation (Mehul et al., 1994, 1995).

Calcium ionophores have previously been reported to induce release of micro-vesicles from cells, notably erythrocytes (Shukla et al., 1978; Iida et al., 1991). However, using a 45 minute pulse and heavy metabolic labelling, a particulate fraction containing 70-80% of newly exported galectin 3 was also isolated from culture supernatants of J774.2 cells in the absence of ionophore A23187 (Fig. 8d). After a 2-4 hour chase the proportion of lectin in the particulate fraction fell to 10-20% of total and the lectin was largely in the soluble fraction. In this experiment, galectin 3 migrated as a closely spaced doublet comprising a major 35 kDa band and a minor band migrating somewhat faster (Fig. 8d). Partial proteolytic processing of the N-terminal domain of galectin 3 has been noted previously in cell lysates including J774.2 cells (Sato and Hughes, 1994).

DISCUSSION

Galectin 3, as shown definitively for galectin 1 (Wilson et al., 1989), is most likely synthesised on free cytoplasmic ribosomes. It lacks a signal sequence for translocation into the ER and is N-acetylated, a marker for cytoplasmic proteins. Targetting of newly synthesised lectin to plasma membranes appears to be a rate-limiting step in secretion as shown by the enhanced secretion of Nt-p56^{lck}-galectin 3 compared with wild-type lectin. The mechanism of transport of p56^{lck} and related proteins to plasma membrane domains is unknown but appears not to involve intracellular vesicles (Rodgers et al.,

