III.1. Late endocytic compartments are major sites of annexin 6 localisation in NRK fibroblasts and polarised WIF-B hepatoma cells

III.1.1. Abstract

Annexin 6 is an abundant calcium- and phospholipid-binding protein whose intracellular distribution and function is still controversial. Using a highly specific antibody, we have studied the distribution of annexin 6 in NRK fibroblasts and the polarised hepatic cell line WIF-B by confocal microscopy. In NRK cells, annexin 6 was almost exclusively found associated with endocytic compartments, which were defined by their ability to receive fluid phase marker internalised from the cell surface. However, extensive colocalisation of annexin 6 and the endocytic marker was only observed after about 45 min indicating that annexin 6 was primarily in late endocytic compartments or (pre)lysosomes. Consistent with this, annexin 6 was predominantly seen on structures that contained the lysosomal protein lgp120, although not on dense core lysosomes by electron microscopy. Two major populations of annexin 6-containing structures were present in polarised WIF-B hepatocytes. One correlated to lgp-120-positive (pre)lysosomes and was still observed after treatment with Brefeldin A (BFA), while the other appeared to be partially associated with Golgi membranes and was BFA-sensitive. The striking association with prelysosomal compartments in NRK and WIF-B cells suggests that annexin 6 could play a role in fusion events in the late endocytic pathway, possibly by acting as tether between membranes.

III.1.2. Introduction

Annexins are a family of widely distributed proteins which are characterised by their Ca^{2+}-dependent binding to phospholipids via highly conserved ‘annexin repeats’ contained in their sequence (Gerke and Moss, 1997). Although annexins have been

implicated to play a role in a variety of biological processes, their physiological tasks are poorly understood (Raynal and Pollard, 1994). The common denominator of otherwise diverse functions suggested for the annexins is to mediate the physical interaction of intracellular structures, e.g. two membranes or a membranous compartment and the cytoskeleton, based on their amphiphatic protein structure. In recent years, the involvement of annexins in membrane traffic has emerged as one of their predominant functions (Gruenberg and Emans, 1993), (Gerke and Moss, 1997). A subset of annexins, including annexins 1, 2, and 6, are widely expressed in mammalian tissues and have been found to distribute over membranes of the exocytic and/or endocytic pathway in patterns specific to each annexin functions (Gruenberg and Emans, 1993), (Gerke and Moss, 1997).

In contrast to the examples of annexins with, at least partially, defined roles in endocytic trafficking, such as annexin 1, 2 and 13 (Futter et al., 1996), (Emans et al., 1993), (Mayorga et al., 1994), (Fiedler et al., 1995), the importance of annexin 6 in endocytosis has remained elusive. It has been recently suggested that annexin 6 is involved in the remodeling of the spectrin cytoskeleton at the cell surface during endocytosis and is thereby facilitating the release of clathrin-coated vesicles from the plasma membrane (Kamal et al., 1998), although not being instrumental in the budding event itself as previously hypothetised (Lin et al., 1992). This is in accord with the observation that annexin 6 is present on clathrin-coated vesicles isolated from adrenocortical tissue (Turpin et al., 1998). However, there is apparently no absolute requirement for annexin 6 for internalisation, since spectrin-dependent internalisation can be bypassed by an annexin 6- and spectrin-independent mechanism (Kamal et al., 1998), and internalisation in A431 cells, which do not express annexin 6, occurs normally (Smythe et al., 1994). The primary function and subcellular distribution of annexin 6 may depend on the cell type. Although ubiquitously expressed, the intracellular concentrations of annexin 6 and its specific membrane association have been reported to be developmentally (Clark et al., 1991), (Giambanco et al., 1993), (Fan et al., 1995) and metabolically (Inui et al., 1994), (Barwise and Walker, 1996), (Francia et al., 1996) regulated in some tissues. Moreover, the two alternative splice forms of annexin 6 may be regulated differently

(Kaetzel et al., 1994) and appear to have different functions (Fleet et al., 1999). Posttranslational modifications of annexin 6 have also been described (Dubois et al., 1995), (Edwards and Moss, 1995).

In many tissues, annexin 6 has been found on endocytic and/or exocytic compartments and at the plasma membrane by morphological and biochemical approaches (Gerke and Moss, 1997). We have previously shown that the highest concentration of annexin 6-positive endocytic membranes is in the pericanalicular region of hepatocytes and that there is a significant overlap between the distribution of annexin 6 and Rab5 (Ortega et al., 1998). Massey-Harroche et al. also found some annexin 6 on vesicles close to bile canaliculi (Massey-Harroche et al., 1998). These results implied that, in hepatocytes, annexin 6 could be a marker for a subapical early endosomal compartment. However, many compartments are concentrated in the perinuclear/subapical region of polarised cells, including the apical recycling compartment described in MDCK cells (Apodaca et al., 1994), (Barroso and Sztul, 1994) and the transcytotic subapical compartment (SAC) of hepatic cells (Barr and Hubbard, 1993), (Barr et al., 1995), (Ihrke et al., 1998). The relationship between these and annexin 6-positive compartments needs further clarification. Moreover, subcellular fractionation of rat liver (Jackle et al., 1994), (Pol et al., 1997) and BHK cells (Seemann et al., 1996) showed that annexin 6 is not only a prominent component of early endosomes but also of late endosomes. Another hint that annexin 6 may function in the late stages of endocytosis stems from the observation that it is more highly concentrated on late rather than early phagosomes isolated from macrophages (Desjardins et al., 1994).

Current models discriminate between at least four endocytic entities involved in the formation of lysosomes that can be distinguished by electron microscopy and biochemical methods: early endosomes, endocytic carrier vesicles (ECVs), a heteromorphous prelysosomal compartment (PLC) (see (Gruenberg and Maxfield, 1995) and (Kornfeld and Mellman, 1989) for review). By immunocytochemical means it is possible to further dissect the PLC into a hydrolase-poor ‘late endosomal’ and a hydrolase-rich ‘prelysosomal’ compartment (reviewed in (Holtzman, 1989) and (Courtoy, 1991)). Late endosomes so defined have relatively low concentrations of

lysosomal membrane proteins (e.g. lgp120) (Geuze et al., 1988), whereas prelysosomal membranes contain up to 50% of total lgp120 visualised by immunoEM (Griffiths et al., 1990). Recent results from in vivo (Bright et al., 1997) and in vitro-studies (Mullock et al., 1998) are consistent with a model in which the protease- and lgp120-rich PLC originates by direct fusion between late endosomes and dense core lysosomes.

Here, we have taken a morphological approach to define the major locations of annexin 6 in cultured non-polarised and polarised cells (NRK and WIF-B, respectively). The WIF-B cell line is an excellent in vitro model for polarised hepatocytes, in which various endocytic compartments have already been characterised (Cassio et al., 1991), (Ihrke et al., 1993), (Tuma et al., 1999). We have utilised a highly specific antibody raised to membrane-bound annexin 6 and antibodies to various markers of intracellular compartments in conjunction with pharmacological manipulation of the endocytic membrane system to systematically map and compare the distribution of annexin 6 in the two cell systems. Our goal has been to narrow the gap between previous work in hepatocytes in situ and cultured cells in vitro in order to provide a better baseline for functional studies.

III.1.3. Results

Specificity of affinity-purified anti-annexin 6 antibody in NRK and WIF-B cells

Since annexins are highly homologous, the generation of antibodies specific to a single annexin has often proved difficult. In this study, we have used the same affinity-purified polyclonal antibody to annexin 6 as for our previous ultrastructural work in rat hepatocytes (Ortega et al., 1998). Immunoblotting of endosomal fractions from rat liver (Ortega et al., 1998) or whole NRK cell lysates (Fig. 1A) demonstrated that this antibody specifically reacted with a narrow-spaced doublet of approx. 68 kDa which is characteristic for annexin 6. No crossreaction with bands in the 35 kDa region (typical for other annexins) could be detected, although several annexins are expressed in both cell types (Desjardins et al., 1994), (Pol et al., 1997), including

annexins 5 (Giambanco et al., 1993), a close relative of annexin 6. In WIF-B cells, it was necessary to concentrate annexin 6 by immunoprecipitation prior to Western blotting to yield a detectable signal, presumably since these cells express the protein at somewhat lower levels. Again, the only protein precipitated and detected by immunoblotting was annexin6 as judged by its molecular weight (Fig. 1B).

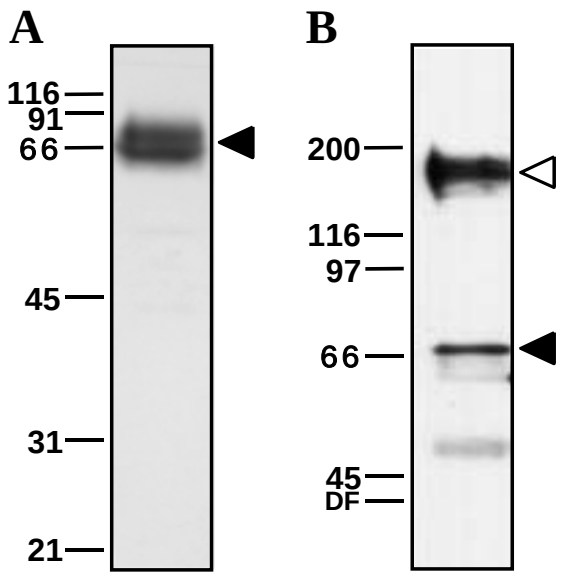


Fig. 1. Affinity-purified pAb to annexin 6 recognises no other annexin by immunoblotting. (A) Total NRK cell lysates and (B) annexin 6-immunoprecipitates from WIF-B cell lysates (using the same anti-annexin 6 pAb as for blotting) were separated on a 10% (reducing) and 7.5% (non-reducing) SDS-polyacrylamide gel, respectively, and immunoblotted with the affinity-purified anti-annexin 6 pAb. Solid arrowheads indicate annexin 6 (doublet in A, single band in B); the open arrowhead in (B) indicates IgG from the immunoprecipitation. Note that the antibody does not recognise any bands in the 30-40 kDa region [i.e., at the dye front (DF) in B] indicative of other annexins.

Localisation of annexin 6 to a prelysosomal compartment in NRK cells

Using confocal microscopy, the localisation of annexin 6 was examined in a variety of cultured cell lines, including NRK, PC12, HepG2 and WIF-B cells. In all cell types, our antibody labelled punctate structures, which were mainly concentrated in the perinuclear region with some additional structures found throughout the cells and at the cell periphery (NRK cells shown in Fig. 2 and 3). We chose to further characterise the distribution of annexin 6 in NRK cells, since they abundantly express this protein and have been previously used for the study of annexins (Desjardins et

al., 1994). To test whether the perinuclear structures were in the endocytic pathway, we performed double immunofluorescence experiments with antibodies to proteins enriched in different endosomal compartments. When using a mAb to the (pre)lysosomal membrane protein lgp120 (GM10) in combination with the anti-annexin 6 pAb, the observed patterns of both proteins were largely identical (Fig. 2A and A'), although the intensity of labelling over single structures varied to some extent. Most structures containing both proteins were in the perinuclear area. However, annexin 6 appeared to have a somewhat wider distribution compared with lgp120 since some structures were only annexin6-positive (arrow in Fig. 2A-A"); these were often more peripheral and included some relatively small structures. A minor population of structures was lgp120-positive and annexin 6-negative (open arrowhead in Fig. 2A-A"). The direct comparison of annexin 6 with late endosomal markers such as the M6PR by double labelling was complicated by the fact that only antibodies developed in the same species were available. However, there was much less overlap between M6PR and lgp120 than observed for annexin 6 and lgp120 (compare Fig. 2B-B" with 2A-A"). To assess the presence of annexin 6 in early endosomes, we internalised fluorescently labelled transferrin for 30 min before fixation and staining with antibodies (Fig. 2C and C'). There was clearly some coincidence of the two proteins; however, structures containing annexin and transferrin were few compared to those containing annexin and lgp120. Essentially no overlap was observed between transferrin and lgp120 (data not shown). Altogether, these findings indicate that annexin 6 is located predominantly in late endocytic compartments of NRK cells, the majority of which appeared to be 'down-stream' from M6PR-positive compartments, i.e. were more prelysosomal or lysosomal in character.

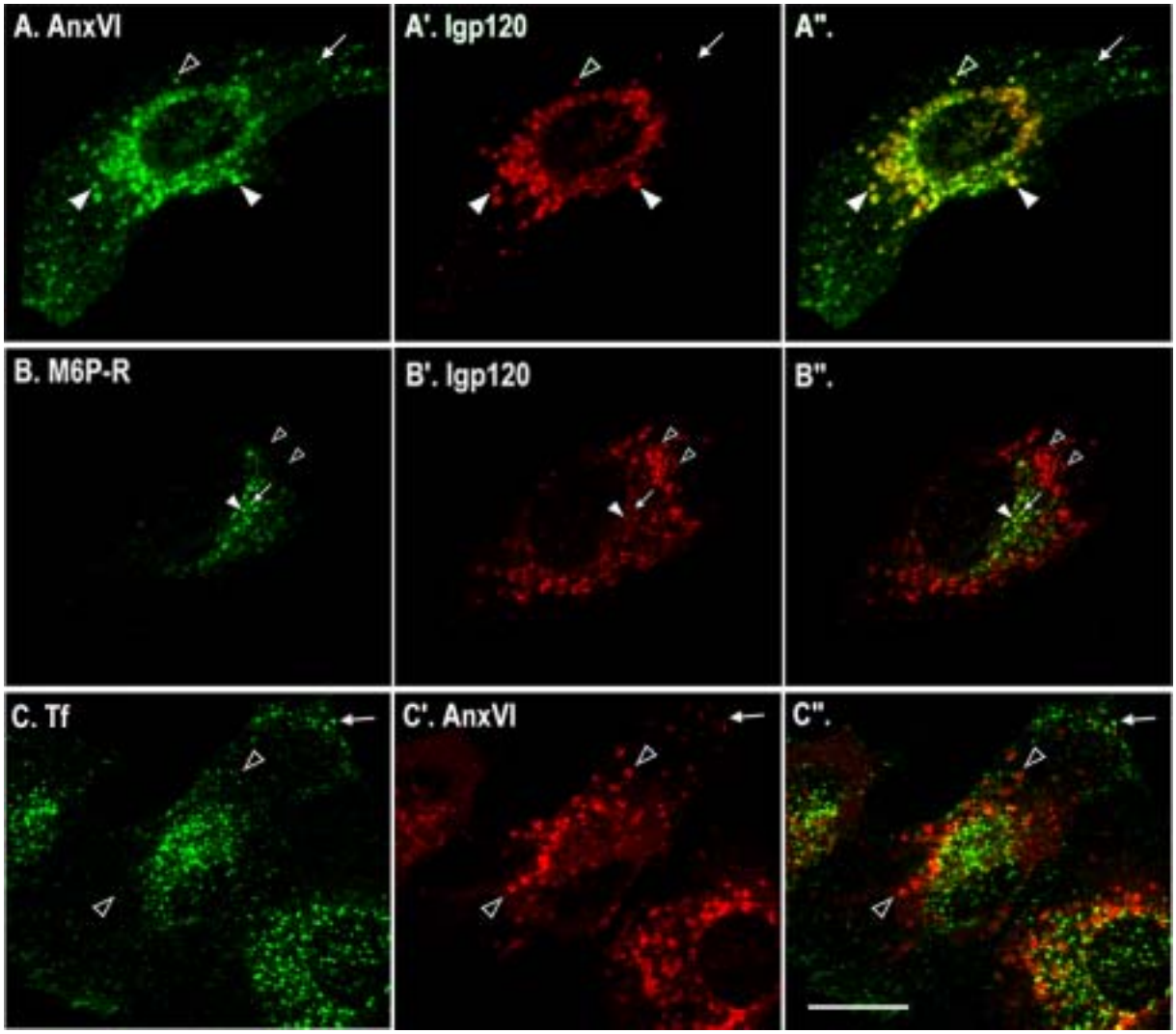


Fig. 2. In NRK cells, annexin colocalises to a high degree with lgp120 by confocal microscopy, but little with M6PR or transferrin. Fixed and permeabilised cells were double-labelled with antibodies to annexin 6 and lgp120 (A/A') and M6PR and lgp120 (B/B'), or with anti-annexin pAb in cells that had internalised FITC-transferrin (20 μg/ml) for 30 min before fixation (C/C'). Antigens in panels A and B (left) and A' to C' (middle) were visualised by FITC- and TRITC-conjugated secondary antibodies, respectively. Panels A" to C" (right) show the corresponding merged images. White arrowheads indicate structures that contain both antigens, white arrows point to (green) structures that carry only annexin 6 (A), M6PR (B), and open arrowheads point to (red) structures that contain only lgp120 (A' and B') or annexin 6 (C').

To confirm that annexin 6 was primarily in late endocytic compartments, we performed uptake experiments with the fluid phase marker FITC-dextran (mol wt 10,000) and determined the degree of colocalisation with annexin 6 over time. After a 5 min-pulse with FITC-dextran cells were washed and chased for various lengths of time in the absence of the fluid phase marker. As expected, when internalised (chased) for very short periods of time (e.g. 5 min), most of the FITC-dextran was observed in peripheral endocytic structures corresponding to early endosomes (Fig. 3A). Little annexin 6 was found in the same structures (Fig. 3A'). Although after 15 min many dextran-containing structures had moved to a more perinuclear position, there was still little overlap with annexin 6 (Fig. 3B and B'). A different result was obtained when FITC-dextran was internalised for 45 min or longer. Now, the majority of structures that contained dextran, i.e. late endosomes and (pre)lysosomes, also contained annexin 6 (Fig. 3C and C'). Moreover, essentially all annexin 6-containing structures had received the fluid phase marker indicating that annexin 6 chiefly associates with late endosomes/(pre)lysosomes.

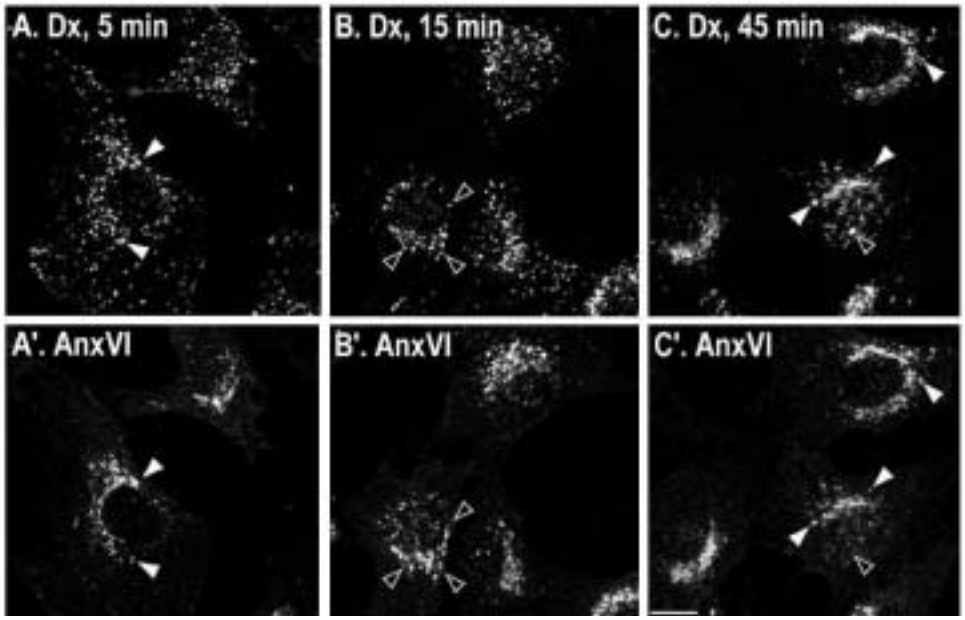


Fig. 3. Annexin 6-positive structures receive fluid-phase marker at late stages of endocytosis.
Cells were incubated with FITC-dextran (mol wt 10,000) for 5 min and then chased in normal medium for 5 min (A), 15 min (B), or 45 min (C). After fixation and permeabilisation, the cells were labelled with anti-annexin 6 (A', B', and C') followed by TRITC-conjugated secondary antibody. White arrowheads indicate structures that contain both dextran and annexin 6, while open arrowheads point to structures that contain either only dextran or annexin 6. Bar is 10 μm.

Perturbation of the 'annexin 6-compartment' in NRK cells: effect of chloroquine, BFA and wortmannin

To gain further insight into the properties of the endocytic annexin 6-positive compartment, we next examined the distribution of annexin 6 in double labelling experiments with lgp120 after perturbation of normal endocytic traffic in multiple ways. It has been previously shown that chloroquine, the phosphatidylinositol 3-kinase inhibitor wortmannin, and BFA, although perturbing endosomal membrane organisation differently, can all be used as diagnostic drugs to differentiate between endosomal and lysosomal membrane systems (Brown et al., 1986), (Reaves et al., 1996), (Wood and Brown, 1992). When NRK cells were incubated for 1 hr with 40 μM chloroquine, annexin 6 and lgp120 were found together in large vacuolar structures (Fig. 4A-A") with the number of labelled structures being reduced compared to controls. While the extent of colocalisation between annexin 6 and lgp120 appeared to be even higher than under control conditions, the M6PR was found in small and large punctate structures distinct from the lgp120-containing compartment with few exceptions (Fig. 4B-B").

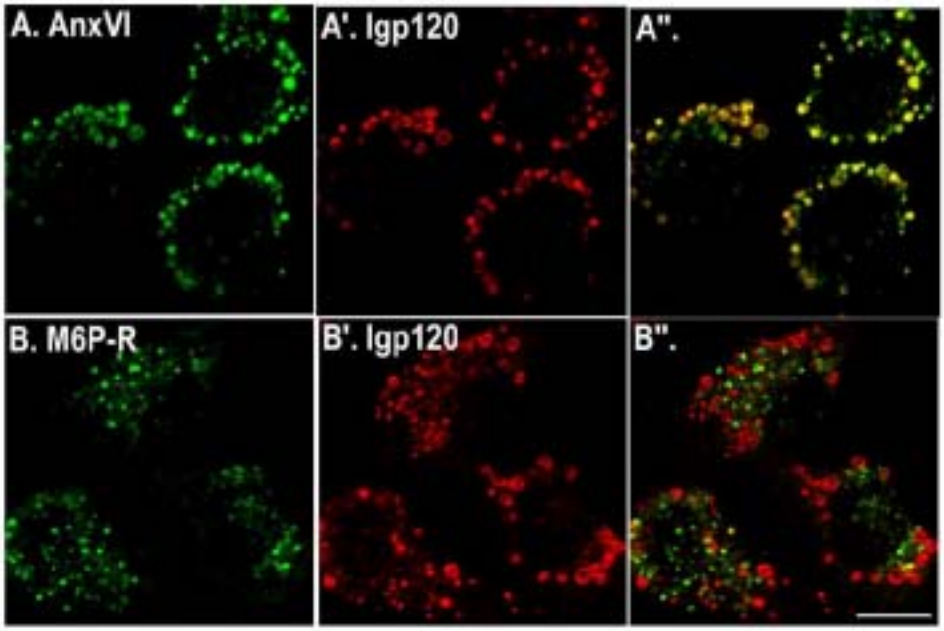


Fig. 4. Annexin 6 and M6PR localise to different compartments after chloroquine treatment of NRK cells. Cells were incubated with 40 μM chloroquine for 1 hr, then fixed, permeabilised and double-labelled with antibodies to annexin 6 and lgp120 (A/A') or M6PR and lgp120 (B/B'). Panels A" and B" show the corresponding merged images. Bar is 10 μm.

This general finding, codistribution of annexin 6 with lgp120 and separation of M6PR from lgp120, was confirmed when NRK cells were treated with wortmannin or BFA (Fig. 5). Wortmannin (1 μM for 1 hr) induced swollen lgp120-positive vacuoles (Fig. 5A'), whereas exposure to BFA (5 μg/ml for 1 hr) caused extensive tubulation of the lgp120-containing compartment (Fig. 5B'), as previously described ((Reaves et al., 1996) and (Lippincott-Schwartz et al., 1991), respectively). In both cases, annexin 6 was found on the same structures as lgp120 (Fig. 5A and B) while M6PR was not (data not shown). These results give further evidence that, in NRK cells, annexin 6 was preferentially associated with (pre)lysosomes rather than with any endosomes earlier in the lysosomal pathway, including M6PR-containing late endosomes.

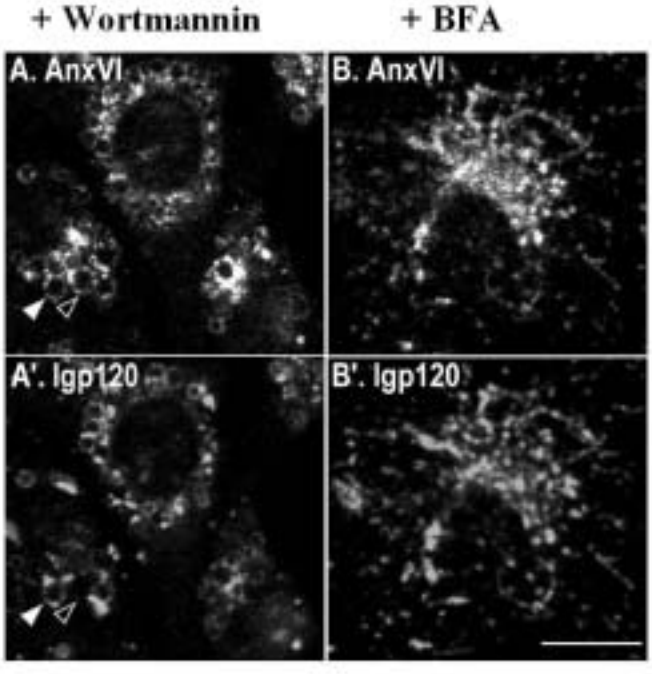


Fig. 5. Annexin 6 and lgp120 colocalise after treatment with BFA or wortmannin in NRK cells. Cells were incubated with 1 μM wortmannin (A/A') or 5 μg/ml BFA (B/B') for 1 hr, then fixed, permeabilised and double-labelled with antibodies to annexin 6 (A and B) and lgp120 (A' and B'). White arrowheads in (A/A') point to one of the swollen vacuoles which are both annexin 6- and lgp120-positive; rarely the swollen vacuoles contained only annexin 6 (open arrowheads). Bar is 10 μm.

To examine whether the association of annexin 6 with (pre)lysosomal membranes depended on cytoskeletal components, we analysed its localisation after disruption of actin filaments (using cytochalasin D or latrunculin A) or microtubules (using nocodazole). The degree of colocalisation between lgp120 and annexin 6 was unchanged in both cases, although nocodazole treatment caused an altered intracellular distribution of labelled structures, which appeared to form large unevenly chaped clusters (data not shown).

Colocalisation of annexin 6 with lgp120 at the ultrastructural level

To analyse the intracellular distribution of annexin 6 in greater detail, NRK cells were processed for cryoimmuno-EM and double-labelled with anti-annexin and anti-lgp120 antibodies (Fig. 6) followed by incubation with the appropriate gold-labelled secondary antibodies. The highest density of gold particles labeling annexin 6 was

found over vacuolar structures of heterogeneous morphology in the perinuclear region of the cells. The size of these structures ranged from 350 nm to 2 μm in diameter; however, most of them had a diameter between 500 nm and 1 μm. Their contents were homogenous, multivesicular, or consisted of concentric membrane swirls, but generally had a low electron density (Fig. 6A-C). The anti-annexin 6 antibody decorated almost exclusively the outer limiting membrane of all labelled structures. The vast majority of annexin 6-positive structures also contained lgp120. In contrast, we only found examples of small electron dense structures (200-500 nm) which were decorated with the anti-lgp120 antibody alone, indicating that dense core lysosomes contained little if any annexin 6.