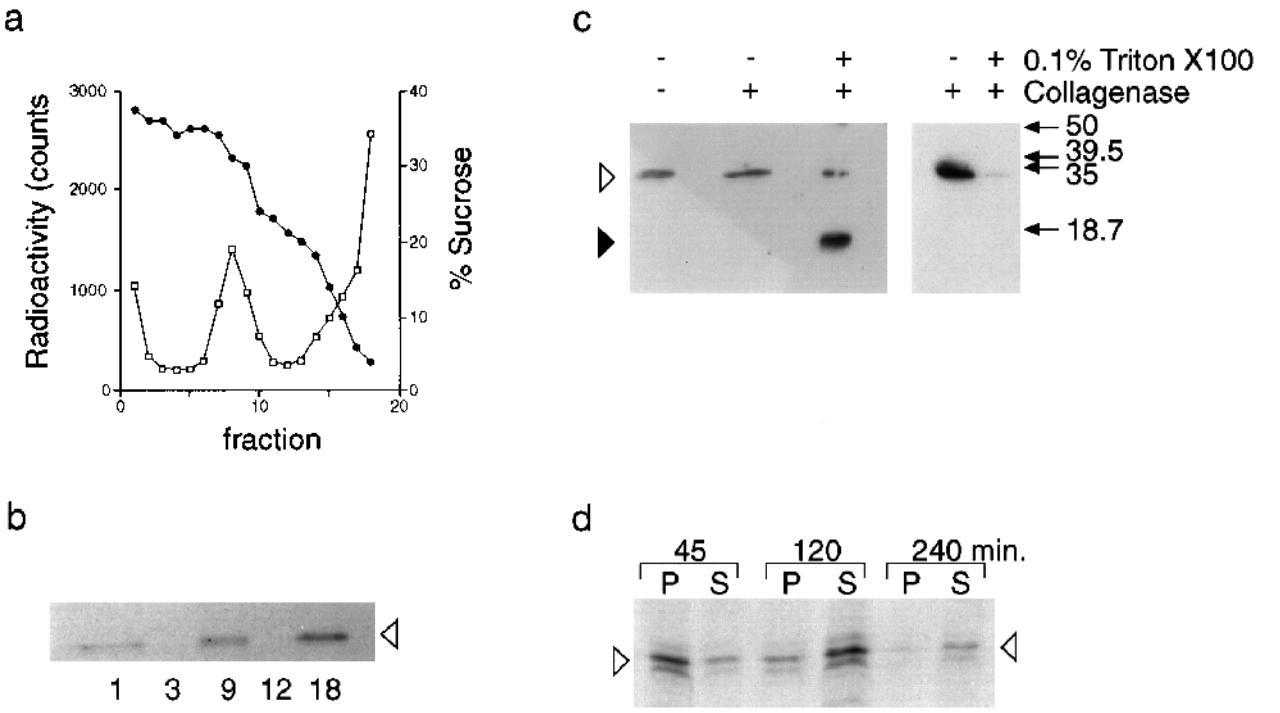

Fig. 8. Galectin 3 loaded vesicles released from J774.2 macrophages. (a) Cells methionine-labelled for 3 hours were chased for 6 hours in the presence of calcium ionophore A23187 and the conditioned medium was fractionated by sucrose gradient centrifugation as described in Fig. 5. (b) Equal samples of selected gradient fractions from a were immunoprecipitated with antibodies against intact galectin 3. Galectin 3 (open arrowhead) was detected by fluorography. (c) Western blotting of galectin 3 (open arrowhead) and CRD (closed arrowhead) in vesicles (gradient fractions 7-10 from a). The vesicles were treated as indicated with Triton X-100 and collagenase as in Fig. 6 except incubation at 37°C was for 4 hours. Blotting was done with antibodies against galectin 3 CRD that cross react with intact galectin 3 (left hand panel), or antibodies against N-terminal domain epitopes that react with intact galectin 3 but not the CRD fragment (right hand panel). The migration of protein standards is indicated by the arrows. (d) Cells were pulsed for 45 minutes and chased as indicated in medium without calcium ionophore. Cell-free culture supernatants were centrifuged at 90,000 ***g*** for 2 hours and galectin 3 was immunoprecipitated from the pelleted (P) and soluble (S) fractions. Fluorography after SDS-PAGE showed the distribution of the murine 35 kDa galectin 3 (open arrowhead) during the chase period.

1994; Shenoy-Scaria et al., 1994). It seems likely therefore that chimaeric Nt-p56^{lck}-galectin 3, and presumably endogenous galectin 3, were transported directly to the plasma membrane prior to secretion. An alternative mechanism involving an intermediate translocation of lectin into Golgi compartments followed by transport within secretory vesicles to the cell surface is unlikely since fusion of intracellular secretory vesicles with plasma membranes would release extracellular galectin 3 in a soluble, collagenase-sensitive form.

Following fatty acid acylation p56^{lck} and related proteins are transported to plasma membrane domains enriched in glycolipids, GPI-linked glycoproteins and caveolin (Rodgers et al., 1994; Shenoy-Scaria et al., 1994; Anderson, 1993). It could be argued that such domains are specifically adapted for protein translocation or for membrane evagination, explaining the efficient secretion of Nt-p56^{lck}-galectin 3 in transfected Cos-7 cells. The failure of transfected cells to secrete p56^{lck} protein and Nt-p56^{lck}-CAT make this unlikely. Furthermore, the lectin-loaded structures seen blebbing from the surface of Nt-p56^{lck}-galectin 3-transfected cells are remarkably similar to surface membrane evaginations seen in cells secreting endogenous galectin 3 (Sato et al., 1993) or the related galectin 1 (Harrison and Wilson, 1992). However, we cannot exclude the possibility that glycolipid-enriched membrane domains do assist in protein secretion in conjunction with other factors. Perhaps normally in cells the variable secretion of endogenous galectin 3 occurs preferentially at such sites. Comparisons of the compositions of galectin 3-loaded vesicles released from Cos-7 cells and macrophages may help resolve this question.

Our results indicate that lectin secretion is induced by signals contained in the amino acid sequence of galectin 3. Similar conclusions have been proposed for secretion from transfected Cos-1 cells of the 18 kDa bFGF isoform and its fusion product with normally cytoplasmic proteins (Florkiewicz et al., 1995). Further work is needed to define the structural motifs of galectin 3 that determine the fate of the lectin after transport to plasma membrane domains. In particular the known role of N-terminal domains of galectin 3 in oligomerization (Mehul et al., 1994) may be an important factor, for example in aggregation of lectin molecules at cytoplasmic sites of blebbing plasma membrane domains.

Membrane blebbing or ectocytosis (Stein and Luzio, 1991) is a common feature at the periphery of many cell types, including fibroblasts (Lee et al., 1993), neutrophils (Stein and Luzio, 1991) and chondrocytes (Hale and Wuthier, 1987) and in some cases is regulated by intracellular calcium (Shukla et al., 1978; Iida et al., 1991) as mentioned before. In the vesicles containing Nt-p56^{lck} galectin 3 released from transfected COS

cells and endogenous galectin 3 released from macrophage J774.2 cells, the lectin is protected from collagenase degradation, at least in the short term, unless the vesicles are disrupted by Triton X-100 treatment. The very limited degradation of galectin 3 by collagenase in the absence of detergent (Figs 6, 8c) is expected since as Fig. 7 shows the vesicles do break down during incubation at 37°C with release of soluble collagenase-sensitive lectin. Although these data and the results of pulse-chase analysis (Figs 7, 8d) suggest an intermediate role of extracellular vesicles in export of both Nt-p56^{lck} galectin 3 from COS cells and endogenous galectin from macrophage J774.2 cells, they do not exclude the possibility that at least part of the lectin may be translocated from plasma membrane domains directly into the extracellular medium. Inspection of Fig. 7 shows a small but definite amount of lectin is present in the soluble fraction of conditioned medium obtained from Nt-p56^{lck} galectin 3-transfected COS cells at the begining of the chase period. Similarly, at the earliest time of chase examined (45 minutes), 20-30% of the endogenous lectin exported from J774.2 cells is soluble (Fig. 8d) suggesting as one possibility that some lectin may have been released directly from the cells.

Cytoplasmic vesicles containing bFGF (Yu et al., 1993) and IL-1β (Rubartelli et al., 1990) have been described. In some instances bFGF vesicles were seen to fuse with the plasma membrane, discharging soluble bFGF from the cell. On the other hand, another leaderless cytoplasmic protein, thioredoxin is not translocated into intracellular vesicles before secretion (Rubartelli et al., 1992). The possibility that thioredoxin is released from externalized vesicles as is galectin 3 was not examined. These data, as well as significant differences in the effects of various drugs on the secretion of leaderless proteins (Sato et al., 1993; Mignatti et al., 1992; Rubartelli et al., 1990; Lindstedt et al., 1993; Sloan et al., 1994) including galectin 3, suggest different pathways or different rate-limiting steps for non-classically secreted proteins.

The factors involved in release of soluble, collagenase-sensitive galectin 3 from secreted vesicles remain to be elucidated. Although we cannot at present exclude the possibility that spontaneous vesicle degeneration is sufficient for lectin release, other mechanisms can be considered. Cytoplasmic translocation of proteins often requires association with chaperones such as *hsp*70 and is favoured by unfolded or partially unfolded conformations of the transported polypeptides (Van der Goot et al., 1992; Isenman et al., 1995; Sloan et al., 1994). Extracellular vesicles shed by reticulocytes and containing transferrin receptors also contain at least one *hsp* component suggesting extra-cytoplasmic roles for some of these proteins (Davis et al., 1986). Our findings implicating the N-terminal domains of galectin 3 in one or more steps of the secretory pathway, may be relevant. Physical studies using circular dichroism and tryptophan fluorescence (Mehul et al., 1994) as well as NMR spectroscopy (B. Birdsell, J. Feeney, B. Méhul and R. C. Hughes, unpublished results) show that the N-terminal domain is largely unfolded, both as a recombinant fragment and also as part of intact galectin 3. Such unfolded segments could maximise association of the mature protein with chaperone proteins. Further studies are in progress to determine if chaperones are involved in galectin 3 secretion and the mechanism of release from secreted vesicles.

We thank Drs P. Zlatkine and A. I. Magee for stimulating discussion and help in chimaera construction, Dr R. Menon for additional experiments and Dr Q. Bao for advice on confocal laser scan microscopy.