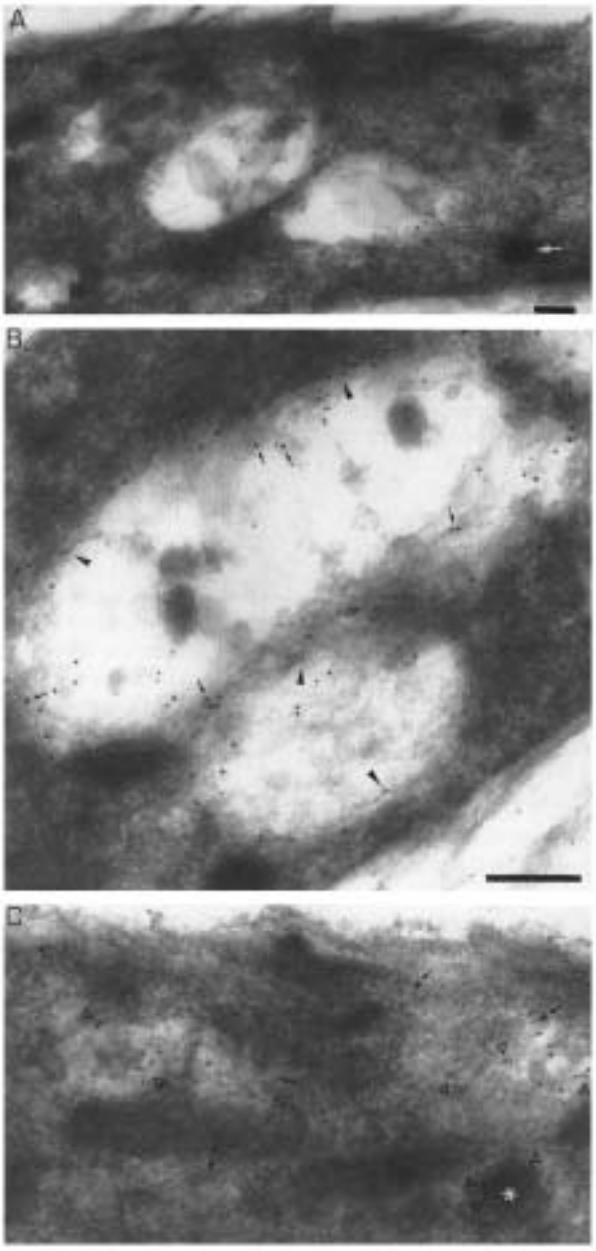


Fig. 6. Annexin 6 and lgp120 colocalise over large heteromorphous structures of low electron-density by immuno-EM. (A-C) Ultrathin cryosections of NRK cells were double-labelled with anti-annexin 6 (15 nm-gold; black arrows in B and C) and anti-lgp120 (10 nm-gold; filled or open arrowheads in B and C, respectively). Note that smaller dense vacuolar structures (lysosomes; white arrow in A) were annexin 6-negative, although often positive for lgp120 (asterisk in C). Bars are: 100 nm in (A) and 200 nm in (B) and (C).

Thus, these results confirmed the high degree of colocalisation of annexin 6 with lgp120 as suggested by confocal microscopy, and furthermore showed that annexin 6 seems to be restricted to prelysosomes or larger, less dense lysosomes. Some annexin 6 appeared also to be localised to other membranes including smaller vesicles and tubulo-vesicular structures, but the gold particle density over these membranes was significantly lower compared to that of (pre)lysosomal structures. Double immunogold labelling with anti-annexin 6 and anti-Rab5 showed some degree of colocalisation in peripheral structures (data not shown).

Partial codistribution of annexin 6 with lgp120 on a Percoll density gradient

Due to their higher density, lysosomal membranes can be relatively easily separated from other membranes, including M6PR-containing late endosomes, early endosomes, Golgi and plasma membrane, by subcellular fractionation using density gradient centrifugation (Griffiths et al., 1990). Therefore, we used this technique to verify the close association of annexin 6 with (pre)lysosomal compartments indicated by our morphologial data. NRK cells were homogenised and organelles fractionated on a 27% isopycnic Percoll gradient. The various density fractions were analysed for β-hexosaminidase specific activity and by immunoblotting with antibodies to annexin 6, lgp120 and Rab5 (Fig. 7). As expected, both the β-hexosaminidase and lgp120 distributions peaked in the dense region of the gradient with lgp120 being slightly shifted towards lighter densities compared to the lysosomal enzyme. The distribution of lgp120 even spread into the light membrane fractions where all non-lysosomal

membranes are expected to collect (see lgp120 "shoulder" in Fig. 7). Annexin 6 was also found in both dense and light fractions with a more pronounced bimodal distribution than lgp120. About 50% was found in the light membrane peak, i.e. the same fractions that contained rab 5. This analysis clearly substantiated the association of annexin 6 with (pre)lysosomes. However, it also indicated that on this type of gradient some lighter (pre)lysosomal membranes do not band together with the bulk of dense membranes but with the rest of intracellular membranes, making it difficult to determine how much annexin 6 was associated with non-lysosomal compartments as opposed to 'light' lgp120-containing membranes.

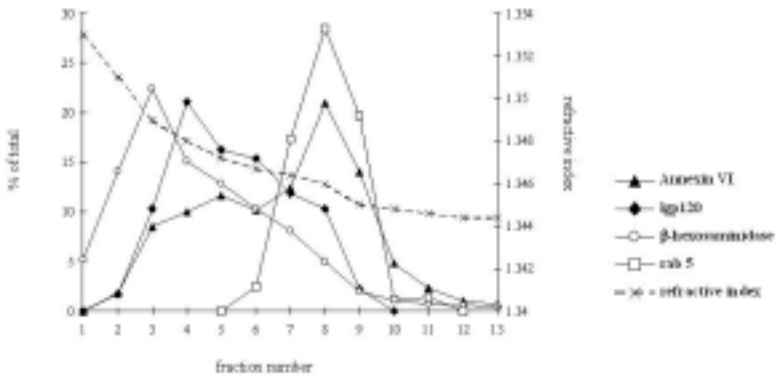


Fig. 7. Annexin 6 is found in high and low density fractions after isopycnic density gradient centrifugation. Post-nuclear supernatants from homogenised NRK cells were fractionated on a continous 27% Percoll gradient; the distribution of annexin 6 (▲), lgp120 (◆), and Rab5 (□) was determined by immunoblotting and that of ß-hexosaminidase (○) assaying the enzymatic activity. Western blots were quantified densitometrically and the results of three independent experiments were plotted together with the density profile (×).

Two major membrane-associated pools of annexin 6 in polarised WIF-B cells

Since the distribution of annexin 6 in cultured fibroblasts may misrepresent its localisation in vivo and in particular in polarised cells like hepatocytes, we extended our studies to the WIF-B cell line. The apical plasma membrane domains of two

adjacent cells together form a bile canalicular-like space (BC) and can be easily distinguished from basolateral domains by confocal microscopy in single optical sections. Many endosomal structures (e.g. recycling and late endosomes) are concentrated in the region between the nucleus and the apical cell surface, i.e., in close proximity to the Golgi apparatus (Ihrke et al., 1998). Lysosomes occupy a large perinuclear region mutually exclusive with the Golgi area. Using TGN38 as a marker for the Golgi and again lgp120 as lysosomal marker, we compared the distribution of annexin 6 relative to these compartments in WIF-B cells (Fig. 8A-A" and 8C-C", respectively). Similar to our results in NRK cells, annexin 6 colocalised in many cases with the lysosomal marker lgp120 indicating that a part of it was associated with lysosomes (white arrows in Fig. 8A-A"). Interestingly, we often found examples where annexin 6 did not coincide precisely with lgp120, but appeared to label only one or more spots along the limiting membrane of the corresponding structure. By comparison, M6PR did not colocalise to a significant degree with lgp120, but was mainly concentrated around the Golgi apparatus (Fig. 8B-B" and 8D-D", respectively). However, similar to M6PR in location and appearance, annexin 6 was also found on structures that resided close to the Golgi apparatus (Fig. 8C-C" and 8D-D", respectively); these structures were about equal in number and labelling intensity compared to the lgp120-positive structures. Occasionally, annexin 6 was seen in peripheral structures but at levels close to our detection limits.

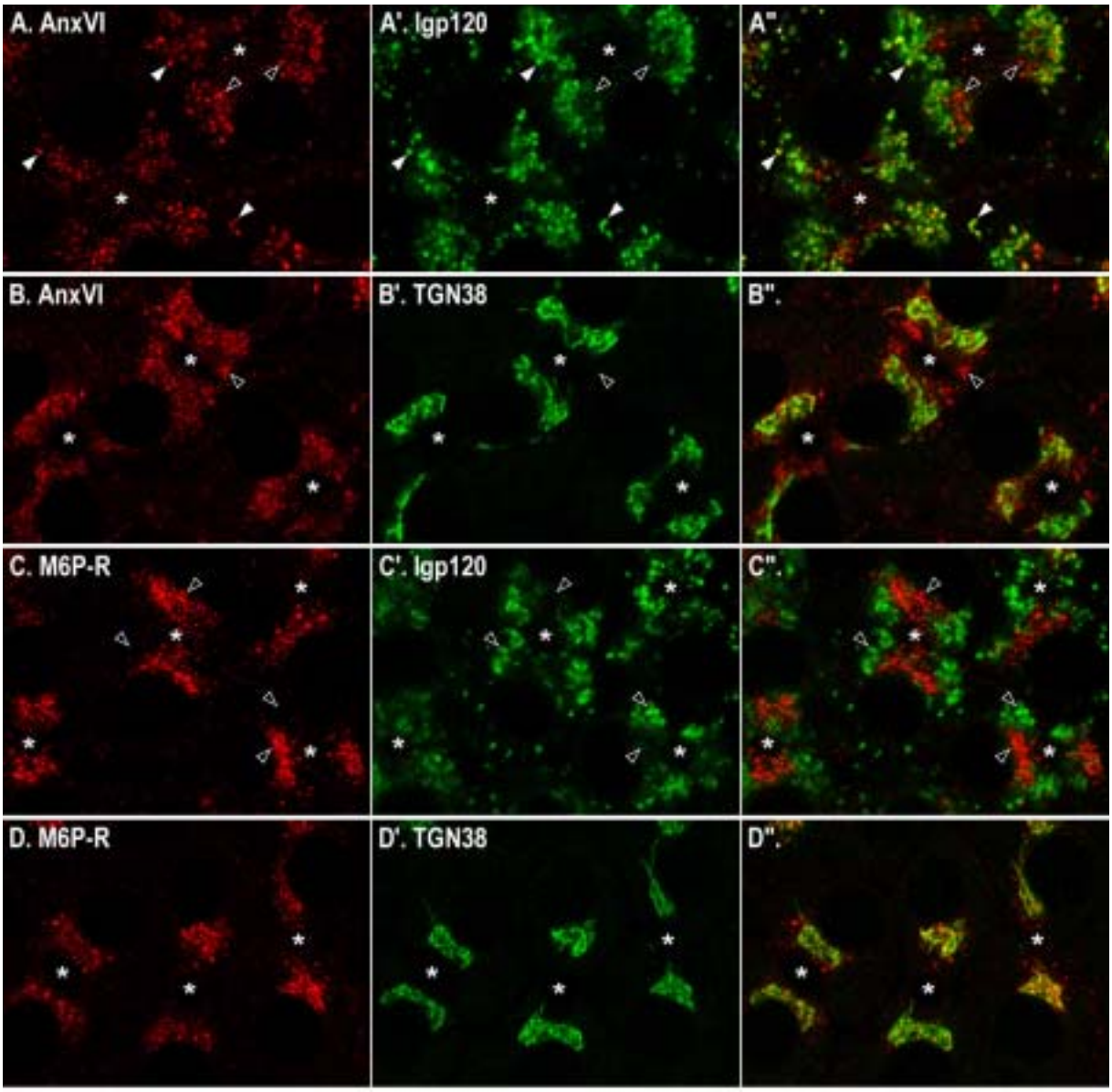


Fig. 8. Two pools of annexin 6 are present in WIF-B cells. Fixed and permeabilised cells were double-labelled with antibodies to the following pairs of antigens and analysed by confocal microscopy: (A/A') annexin 6 and lgp120; (B/B') annexin 6 and TGN38; (C/C') M6PR and lgp120; and (D/D') M6PR and TGN38. Antigens in panels A to D (left) and A' to D' (middle) were visualised by Cy3- and FITC-conjugated secondary antibodies, respectively. Panels A" to D" (right) show the corresponding merged images. * mark BC; white arrowheads indicate structures that contain both antigens and open arrowheads structures that contain only one antigen (only annexin 6 and not lgp120 in A-A", but either M6PR or lgp120 in B-B"); note that in C-C" and D-D", white arrows indicate overlap of annexin 6- or M6PR-positive punctate structures with tubules of the TGN/Golgi.

To see whether the non-lysosomal pool of annexin 6 was associated with endosomes, we performed dextran uptake experiments similar to the ones described above. As in NRK cells, there was little coincidence between the fluid phase marker and annexin 6 at early time points (5-15 min uptake and chase), i.e. in basolateral early endosomes (Fig. 9A). However, starting after ~30 min of chase and more so after 45 min (Fig. 9B/B') or 90 min (Fig. 9E/E'), there was a noticable overlap between the two markers in the perinuclear area (white arrowheads). Nonetheless, even at the late time points there was a much greater coincidence between dextran and lgp120 (Fig. 9D/D', 45 min). Surprisingly, and different from NRK cells (data not shown), we detected little overlap between dextran and M6PR at all time points (Fig. 9C/C', 45 min), possibly indicating that in WIF-B cells the M6PR was primarily localised at the TGN or on transport vesicles between TGN and late endosomes. Taken together, these results show that a significant portion of annexin 6 was associated with endosomes or lysosomes.