REFERENCES

Anderson, R. G. W. (1993). Caveolae: Where incoming and outgoing messengers meet. *Proc. Nat. Acad. Sci. USA* **60**, 10909-10913.

Barondes, S. H., Cooper, D. N., Gitt, M. A. and Leffler, H. (1994). Galectins. Structure and function of a large family of animal lectins. *J. Biol. Chem.* **269**, 20807-20810.

Bao, Q. and Hughes, R. C. (1995). Galectin-3 expression and effect on cyst enlargement and tubulogenesis in kidney epithelial MDCK cells cultured in three-dimensional matrices in vitro. *J. Cell Sci.* **108**, 2791-2800.

Davis, Y. Q., Dansereau, D., Johnstone, R. M. and Bennett, V. (1986). Selective externalization of an ATP-binding protein structurally related to the clathrin-uncoating ATPase/heat shock protein in vesicles containing terminal transferrin receptors during reticulocyte maturation. *J. Biol . Chem.* **261**, 15368-15371.

Florkiewicz, R. Z., Majack, R. A., Buechler, R. D. and Florkiewicz, E. (1995). Quantitative export of FGF-2 occurs through an alternative energy dependent non ER-Golgi pathway. *J. Cell Physiol.* **162**, 388-399.

Hale, J. E. and Wuthier R. E. (1987). The mechanism of matrix vesicle formation *J. Biol. Chem.* **262**, 1916-1925.

Harrison, F. L. and Wilson, T. J. (1992). The 14 kDa beta-galactoside binding lectin in myoblast and myotube cultures: localization by confocal microscopy. *J. Cell Sci.* **101**, 635-646.

Iida, K., Whitlow, M. B. and Nussenzweig, V. (1991). Membrane vesiculation protects erythrocytes from destruction by complement. *J. Immunol.* **147**, 2638-2642.

Isenman, L., Liebow, C. and Rothman, S. (1995). Transport of proteins across membranes, a paradigm in transition. *Biochim. Biophys. Acta* **1241**, 341-370.

Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. and Magee, A. I. (1994). Palmitoylation of multiple src-family kinases at a homologous N-terminal motif. *Biochem. J.* **303**, 749-753.

Lee, T.-L., Lin, Y.-C., Mochitate, K. and Grinnell, F. (1993). Stress-relaxation of fibroblasts in collagen matrices triggers ectocytosis of plasma membrane vesicles containing actin, annexins II and VI and β1 integrin receptors *J. Cell Sci.* **105**, 167-177.

Le Marer, N. and Hughes, R. C. (1996). Effects of galectin 3 on the invasiveness of human breast carcinoma cells. *J. Cell Physiol.* **168**, 51-58.

Lindstedt, R., Apodaca, G., Barondes, S. H., Mostov, K. E. and Leffler, H. (1993). Apical secretion of a cytosolic protein by Madin-Darby canine kidney cells. Evidence for polarized release of an endogenous lectin by a nonclassical secretory pathway. *J. Biol. Chem.* **268**, 11750-11757.

Liu, F. T. (1993). S-type mammalian lectins in allergic inflammation. *Immunol. Today* **14**, 486-490.

Mehul, B., Bawumia, S., Martin, S. R. and Hughes, R. C. (1994). Structure of baby hamster kidney carbohydrate-binding protein CBP30, an S-type animal lectin. *J. Biol. Chem.* **269**, 18250-18258.

Mehul, B., Bawumia, S. and Hughes, R. C. (1995). Cross-linking of galectin 3 a galactose-binding protein of mammalian cells, by tissue-type transglutaminase. *FEBS Lett.* **360**, 160-164.

Mignatti, P., Morimoto, S. R. and Rifkin, D. B. (1992). Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the ER-Golgi complex. *J. Cell Physiol.* **151**, 81-93.

Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R. and Rapoport, T. A. (1990). A novel pathway for secretory proteins? *Trends Biochem. Sci.* **15**, 86-88.