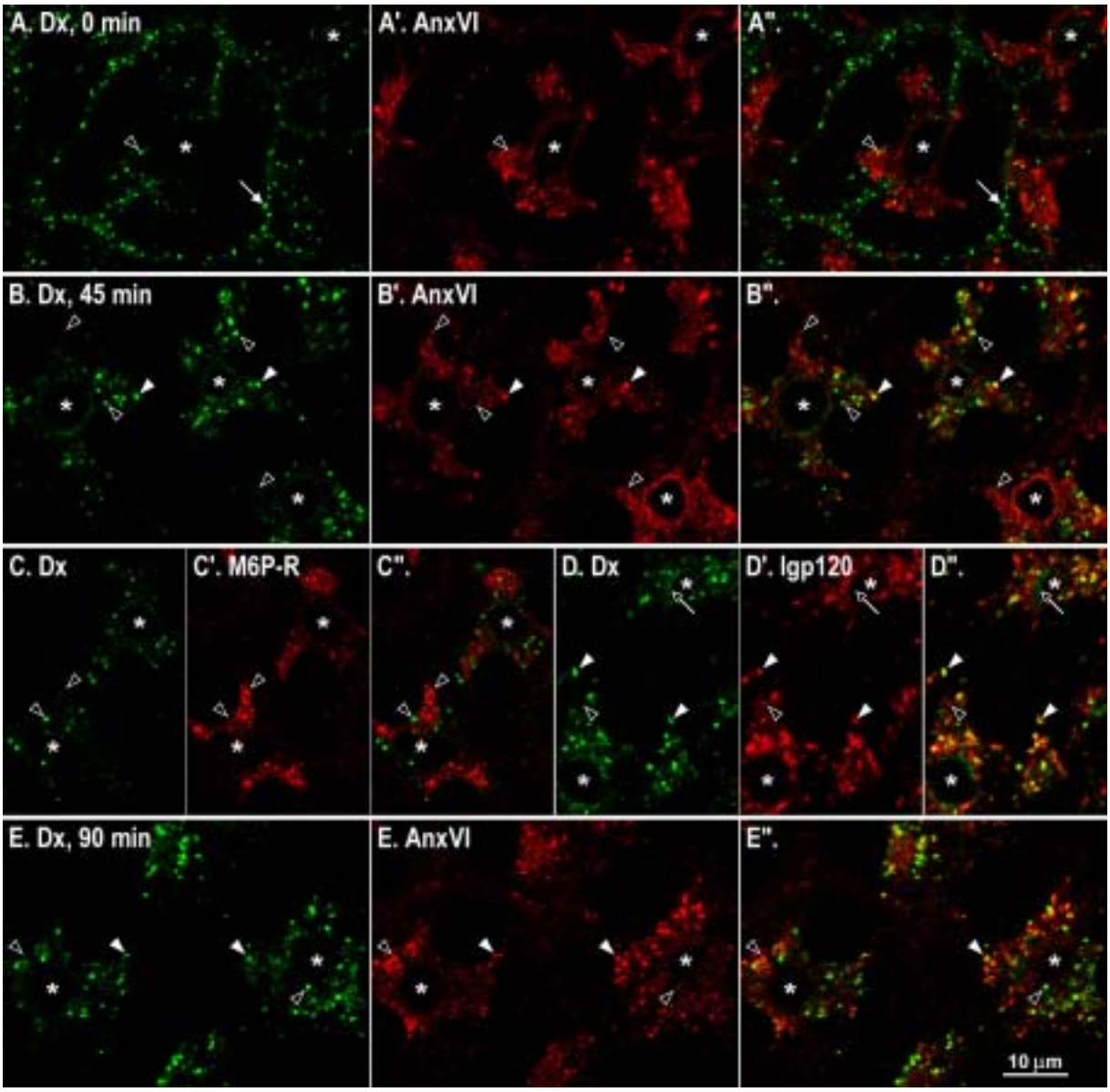


Fig. 9. A subpopulation of annexin 6-positive structures receives fluid-phase marker in WIF-B cells. Cells were incubated with FITC-dextran (mol wt 10,000) for 5 min and then chased in normal medium for 0 min (A), 45 min (B, C, and D), or 90 min (E). After fixation and permeabilisation, the cells were labelled with antibodies to annexin 6 (A', B', and E'), M6PR (C'), or lgp120 (D') followed by Cy3-conjugated secondary antibodies. * mark BC; the arrow in A points to one of many peripheral early endosomes which contain no detectable amounts of annexin 6 (see arrow in A'). White arrowheads indicate structures that contain both dextran and annexin 6 or lgp120, while black arrows point to structures that contain either only dextran or only annexin 6, M6PR, or lgp120. Note that after 45 min, there are few if any structures visible that contain both dextran and M6PR (C/C'), but many lgp120-positive structures that are also dextran-positive (D/D').

When we examined the distribution of annexin 6 after wortmannin treatment (100 nM for 1 hr), we still observed a significant coincidence with lgp120 on vacuoles of various sizes (data not shown). Under these conditions, M6PR also coincided with lgp120 in some cases, but was primarily found on a separate set of vacuoles in proximity to the Golgi apparatus. Different from lgp120 and M6PR, which were found more homogeneously along the limiting membrane of vacuoles, the majority of annexin 6 was still visible as a cloud of puncta with a fine dotty appearance similar to controls (data not shown). Annexin 6 label that was not associated with (pre)lysosomal structures was, similar to the M6PR, located in the area of the Golgi. In contrast, the asialoglycoprotein receptor (ASGPR), a basolateral membrane protein that recycles from peripheral and juxtanuclear early endosomes (Ihrke et al., 1998), was found in vacuoles of different sizes spread over a large area in the presence of BFA. Thus, it is unlikely that annexin 6 is associated with recycling endosomes to any large extent (data not shown).

To further explore the relationship of annexin 6 on structures in the Golgi region and the Golgi apparatus themselves, we used BFA (10 μg/ml for 1 hr) as a mean to redistribute Golgi membranes and examine whether a similar redistribution was seen for annexin 6. High resolution confocal microscopy, as shown in Figure 10, reveals that under control conditions some of the annexin 6 puncta align on or directly next to Golgi tubules labelled with TGN38 (not shown) or the cis-Golgi marker mannosidase II (Fig. 10A and A', respectively). After BFA treatment, the mannosidase II-positive tubular structures had disappeared as expected (Fig. 10B'), presumably due to the relocation of the protein to the ER (see e.g. (Lippincott-Schwartz et al., 1991)). The number of annexin 6-positive structures was reduced (Fig. 10B and C) and most of the remaining structures could be identified as (pre)lysosomal elements by double-labelling with lgp120 (Fig. 10C'). This suggests that some annexin 6 is indeed normally associated with Golgi membranes. Little if any codistribution of annexin 6 with TGN38 was seen, which, different from membrane proteins of the cis- and medial Golgi, is found in a tubular network and/or in dense juxtanuclear aggregations of membranes after BFA treatment (data not shown). A higher "background" level of annexin 6 was evident throughout the cells,

which may indicate that the Golgi-associated annexin 6 pool redistributed to the ER**,** or perhaps was released into the cytoplasm.

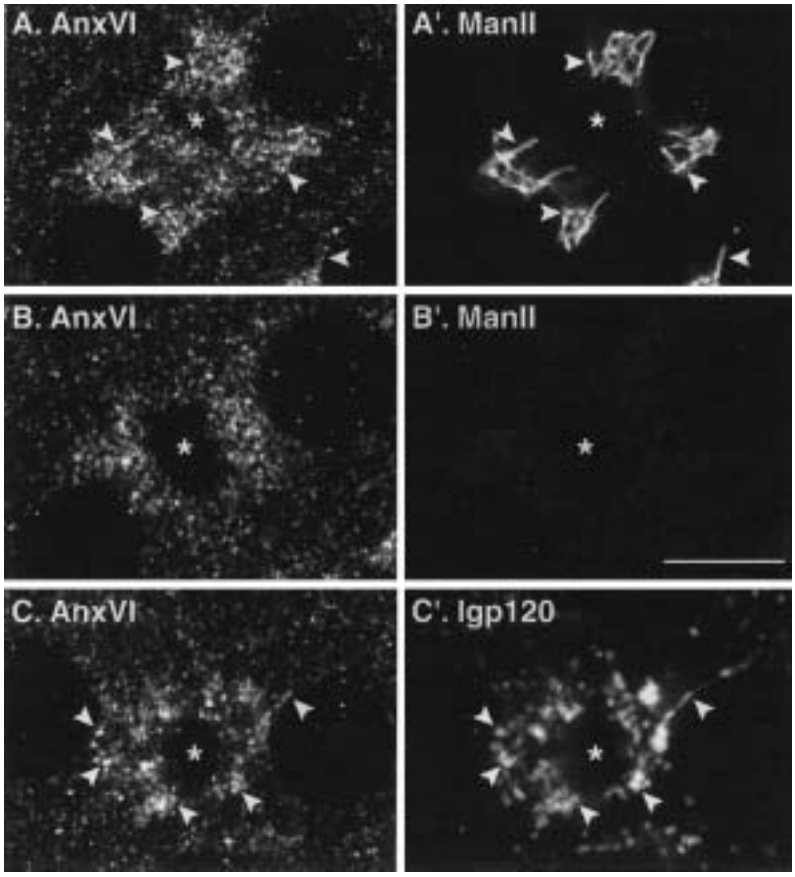


Fig. 10. A subpopulation of annexin 6-positive structures is associated with the Golgi apparatus in WIF-B cells. Cells were incubated in the absence (A/A') or presence (B/B' and C/C) of BFA (10 μg/ml) for 1 hr, then fixed, permeabilised and double-labelled for annexin 6 and mannosidase II (A/A' and B/B') or annexin 6 and lgp120 (C/C'). * mark BC; white arrowheads point to examples where annexin 6-positive structures align with Golgi tubules (A/A') or overlap with lysosomal structures (C/C'); bar is 10 μm.

Annexin 6 is not associated with the subapical transcytotic compartment of WIF-B cells, but with apical early endosomes

Although the localisation of both lysosomes and the Golgi apparatus is shifted towards the apical domain in polarised cells, membranes of neither compartment are usually found directly underneath the apical plasma membrane. However, we frequently observed a minor population of annexin 6-positive structures in this location. The best-studied subapical compartment of WIF-B cells, the socalled "SAC", is part of the basolateral-to-apical transcytotic pathway (Ihrke et al., 1998). We used endolyn-78 as a marker since this membrane protein moves through SAC on its intracellular itinerary. At steady-state, endolyn-78 is primarily located in lysosomes; thus, SAC is best visualised when antibodies to this protein (mAb 501) are bound to the basolateral surface and internalised under pulse-chase conditions such that a wave of the antigen-antibody complexes moving through SAC can be detected (Ihrke et al., 1998). To maximise the signal in this compartment, we incubated WIF-B cells in the continuous presence of antibody for 1 hr, then fixed the cells and co-stained with anti-annexin 6 (Fig. 11A and A', respectively). As expected, the antibody to endolyn-78 labelled many punctate structures within about 2 μm of the apical membrane and some structures deeper in the cells towards the nucleus (Fig. 11A). However, annexin 6 was usually not found on the same subapical structures as endolyn-78.

A distinct set of apical endosomes can be identified by labeling with the early endosomal protein EEA1. This protein colocalises with rab5 on membranes (Callaghan et al., 1999). In WIF-B cells, although significantly colocalising with other early endosomal markers, such as the transferrin receptor (TfR), EEA1 is more predominantly seen on endosomes in the perinuclear region of WIF-B cells, including endosomes underneath the apical surface (G.I. unpublished data). The latter contain little if any TfR and presumably correspond to early endosomes derived from the apical plasma membrane. When we co-labelled WIF-B cells with antibodies to EEA1 and annexin 6, we found some subapical structures that were positive for both proteins (Fig. 11B and B', respectively), although the majority of EEA1-positive structures further inside the cells did not exhibit visible annexin 6 staining.

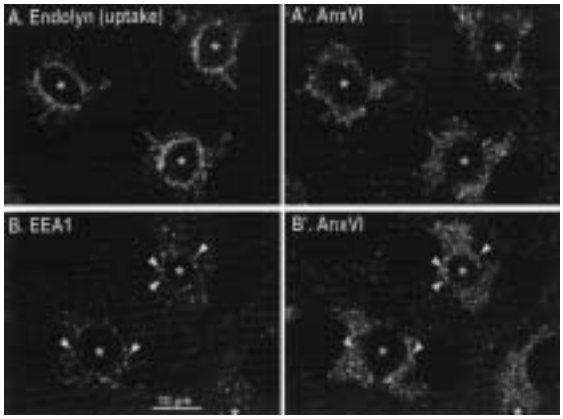


Fig. 11. Some subapical annexin 6-positive structures correspond to apical early endosomes in WIF-B cells. (A/A') Living cells were incubated in medium containing antibodies to endolyn-78 for 1 hr to load the transcytotic SAC underneath the BC membrane. Cells were then fixed, permeabilised and incubated with the anti-annexin 6 antibody, followed by secondary antibodies (FITC, endolyn-78; Cy3, annexin 6). (B/B) Fixed and permeabilised cells were double-labelled with antibodies to the early endosomal marker EEA1 and annexin 6, followed by secondary antibodies (FITC, EEA1; Cy3, annexin 6). Note that there is essentially no overlap between endolyn-78 and annexin 6 in the ring-like zone occupied by SAC (open arrows) or in structures further inside the cells (open arrowheads). However, some subapical structures contain both EEA1 and annexin 6 (white arrowheads). * mark BC.

III.1.4. Discussion

In the present study, we have determined the intracellular distribution of annexin 6 in non-polarised NRK cells and in polarised cells of hepatic origin (WIF-B). By confocal microscopy, high concentrations of annexin 6 were found associated with late endocytic/lysosomal elements, while less label was seen in earlier compartments of the endocytic pathway. Four major lines of evidence indicated the predominant association with (pre)lysosomal compartments: 1) higher coincidence of annexin 6 with internalised fluid phase marker after long compared to short chase times; 2) significant colocalisation with the lysosomal marker lgp120 and comparatively little with early endocytic markers; 3) stronger colocalization of annexin 6 with lgp120 than with the late endocytic marker M6PR after pharmacological perturbation of the

endocytic membrane system (NRK cells) and 4) little coincidence of lgp120 and annexin 6 on dense core lysosomes by electron microscopy. In WIF-B cells, a substantial amount of annexin 6 appeared to be also present in compartments of the secretory pathway (Golgi). However, the marked association of annexin 6 with prelysosomal membranes in both cell types argues for a potential function of annexin 6 in the late endocytic pathway. Since annexin 6 has been shown to mediate the association of membranes in vitro (Zaks and Creutz, 1990), its role could be the tethering of prelysosomal membranes prior to their fusion.

Annexin 6 in endocytic compartments of lysosomal character

There has been some controversy in the literature regarding the subcellular localisation of annexin 6. It has been reported to be at the plasma membrane in rat hepatocytes (Tagoe et al., 1994), (Weinman et al., 1994) and to associate with the actin cytoskeleton in fibroblastic cell lines (Hosoya et al., 1992). The antibody used in this study was raised against membrane-bound annexin 6 (Ortega et al., 1998). Thus it is possible that it recognises primarily certain isoforms or conformations of annexin 6 (Crompton et al., 1988), (Bianchi et al., 1992), (Dubois et al., 1995), (Edwards and Moss, 1995), (Benz et al., 1996), (Kawasaki et al., 1996) which could explain preferential recognition in certain subcellular localisations (e.g. membrane-associated). However, it has been previously established by biochemical means that annexin 6 is a major component of rat liver endosomes (Jackle et al., 1994) and the majority of studies (using several different anti-annexin 6 antibodies) agrees on an endosomal distribution of annexin 6 in many cell types (Desjardins et al., 1994), (Seemann et al., 1996), (Ortega et al., 1998).

NRK cells and hepatoma-derived cell lines (e.g. H_4S or HepG2) have frequently been used as prototypes to study endocytic compartments in the degradative pathway of mammalian cells ((Geuze et al., 1988), (Griffiths et al., 1988), (Griffiths et al., 1990), (Bright et al., 1997) and many others). Recent evidence suggests that a prelysosomal hybrid organelle is formed by direct fusion of late endosomes and lysosomes (Bright et al., 1997), (Mullock et al., 1998). This model agrees well with the hybrid character of the protease-rich PLC described earlier

(Holtzman, 1989), (Courtoy, 1991); (Griffiths et al., 1990), regarding its composition, heteromorphous morphology and buoyant density. It also emphasises that the protease-rich PLC or hybrid compartment is at the transition point between membranes with predominantly endosomal and predominantly lysosomal character, respectively.

Various drugs, including chloroquine, BFA and wortmannin, seem to impair fusion and/or fission processes by which the endosomal and lysosomal branches of the degradative pathway communicate with each other (Brown et al., 1986), (Lippincott-Schwartz et al., 1991), (Wood and Brown, 1992), (Reaves et al., 1996). Despite different underlying mechanisms and specific morphological effects, these drugs have in common that the distributions of the M6PR and lgp120 are more separated from each other compared to normal conditions with the former being more endosomal and the latter more lysosomal.

A caveat concerning the use of the M6PR as late endosomal marker is that its relative distribution between TGN, late endosomes, and carrier vesicles between the two compartments depends on the cell type (Geuze et al., 1988), (Griffiths et al., 1990), (Hirst et al., 1998). This might partially explain why we found significant M6PR label on dextran-labelled late endosomes, while this was not so in WIF-B cells. This discrepancy to previous results in WIF-B cells (Ihrke et al., 1998) may be due to the use of different antibodies to M6PR with distinct binding preferences. However, our histochemical data obtained in both NRK and WIF-B cells all indicate that a major pool of annexin 6 is found on membranes of the protease-rich PLC or hybrid compartment and lysosomes of various stages, but not on dense core lysosomes. The density profile of membranes that had annexin 6 attached was in agreement with the microscopic results, since annexin 6 and lgp120 peaked in the same fractions of the denser region of the Percoll gradient. However, approximately 50% of annexin 6 was associated with lighter membranes, which are primarily non-lysosomal. The apparent discrepancy to the distribution revealed by microscopy can be explained by the following factors. A significant amount of annexin 6 may in fact be associated with relatively light prelysosomal hybrid organelles (Mullock et al., 1998) which could migrate in the second (light) peak as indicated by the presence of a small amount of

lgp120 in this part of the gradient. Moreover, annexin 6 which is presumably present at lower concentrations on most endocytic membranes as well as on exocytic membranes (Jackle et al., 1994) would be collectively detected in the second peak. This is consistent with our microscopic observation that annexin 6 was colocalising to some degree with early endosomal markers in both cell types and also with Golgi membranes in WIF-B cells (see below).

Annexin 6 on non-lysosomal membranes in polarised WIF-B cells

The predominantly prelysosomal pattern of annexin 6 in NRK cells by confocal and electron microscopy may represent an extreme in the spectrum of possible distributions and partially reflect the difference between (some) cultured cells and tissues in vivo. This view was corroborated by our finding that annexin 6 only partially coincided with lysosome-like compartments in hepatic WIF-B cells. Although there was little direct colocalisation in the Golgi area with any other marker tested, annexin 6 label often appeared as small puncta aligning with Golgi tubules. Since this annexin 6 pool was no longer apparent after BFA treatment, it is most likely that a significant amount of annexin 6 was associated with Golgi or TGN membranes themselves or with carrier vesicles derived from these.

Another pool of annexin 6 appeared to be associated with apical early endosomes as evidenced by colabeling with EEA1 in structures close to the apical plasma membrane. Interestingly, we saw less overlap with basolateral early endosomes containing ASGPR (data not shown) or internalised dextran, indicating that annexin 6 might be more strongly associated with early endosomes derived from the apical surface. Since in wortmannin-treated cells, annexin 6 and ASGPR behaved very differently, we assume that the overall contribution of early endosome-associated annexin 6 is relatively small compared to the total pool of membrane-associated annexin 6 in WIF-B cells. However, this may be an underestimate since membranes with very low concentrations of annexin 6 would not be detected by confocal microscopy. Thus, this finding is in general agreement with earlier results demonstrating annexin 6 on membranes in the apical cytoplasm (Massey-Harroche et al., 1998). These endosomes are not identical to the previously described transcytotic

SAC in WIF-B cells (Ihrke et al., 1998). We did not find convincing evidence that annexin 6 associated with SAC, in accord with observations in rat liver in situ, where relatively little pIgAR was found to colocalise with annexin 6 in the subapical region (Ortega et al., 1998).

Irrespective of the location within the cells, we often noticed that annexin 6 had a more dotty appearance compared to the integral membrane proteins used as markers in this study. This was especially evident in wortmannin-treated cells, where annexin 6 retained its fine punctate appearance as if being constrained to a subdomain of the vacuolar membrane it was associated with (data not shown). Such a localisation to discrete membrane regions of endosomes has been demonstrated for annexin II (Harder et al., 1997). It is tempting to speculate that such focal concentrations of annexins constitute putative binding sites with other membranes; though, whilst being consistent with this idea, our data provide no direct proof for this conjecture.

Physiological relevance of annexin 6-binding to prelysosomal membranes

Our main observation that annexin 6 is enriched in prelysosomal compartments of the endocytic pathway in NRK and WIF-B cells agrees with earlier studies in macrophages. Work by Desjardins et al. (Desjardins et al., 1994) and Diakonova et al. (Diakonova et al., 1997) suggest that, although many annexins (1-6) are found on phagosomes, only annexin 4 and 6 are enriched on more mature phagosomes that have a lysosome-like phospholipid composition. Preliminary experiments indicate that annexin 6 also associates with lgp120-positive autophagic vacuoles in the perinuclear region of NRK cells when autophagocytosis is stimulated in these cells by serum starvation (M.P. and C.E., unpublished data). However, the present study shows that the high affinity of annexin 6 to lysosome-like compartments is not restricted to phagosomes. Annexins 1, 2, 3, and 4 seem to preferentially associate with the plasma membrane or early endosomes (Gerke and Moss, 1997), (Diakonova et al., 1997). Although annexin 5 was found on late endosomes, it was at least similarly abundant at the plasma membrane and on early endosomes

(Diakonova et al., 1997). Thus, the only annexin which appears to be specifically enriched on late endocytic/prelysosomal compartments of cultured cells is annexin 6.

The presence of annexin 6 in most tissues and many cell lines suggests that it plays a ubiquitous, yet unidentified role in endosomal/lysosomal membrane traffic. The unique structure of annexin 6, containing two distinct lipid binding domains which assume a coplanar arrangement upon lipid binding (Kawasaki et al., 1996), (Benz and Hofmann, 1997), appears to be especially suitable to mediate the association and perhaps aid the fusion of two separate compartments. This hypothesis is supported by recent observations in regard to annexin II, which is the only other annexin with two lipid binding domains in the functional complex (an annexin II_2-$p11_2$ heterotetramer) (Gerke and Moss, 1997). Annexin II is reportedly competent to form characteristic junctions between lipid layers as well as interactions between plasma membrane and the actin cytoskeleton (Lambert et al., 1997). Interestingly, annexin 2 has been implicated in homotypic fusion between early endosomes (Emans et al., 1993), (Mayorga et al., 1994). It is an intriguing conjecture that annexin 6 may exert similar effects in the late steps of the endocytic pathway.

Because of its high concentration on prelysosomes and lysosomes, we suggest that annexin 6 may facilitate the continuously ongoing fusions between late endocytic compartments. It is imaginable that annexin 6 is a structural component of the filaments that have occasionally been observed between late endosomes and prelysosomes or lysosomes and may attach vacuoles to each other prior to a fusion event (Futter et al., 1996), (Bright et al., 1997). Experiments specifically designed to test this hypothesis, e.g. by determining the effect of exogenously expressed annexin 6 on degradation in cultured cells or the importance of annexin 6 for in vitro fusion of late endosomes and lysosomes, may bring us closer to an understanding of the function of this annexin in the endocytic pathway.

III.1.5. Resum capítol 1

Late Endocytic Compartments Are Major Sites of Annexin VI Localization in NRK Fibroblasts and Polarized WIF-B Hepatoma Cells

Precedents

La localització de l'anx6 ha estat sempre descrita associada a fosfolípids de membrana però el tipus de membrana discerneix segons el tipus cel·lular que s'estudia i l'anticòs utilitzat. En aquest estudi s'ha emprat un anticòs policlonal de conill contra la forma d'anx6 aïllada d'endosomes de fetge de rata. Concretament en la membrana plasmàtica l'han classificada com a proteïna estructural, unint-se per un costat a la membrana i per l'altre al citoesquelet. També s'ha localitzat en compartiments endocítics i exocítics, així com en autofagosomes tardans. El nostre laboratori va trobar que en els hepatòcits de rata l'anx6 colocalitzava amb un marcador d'endosomes primerencs (Rab5) al voltant del canalicle biliar.

OBJECTIUS

1. Distribució de l'annexina 6 en les cèl·lules NRK i WIF-B
2. Caracterització del compartiment on es troba l'annexina 6
3. Aïllament de compartiments subcel·lulars enriquits en annexina 6

Resultats

Mitjançant tècniques d'immunofluorescència indirecta vam poder demostrar que en les cèl·lules NRK l'anx6 es trobava en la regió perinuclear i col·localitza completament amb lgp120, una glicoproteïna resident en prelisosomes i lisosomes. En les cèl·lules hepàtiques polaritzades WIF-B un 50% de l'anx6 es trobava en compartiments lgp120 positius. Per tant l'anx6 es trobava en els últims estadis de la via endocítica, fet que es corroborava amb la localització amb Dextrà-FITC internalitzat tan sols a partir de 45 minuts. L'altre pool d'anx6 de les cèl·lules WIF-B es trobava de forma puntejada en el Golgi o vesícules del Golgi ja que després d'un tractament amb BFA, desapareixia aquest pool.

Per distingir si es trobava en els endosomes tardans o lisosomes, molt a prop físicament, es van tractar les cèl·lules amb cloroquina (modifica els lisosomes i prelisosomes) i es va fer un doble marcatge d'anx6 i un marcador d'endosomes tardans, el receptor de la manosa-6-fosfat. Després del tractament es veia més clarament que l'anx6 no es trobava en els endosomes tardans.

La morfologia del compartiment intracel·lular anx6 es va estudiar per microscòpia electrònica, revelant unes vesícules poc electrodenses marcades per anx6 i lgp120. Tanmateix però, en els lisosomes madurs no es trobava l'anx6, indicant una localització prelisosomal.

Mitjançant un gradent de Percoll vam poder disseccionar dos pools intracel·lulars d'anx6, el més dens dels quals corresponia als prelisosomes i el més lleuger a una fracció formada per endosomes primerencs, membrana plasmàtica i altres organels. Aquests dos pics ens posaven de manifest que l'anx6 en les cèl·lules NRK es pot trobar en altres membranes cel·lulars però l'anticòs utilitzat en les immunocitoquímiques reconeixia específicament l'anx6 que es trobava unida a endosomes.

III.2. Evidence for the involvement of annexin 6 in the trafficking between the endocytic compartment and lysosomes

III.2.1. Abstract

Annexins are a family of calcium dependent-phospholipid-binding proteins, which have been implicated, in a variety of biological processes including membrane trafficking. The annexin 6/lgp120 pre-lysosomal compartment of NRK cells was loaded with LDL (low density lipoprotein) and then its transport from this endocytic compartment and its degradation in lysosomes was studied. NRK cells were microinjected with the mutated annexin 6 ($anx6_{1-175}$), to assess the possible involvement of annexin 6 in the transport of LDL from the pre-lysosomal compartment. The results indicated that microinjection of mutated annexin 6, in NRK cells, showed the accumulation of LDL in larger endocytic structures, denoting retention of LDL in the pre-lysosomal compartment. To confirm the involvement of annexin 6 in the trafficking and the degradation of LDL we used CHO cells transfected with mutated annexin 6_{1-175}. Thus, in agreement with NRK cells the results obtained in CHO cells demonstrated a significant inhibition of LDL degradation in CHO cells expressing the mutated form of annexin 6 ($anx6_{1-175}$). Therefore, we conclude that annexin 6 is involved in the trafficking events leading to LDL degradation.

III.2.2. Introduction

The membrane traffic pathway from early to late endosomes has been studied in detail (Gruenberg and Maxfield, 1995), (Gu and Gruenberg, 1999). In contrast, the

events that govern the trafficking from the “late endocytic compartment” to the lysosome are still poorly characterised. Although most trafficking into the lysosomes comes from the early/sorting endosomes (Mellman, 1996), a subset of different intermediate entities, endocytic carrier vesicles (ECVs), hybrid organelles, late endosomes or pre-lysosomes, have been described morphologically and biochemically (Gruenberg and Maxfield, 1995), (Kornfeld and Mellman, 1989), (Geuze et al., 1988), (Jahraus et al., 1994), (Pons et al., 2000) and the references therein.