Paige, L. A., Nadley, M. J. S., Harrison, M. L., Cassady, J. M. and Geahlen, R. L. (1993). Reversible palmitoylation of the protein tyrosine kinase p56^{lck}. *J. Biol. Chem.* **268**, 8669-8674.

Raz, A., Zhu, D. G., Hogan, V., Shah, N., Raz, T., Karkash, R., Pazerini, G. and Carmi, P. (1990). Evidence for the role of 34-kDa galactoside-binding lectin in transformation and metastasis. *Int. J. Cancer* **46**, 871-877.

Rodgers, W., Crise, B. and Rose, J. K. (1994). Signals determining protein tyrosine kinase and glycosyl phosphatidylinositol-anchored protein targeting to a glycolipid-enriched membrane fraction. *Mol. Cell Biol.* **14**, 5384-5391.

Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. (1990). A novel secretory

pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* **9**, 1503-1510.

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992). Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Chem.* **267**, 24161-24164.

Sato, S. and Hughes, R. C. (1992). Binding specificity of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans, and appropriately glycosylated forms of laminin and fibronectin. *J. Biol. Chem.* **267**, 6983-6990.

Sato, S., Burdett, I. and Hughes, R. C. (1993). Secretion of the baby hamster kidney 30-kDa galactose-binding lectin from polarized and nonpolarized cells: a pathway independent of the endoplasmic reticulum-Golgi complex. *Exp. Cell Res.* **207**, 8-18.

Sato, S. and Hughes, R. C. (1994). Regulation of secretion and surface expression of Mac-2, a galactoside-binding protein of macrophages. *J. Biol. Chem.* **269**, 4424-4430.

Shenoy-Scaria, A. M., Dietzin, D. J., Kwong, J., Link, D. C. and Lublin, D. M. (1994). Cysteine^3 of *src* family protein tyrosine kinases determines palmitoylation and localization in caveolae. *J. Cell Biol.* **126**, 353-363.

Shukla, S. D., Berriman, J., Coleman R., Finean J. B. and Michell, R. H. (1978). Membrane protein segregation during release of microvesicles from human erythrocytes *FEBS Lett.* **90**, 289-292.

Sloan, I. S., Horowitz, P. M. and Chirgwin, J. M. (1994). Rapid release by a non classical pathway of overexpressed mammalian mitochondrial rhodanase. *J. Biol. Chem.* **269**, 27625-27630.

Stein J. M. and Luzio J. P. (1991). Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles *Biochem. J.* **274**, 381-386.

Van Der Goot, F. G., Lakey, J. H. and Pattus, F. (1992). The molten globule intermediate for protein insertion or translocation through membranes. *Trends Cell Biol.* **2**, 343-348.

Wilson, T. J. G., Firth, M. N., Powell, J. T. and Harrison, F. L. (1989). The sequence of mouse 14 kDa β galactoside-binding lectin and evidence for its synthesis on free cytoplasmic ribosomes. *Biochem. J.* **261**, 847-852.

Yamaoka, K., Ohno, S., Kawasaki, H. and Suzucki, K. (1991). Overexpression of a β-galactoside-binding lectin causes transformation of Balb 3T3 cells. *Biochem. Biophys. Res. Commun.* **179**, 272-279.

Yu, Z. X., Biro, S., Fu, Y. M., Sanchez, J., Smale, G., Sasse, J., Ferrans, V. J. and Casscells, W. (1993). Localization of basic fibroblast growth factor in bovine endothelial cells: Immuno-histochemical and biochemical studies. *Exp. Cell Res.* **204**, 247-259.

Zlatkine, P., Mehul, B. and Magee, A. I. (1997). Retargeting of cytosolic proteins to the plasma membrane by the lck protein tyrosine kinase dual acylation motif *J. Cell Sci.* **110**, 673-679.

Zuberi, R. I., Frigeri, L. G. and Liu, F. T. (1994). Activation of rat basophilic leukemia cells by BP, an IgE-binding endogenous lectin. *Cell. Immunol.* **156**, 1-12.

(Received 5 September 1996 – Accepted 10 March 1997)