Among the molecules that might be involved in these steps of membrane trafficking are the low molecular weight GTPases, the Rabs (Novick and Zerial, 1997), and the membrane fusion proteins of the vesicle-associated membrane protein (VAMP), syntaxin and synaptosomal-associated protein of the 25 kDa (SNAP-25) families and, the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs) (Advani et al 1999). Whereas Rab 7 and 9 (Chavrier et al., 1990), (Lombardi et al., 1993) are associated with late endosomes few SNAREs have been shown to specifically function within the endosomal pathway. Althought several SNAREs, including cellubrevin, VAMP8/endobrevin, syntaxin 13, and syntaxin 7, have been localised in endosomal membranes, their precise location, interactions and function remain to be clarified (see a recent review on polarised cells by Mostov et al. 2000) (Mostov et al., 2000); we showed that cellubrevin/VAMP-3 was present in the basolateral “early” endocytic compartment of hepatocytes and was involved in the transcytosis of pIgA (Calvo et al., 2000). Recently, VAMP-7 (TI-VAMP) (Galli et al., 1998) was shown to mediate the vesicular transport from endosomes to lysosomes (Advani et al., 1999) and syntaxin 7 and VAMP 8 were reported in the late endocytic compartment and shown to be necessary for the fusion to lysosomes (Mullock et al., 2000).

However, the encounter (kiss and run) and/or the final fusion between two compartments (endosomes and lysosomes) (Storrie and Desjardins, 1996), (Luzio et al., 2000) require not only proteins of the docking and fusion machinery (Rabs and SNAREs) but a variety of effectors, adapters, GTP-binding proteins, Ca^{2+}/calmodulin and tethering proteins (Pryor et al., 2000). We proposed that annexin 6 acts as a

tether between membranes at the last stage of the endocytic route (Pons et al., 2000).

In this study we will highlight the importance of the subcellular location of annexin 6, and its specific local interactions with spectrin, as a baseline to understand the function of annexin 6 in the “late endocytic compartment”. However, the ability of annexin 6 (like annexin II) to change its location according to signals which involve intracellular Ca^{2+} mobilisation, should also be considered (Babiychuk et al., 1999), (Babiychuk and Draeger, 2000).

In recent years, the involvement of annexins in membrane traffic has emerged as one of their predominant functions (Gerke and Moss, 1997), (Gruenberg and Emans, 1993). Annexin 6 was first reported at the plasma membrane of rat hepatocytes (Tagoe et al., 1994), (Weinman et al., 1994), of erythrocytes (Bandorowicz et al., 1992) or mammary tissue (Lavialle et al., 2000) but also associated with the actin cytoskeleton in fibroblastic cell lines (Hosoya et al., 1992), in phagosomes of J774 macrophages (Desjardins et al., 1994) or in structures of the endocytic compartment (Ortega et al., 1998), (Seemann et al., 1996), (Massey-Harroche et al., 1998)]. By biochemical means, we demonstrated that annexin 6 was a major component of rat liver endosomes (Jackle et al., 1994), (Pol et al., 1997) and we have shown the prominent intracellular location of annexin 6 in the peri-nuclear, pre-lysosomal compartment of NRK and WIF-B cells (Pons et al., 2000).

At the plasma membrane (depending on Ca^{2+} mobilisation) annexin 6 may be involved in receptor-mediated endocytosis and perhaps in the remodelling of the spectrin cytoskeleton at the cell surface during endocytosis, thus facilitating the release of clathrin-coated vesicles from the plasma membrane. The binding of annexin 6 to spectrin (and to actin), mediate the action of calpain I which, in turn may cleave the spectrin-actin cytoskeleton allowing endocytosis, (Kamal et al., 1998). The fact that calpain I was located in clathrin coated vesicles (Sato et al., 1995) together with the finding that annexin 6 binds to spectrin (Watanabe et al., 1994) and is required for budding in vitro (Lin et al., 1992) indicates that a reorganization of the spectrin cytoskeleton might be necessary to allow the budding at the plasma membrane.

Interestingly, spectrin is also present in the membranes of Golgi (Beck et al., 1994), in a variety of poorly characterised intracellular vesicles (De Matteis and Morrow, 1998), (Stankewich et al., 1998) and in macropinosomes of NIH 3T3 cells (Xu et al., 2000). Supporting the role of annexin 6 at the plasma membrane, we showed the involvement of annexin 6 in the endocytosis and trafficking of LDL (Grewal et al., 2000).

Here we demonstrate that the mechanism proposed, by Kamal and co-workers (1998) (Kamal et al., 1998) for annexin 6 at the plasma membrane, can also operate in structures of the late endocytic compartment, which is the priority location of annexin 6 in NRK cells (Pons et al., 2000). Our results indicate that, in NRK and CHO cells, annexin 6 is involved in the trafficking events occurring at the endosomal compartment, and therefore may modulate the exit of LDL from the pre-lysosomal compartment and eventually its degradation in lysosomes.

III.2.3. Results

Annexin 6 and spectrin in the prelysosomal compartment of NRK cells

In NRK cells the structures with the highest concentrations of annexin 6 were mostly positive for lgp120 (Fig.1a,b,c), and devoid of M6P-R, a late endosomal marker (Pons et al., 2000). Furthermore, it was observed that annexin 6 also co-localises, to some extent, with spectrin in the peri-nuclear region of NRK cells (Fig. 1d, e, f). However, the spectrin observed in the peri-nuclear location, which co-localise with annexin 6, does not correspond to the Golgi spectrin, since annexin 6 did not overlap with the Golgi structures, labelled with anti-Golgi 58k antibody (Fig.1g, h, i). This indicated that the annexin 6-pre-lysosomal compartment also contains spectrin.

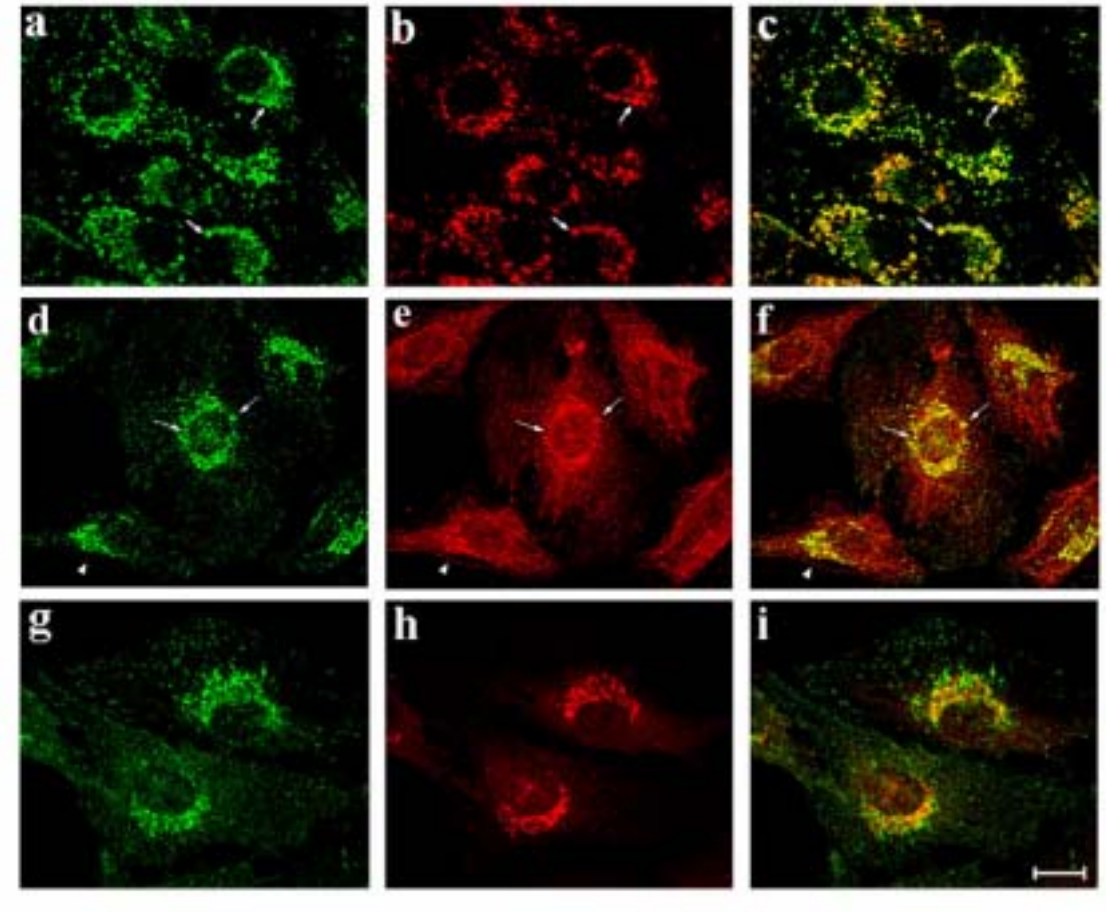


Figure 1. Annexin 6 co-localises with spectrin in the peri-nuclear region of NRK cells. Immunofluorescence analysis of annexin 6 and spectrin location in NRK cells showed in a double labelling immunocytochemistry. Anti-annexin 6 (a, d, g) and anti-lgp120 (b) co-localise in the peri-nuclear region of cells (c, merge); when cells were labelled with a polyclonal anti-spectrin antibody (e) it can be observed that in addition of spectrin at the cell surface (arrowheads) there was intense labelling at different intracellular locations (arrows) some of these co-localised with annexin 6 (f, merge); finally, the labelling of annexin 6 (g) and the Golgi marker (anti-Golgi 58k) (h) was compared. Despite the staining is in the same region of the cells no significant co-localisation was observed (i, merge). Bar is 10 μm.

The interaction of annexin 6 with spectrin was confirmed, in vitro, by pull-down experiments with GST-annexin 6 using a pre-lysosome/late endosomal fraction from NRK cells, and also in rat liver endosomes. Fig. 2 shows the Western blotting, with

anti-spectrin polyclonal antibody, after the pull-down with GST-annexin 6 (GST-anx6$_{wt}$) or GST, as control; spectrin was positively recognised in both subcellular fractions.

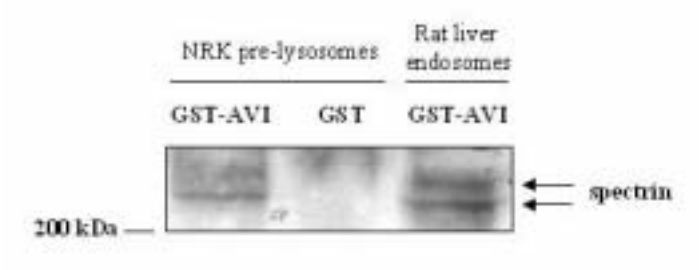


Figure 2. Interaction of GST-annexin 6 and spectrin. GST-annexin 6 was used to study the interactions of annexin 6 with other proteins in subcellular fractions of NRK cells and in an endosomal fraction from rat liver. A pre-lysosomal fraction, corresponding to the peak of annexin 6 and lgp120 of the Percoll gradients (Pons et a., 2000) or a crude endosomal fraction from rat liver were incubated with GST-annexin 6-Sepharose for 2 h and then bound proteins were collected and analysed by Western-blotting with anti-spectrin antibody. Control with GST alone shows no binding to spectrin.

Annexin 6 is involved in the trafficking to lysosomes

In this study two different approaches that interfere with the removal of spectrin from membranes were considered: overexpression of anx6$_{1-175}$ by microinjection of the pcDNA-anx6$_{1-175}$ into the nucleus of NRK cells (NRK cells showed very low efficiency of transfection) and incubation of cells with ALLN, a calpain inhibitor. Overexpression of anx6$_{1-175}$ was shown to inhibit the LDL internalisation, by clathrin-coated pits and inhibit the loss of spectrin in isolated membranes in vitro (Kamal et al., 1998).

Cells were first microinjected with the pcDNAanx6$_{wt}$ vector or cDNA corresponding to the mutated anx6$_{1-175}$, allowed to recover for 1 hour and incubated with DiI-LDL for 1 hour and chased overnight. This treatment provides the accumulation of DiI-LDL into the annexin 6 pre-lysosomal/late endocytic compartment, before the expression of microinjected cDNAs (see also Fig.4a, b). From this pre-lysosomal compartment we now investigated whether LDL is able to reach the lysosomes for degradation.

The distribution of DiI-LDL in cells microinjected with anx6$_{wt}$, showed no difference with non-injected cells (vesicular structures of 200-400 nm average size in the Golgi/lysosome region) (Fig. 3a); however, those cells microinjected with the mutated anx6$_{1-175}$ showed vesicles with DiI-LDL, which were larger (approx. 500-700 nm) (Fig. 3b) (70% of anx6$_{1-175}$ microinjected showed the enlarged LDL containing endosomes).

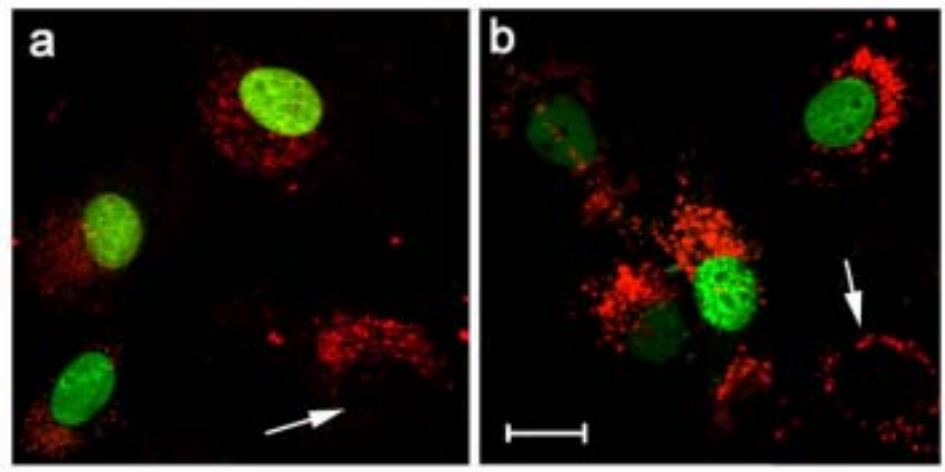


Figure 3. Uptake and transport of LDL in microinjected NRK cells. Representative fields of NRK cells co-microinjected into the nucleus with the pcDNA of annexin 6 wild type (a) or the pcDNA of the mutated form of annexin 6 (anx6$_{1-175}$) (b) and dextran-FITC. Cells were and then incubated with DiI-LDL for 1 hour, chased overnight and 6sualised with confocal microscopy. In (a) cells overexpressing wild type annexin 6, (green nucleus) show no differences in the pattern of DiI-LDL internalisation. In (b) DiI-LDL accumulates in large endocytic structures of microinjected cells. White arrow (a,b) shows a non-injected cell for comparison. Bar is 10 μm.

Immunocytochemical analysis of annexin 6 expressing the truncated form (anx6$_{1-175}$) showed a diffuse cytoplasmic staining, similar to those cells overexpressing the wild type (not shown). The efficiency of microinjection was 85% for the wild type (anx6 wt) and 71% for the mutated form (anx6$_{1-175}$ mutant).

In the second approach, the annexin 6 compartment was loaded with DiI-LDL for two hours, in NRK cells induced to express LDL receptors by replacing the fetal calf serum with human lipoprotein deficient serum as previously described (Goldstein et al., 1983). Then, cells were chased for 2 hours in the presence of DMSO or ALLN. Fig. 4 (a, b) shows the co-localisation of internalised LDL and annexin 6 and also the effect of ALLN on the endocytic compartment; it can be also observed an enlargement of endocytic structures at the peri-nuclear region after the treatment with the calpain inhibitor (a similar effect was observed in cells treated with chloroquine) (Pons et al., 2000). The enlargement of LDL-containing vesicles, after the ALLN treatment, may be caused by an accumulation of LDL in pre-lysosomes, with a concomitant inhibition of degradation in the lysosomes.

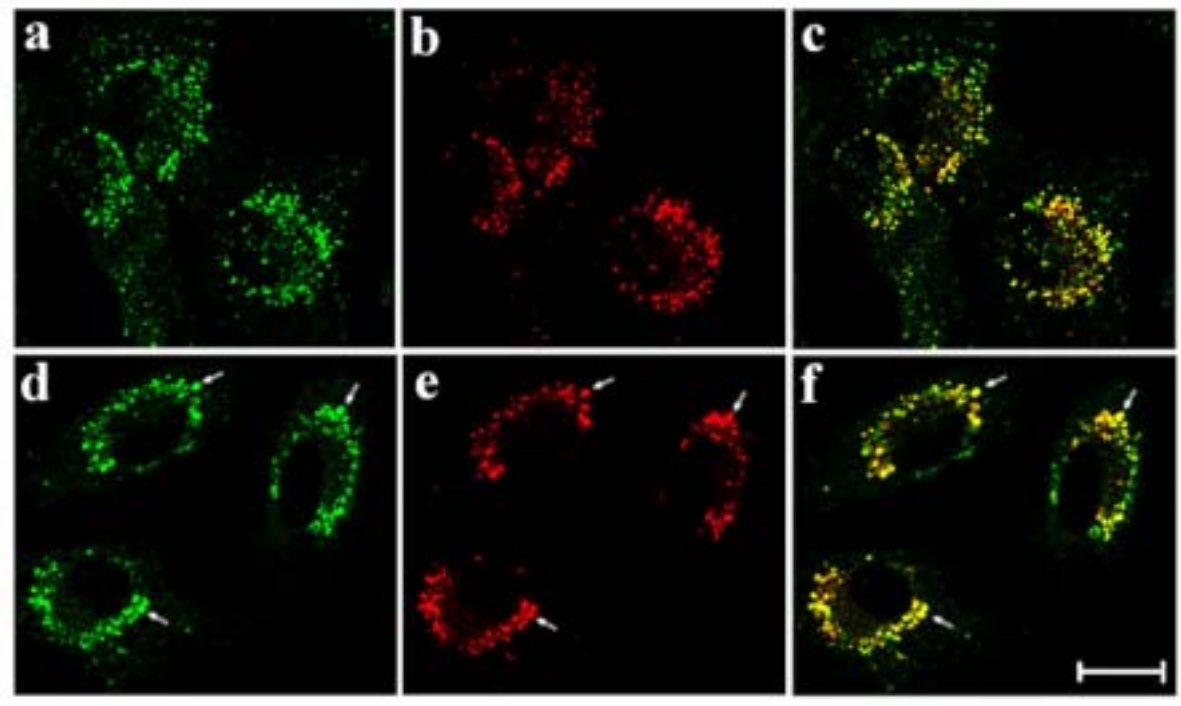


Figure 4. Co-localisation of annexin 6 and internalised LDL-DiI in ALLN treated cells. Confocal microscopy analysis of NRK cells labelled with anti-annexin 6 (a, d) after 2 hours DiI-LDL internalisation in control cells (DMSO) (b) or cells treated with ALLN (e). In c and f, show the co-

localisation. Arrows in d, e and f indicated the enlargement of annexin 6 structures containing LDL. Bar is 10 μm.

To find out whether this enlargement of endocytic structures was attributable to an inhibition of LDL degradation, we repeated the experiment, detailed in Fig. 4, but now using ^{125}I-LDL. Medium was collected after 2, 4, 6 and 8 hours DMSO/ALLN treatment and the amount of free ^{125}I in the supernatant after TCA-precipitation was measured. Fig. 5 shows the inhibition of ^{125}I-LDL degradation, at all time points, in cells treated with ALLN compared with control samples (DMSO).

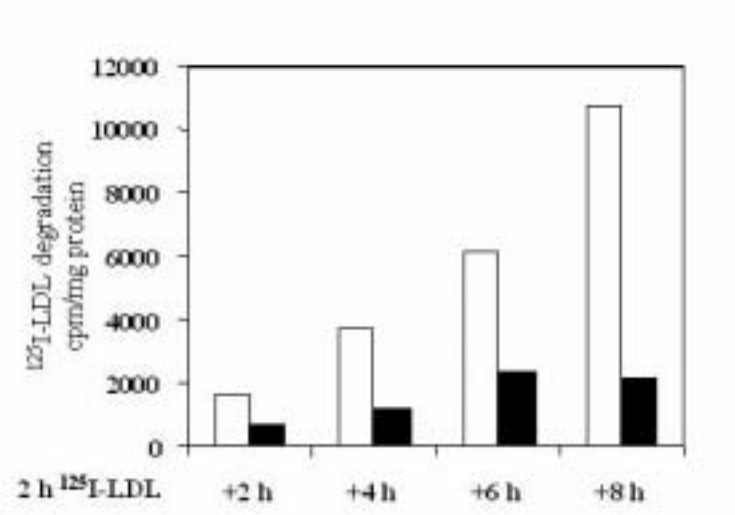


Figure 5. **Degradation assay in ^{125}I-LDL NRK cells treated with ALLN.** NRK cells were internalised with I^{125}-LDL for 2 hours and then chased for 2, 4, 6 or 8 hours in the presence of DMSO (open bars) or ALLN (black bars) and the degradation of LDL was compared. A significant inhibition of degradation can be observed in those samples treated with ALLN. Results are from 3 independent experiments (in triplicates) and values did not vary more than 10%.

Since it has been described that ALLN may also inhibit cathepsins B and L, we tested the effect of cathepsin inhibitor I using the same conditions. No morphological

changes in the pattern of labelling of the endocytic compartment were observed (not shown).

Annexin 6 is also involved in the trafficking and degradation of LDL in CHO cells

Since NRK cells showed low efficiency in transfection and the analysis of the expression and location of mutated annexin 6 (annexin $6_{1\text{-}175}$), in microinjected NRK cells was difficult, we have used CHO cells shown to have very low levels of endogenous annexin 6 (Grewal et al., 2000). Transiently transfected CHO cells with wild type annexin 6 or the mutated annexin $6_{1\text{-}175}$ were characterised. Cells were transiently co-transfected with LDL-R and annexin 6 wild type or the mutated anx$6_{1\text{-}175}$ and prepared for immunofluorescence, after the incubation of LDL-DiI for 1 hour. Fig. 6 shows the pattern of intracellular wild type annexin 6 (a) distributed throughout the cell but concentrated in structures underneath the plasma membrane (arrows); on the other hand, the mutated annexin $6_{1\text{-}175}$ (c) showed a more diffuse intracellular distribution, being more abundant in the perinuclear region (arrows). In both, internalised LDL-DiI was concentrated in endocytic structures at the perinuclear region (b, d).

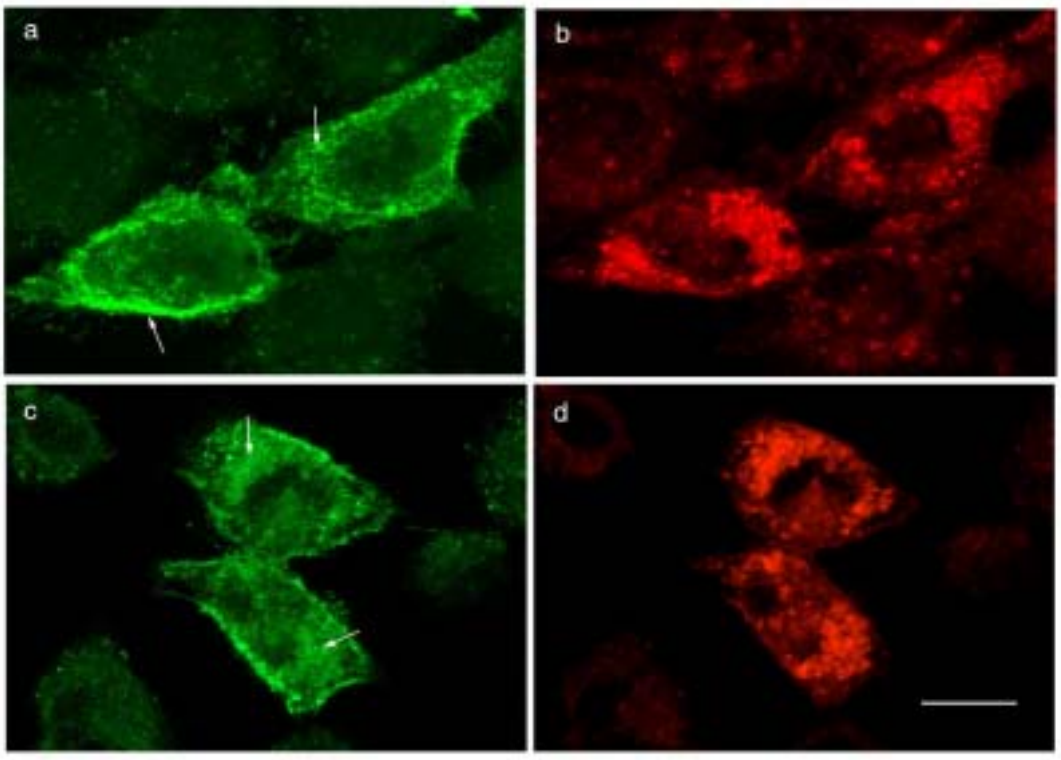


Figure 6. Immunofluorescence analysis of annexin 6 in CHO cells. Confocal immunofluorescence analysis of CHO cells transiently co-transfected with LDL-R plus annexin 6 wild type (a) or LDL-R and mutated annexin $6_{1\text{-}175}$ (c) (in green), using anti-annexin 6 antibody; co-transfected cells were incubated with LDL-DiI for 1 hour (in red); wild type CHO cells (b) and CHO-anx$6_{1\text{-}175}$ cells (d). Arrows indicate the preferential location of wild type or mutated annexin 6 in transfected CHO cells. Bar 5 μm.

In order to assess whether the mutated annexin $6_{1\text{-}175}$ was bound to membranes, cells were lysed and then lysates centrifuged. Pellets and supernatants were analysed by Western-blotting with anti-annexin 6 antibody. Fig. 7 shows that whereas the wild type annexin 6 was almost 100% membrane-bound, significant amounts of annexin $6_{1\text{-}175}$ (approx. 50%) was detected in the cytosolic fraction.

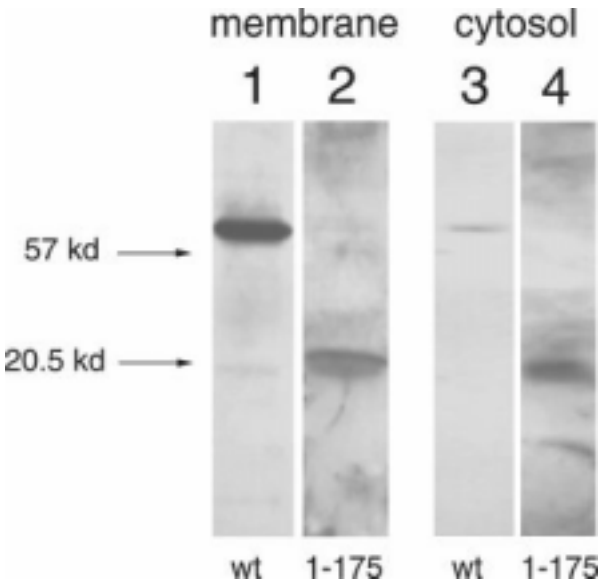


Figure 7. Western-blot analysis of annexin 6 distribution in transiently transfected CHO cells. CHO cells were transfected with wild type annexin 6 or the deletion mutant annexin $6_{1\text{-}175}$ as indicated. 24 hours after transfection cells were lysed and centrifuged for 15 min at 13,000 rpm. Pellets (membrane) and supernatants (cytosol) were cleared for (1 hour, 100,000 rpm). 20 μg of protein was loaded and the expression of annexin 6 was analysed by Western blotting using an affinity purified polyclonal anti-annexin 6 antibody.

In some experiments CHO cells were homogenised and the post-nuclear-supernatant fraction (PNS) subjected to cellular fractionation on sucrose gradients, as characterised in detail in Grewal et al. (2000) (Grewal et al., 2000). Fractions from the gradient were separated in PAGE-SDS and then analysed for the presence of spectrin and annexin 6. Spectrin was detected in the fractions (3 - 5 of the sucrose gradient) corresponding to endosomal markers and annexin 6 (not shown).

Finally, we used the CHO cells co-transfected with annexin 6, wild type or mutated form, and the LDL-R to analyse the uptake and the degradation of I^{125}-LDL. In Fig. 8 it is shown that there was a significant decreased degradation of I^{125}LDL (32%) in the cells expressing the mutated annexin 6 (LDL-R plus $anx6_{1-175}$), compared with LDL-R plus $anx6_{1-175}$. Data of uptake also showed that, in agreement with Kamal et al. (1998) (Kamal et al., 1998), there was a slightly diminution in the uptake in cells transfected with the mutated $anx6_{1-175}$, compared with $anx6_{wt}$.

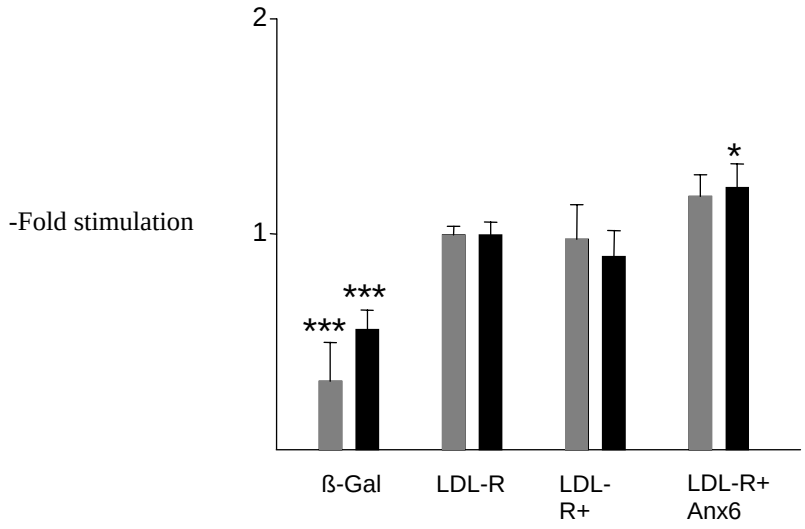


Figure 8. Uptake and degradation of I^{125}LDL in CHO transfected cells. 3 $x10^5$ CHO cells were transfected with β-galactosidase (β-gal), LDL-R, LDL-R + $anx6_{1-175}$ or with LDL-R + $anx6_{wt}$. The values for I^{125}LDL uptake (grey bars) and degradation (black bars) are represented with standard deviations. A reduction of 32% of LDL degradation in cells transfected with $anx6_{1-175}$ compared with to the $anx6_{wt}$ was observed. This is statistically significant for 5 independent experiments with triplicate samples (±

standard deviation). A student t-Test was performed: p = 0.14 for LDL-R and LDL-R+ anx6_{1-175}; p> 0.02 for LDL-R and LDL-R+ anx6 (*); p> 0.0004 for LDL-R and β-gal (***).

III.2.4. Discussion

In a recent paper by Anderson and coworkers a fascinating new mechanism for the regulation of receptor-mediated endocytosis was envisaged (Kamal et al., 1998). It is based on the interaction of spectrin with annexin 6, and the removal of spectrin, from the plasma membrane, mediated by the calcium-dependent cysteine protease, calpain. Upon annexin 6 binding to spectrin, calpain I cleaves spectrin and "opens" the actin cytoskeleton facilitating the endocytosis. It is assumed that annexin 6 was located at the clathrin-coated pits on the plasma membrane.

Using an antibody prepared by purification of annexin 6 from isolated rat liver endosomes (Jackle et al., 1994) we demonstrated a precise intracellular staining of endocytic structures, by confocal and electron microscopy, in hepatocytes, WIF-B and in NRK cells (Ortega et al., 1998), (Calvo et al., 2000), (Pons et al., 2000), in agreement with its proposed role in intracellular trafficking (Gerke and Moss, 1997), (Massey-Harroche et al., 1998), (De Matteis and Morrow, 1998), (Grewal et al., 2000).

In NRK cells annexin 6 was almost exclusively found associated with the pre-lysosomal compartment (Pons et al., 2000), This intracellular location does not rule out its presence at other sites such as the plasma membrane, and most probably reflects the various antibodies used, which may recognise different isoforms or conformations of annexin 6, or different cells analysed. Besides, annexins 1, 2, 4, 5 and 6 relocate in response to rises in intracellular calcium; particularly, annexin 6 was shown to move from the peri-nuclear region to a more homogeneous distribution on the plasma membrane, in human fibroblasts (Seemann et al., 1996), (Barwise and Walker, 1996), or to the sarcolemma to serve as a link of the cytoskeleton, in smooth muscle cells (Babiychuk and Draeger, 2000). In addition, we now have evidences that annexin 6 may undergo ligand-induced translocation (Grewal et al., 2000).

Thus, considering the priority intracellular location of annexin 6, we investigated whether a similar mechanism postulated for annexin 6 at the plasma membrane also occur between the endocytic compartment and lysosomes.

In this study we show the presence of spectrin in the annexin 6 intracellular compartment and demonstrate that both proteins interact. The annexin 6 compartment of NRK cells was loaded with LDL and then the transport from this pre-lysosomal compartment and the degradation of LDL was analysed. NRK cells were microinjected with anx6wt or a mutated anx$6_{1\text{-}175}$ pcDNAs, to assess the possible involvement of annexin 6–spectrin interactions in the trafficking of LDL to the lysosomal compartment. The microinjection of mutated annexin 6 showed an accumulation of LDL in larger endocytic structures which resemble those observed after ALLN (calpain I inhibitor) treatment. Alternatively in CHO cells, which showed very low levels of endogenous annexin 6, the LDL degradation in transient transfected annexin $6_{1\text{-}175}$ cells was significantly decreased, compared with cells expressing the wild type annexin 6.

It has been described that calpains are involved in the formation of coated vesicles and /or vesicle fusion to endosomes (Sato et al., 1995). These calcium-dependent proteases associate with coated vesicles and modulate the interactions of membranes and cytoskeleton (calpains degrade membrane-lining proteins that connect membrane proteins and cytoskeletal proteins) with a concomitant effect on the disorganisation of the lipid asymmetry of membranes, which is a necessary step for membrane fusion. The location of both, calpain and annexin 6 to membranes is Ca^{2+} -dependent, indicating that calpain might be involved in the budding through the modulation of annexin 6; also, it was shown that vesicle-bound annexin 6/calpain complexes were involved in the fusion of intracellular membranes (Sato et al., 1995). At least in NRK cells, at the steady state annexin 6 seems associated with spectrin, in the pre-lysosomal compartment however, changes of intracellular Ca^{2+} , which may be induced by ligand transport (Grewal et al., 2000) or from signal transduction up-stream events, may recruit calpain I to cleave spectrin and allowing the fusion with lysosomes.

From the results of this study we conclude that - although annexin 6 might be dispensable (see work in knockout mice by Hawkins et al. 1999) (Hawkins et al., 1999) for adult structure or development and/or even for those cellular aspects in which annexin 6 has been involved - in cells expressing annexin 6 it seems that it can be involved in the intracellular trafficking from the endocytic compartment to the lysosomes.

III.2.5. Resum capítol 2

Evidence for the involvement of annexin 6 in the trafficking between the endocytic compartment and lysosomes

Precedents

Encara que existeixen moltes discrepàncies en la funció de l'anx6, totes coincideixen en el denominador comú de mediar interaccions d'estructures intracel·lulars, ja siguin entre membranes o membrana-citoesquelet. Ja s'havia descrit la interacció de l'anx6 amb membres del citoesquelet com l'actina o l'espectrina.

Més recentment es va trobar l'anx6 en els sots de clatrina, on es va demostrar la importància de l'anx6 per la internalització de la vesícula de clatrina. Perquè la vesícula s'alliberi de la membrana i entri dins la cèl·lula és necessari que l'espectrina que recobreix la cara interna de la membrana plasmàtica es trenqui per la proteasa Calpaina I. Mitjançant assaigs in vitro van demostrar que l'anx6 havia d'interaccionar amb l'espectrina perquè tingués lloc el seu trencament i la subseqüent internalització de la vesícula. Un mutant de l'anx6 inhibia la internalització de la vesícula de clatrina i bloquejava l'entrada de LDL, tanmateix però, al cap d'una hora el sistema trobava una via independent d'anx6 i espectrina per internalitzar LDL.

OBJECTIUS

2. Col·localització d'anx6 i espectrina en els pre-lisosomes de cèl·lules NRK
3. Interacció de l'espectrina dels pre-lisosomes amb GST-anx6
4. Estudiar si la funció de l'anx6 i espectrina descrita a la membrana plasmàtica té lloc a nivell dels pre-lisosomes

Hipòtesi: l'espectrina es troba rodejant els organels per mantenir la seva estructura i perque es doni la fusió entre vesícules, l'anx6 unida a espectrina fa que la calpaïna trenqui l'espectrina, obrint aquesta xarxa de citoesquelet.

Resultats

Un primer indici per una funció conjunta de l'anx6 i espectrina va ser la col·localització d'aquests en la regió perinuclear. Mitjançant assaigs de pull down també vam poder demostrar que l'espectrina de fraccions aïllades d'un gradent de percoll corresponent als pre-lisosomes s'unia a la proteïna de fusió GST-anx6.

Degut a la baixa eficiència de transfecció de les cèl·lules NRK, vam microinjectar el cDNA de l'anx6 wt i de l'anx6$_{1\text{-}175}$ la qual li faltaven les 6 últimes repeticions de l'extrem carboxil. Després d'internalitzar LDL-DiI vam comprovar que en les cèl·lules microinjectades amb el mutant, la LDL es quedava acumulada en unes vesícules inflades perinuclears. Aquest efecte es va veure també després de tractar les cèl·lules amb l'inhibidor de la calpaina (ALLN), mostrant que aquestes vesícules plenes de LDL eren anx6 positives. Realitzant aquest mateix experiment amb LDL-^{125}I vam poder mesurar que aquesta acumulació de LDL en els pre-lisosomes es traduïa en una inhibició de la degradació.
També es van transfectar cèl·lules CHO amb el mutant l'anx6$_{1\text{-}175}$ i seguidament es va internalitzar LDL. En aquestes cèl·lules es va poder veure el mateix efecte morfològic i una disminució de la degradació respecte el control.

Això ens suggereix que l'anx6 té un paper important en la sortida de lligands dels pre-lisosomes per la seva posterior degradació, i sembla ser que podria actuar conjuntament amb l'espectrina.

III.3. Activation of Raf-1 and Ras is defective in annexin 6 overexpressing Chinese hamster ovary cells

III.3.1. Abstract

Annexin 6 is a Ca^{2+} -dependent phospholipid-binding protein involved in membrane trafficking mediating reversible interactions between membranes and cytoskeleton. Following changes in intracellular calcium annexin 6 translocates to the plasma membrane, where it may be involved in endocytosis or signal transduction. Pull-down experiments demonstrate the interaction of Raf-1 with recombinant rat annexin 6; this interaction is most likely to occur in the C-terminal region of annexin 6 since a truncated GST-annexin 6_{1-175} (lacking six of the eight repeats) fusion protein does not bind to Raf. Raf-annexin 6 interaction was shown to be independent of cell activation by epidermal growth factor (EGF) or phorbol esters (TPA). A stable CHO-anx6 cell line overexpressing annexin 6 was established to examine the function of annexin 6. In these cells, no increase of Ras-GTP levels, induced by EGF or TPA, were detected. In addition, the activity of Raf was completely inhibited, whereas the MAPK-P was unaffected. This study shows that annexin 6 overexpression inhibits the TPA or the EGF-induced Ras signaling pathway, possibly through its interaction with the GAP/PKC/Pyk2 protein complex.

III.3.2. Introduction

The annexins are a family of widely expressed calcium/phospholipid-binding proteins that are highly conserved (Gerke and Moss, 1997). In recent years, annexins 1, 2, 4, 6, 7 and 13b have been implicated in membrane trafficking (Gerke and Moss,

1997), (Gruenberg and Emans, 1993), (Donato and Russo-Marie, 1999), (Grewal et al., 2000), (Pons et al., 2000) and references therein).

Annexin 6 was first reported at the plasma membrane (Tagoe et al., 1994), (Weinman et al., 1994), (Bandorowicz et al., 1992) and later in vesicles of the endocytic compartment (Jackle et al., 1994), (Ortega et al., 1998), (Seemann et al., 1996), (Massey-Harroche et al., 1998), (Pol et al., 1997). More recently, annexins 2 and 6 have been described in isolated subcellular fractions enriched in caveolin, from endothelial cells (Schnitzer et al., 1995) as well as from rat liver (Pol et al., 1999). The presence of annexins and other components of the budding and fusion machinery (i.e. SNARE's) have been associated with the dynamic internalization of caveolae and their subsequent interaction with the endocytic compartment (Pol et al., 2000), (Pol et al., 2000). Although annexin 6 does not contain the well conserved predicted caveolin-binding motif, shown by other molecules reported to interact with caveolin (Okamoto et al., 1998), it may interact indirectly through proteins such as PKCα (Smart et al., 1994), Fyn (Wary et al., 1998), filamin (Langanger et al., 1984), dynamin (Henley et al., 1998), H-Ras (Song et al., 1996) or as shown in this study through Raf. Moreover, direct interaction of annexin 6 and the lipids of the cytoplasmic face of caveolae (e.g. phosphatidylserine) cannot be ruled out; indeed, annexin 6 and PKC are both calcium-dependent phospholipid binding proteins, and their association - in the caveolae - probably involves specific protein-protein interactions.

Recently, it was demonstrated that annexin 6 interacts directly with the C2 domain of key Ras regulatory p120-GTPase activating protein ($p120^{GAP}$) which forms part of a protein complex containing Fyn and Pyk2 (Chow et al., 2000). This was an additional indication that annexin 6 could be involved in the regulation of signal transduction, through the "bridging" of a protein complex formation with Fyn and Pyk2, two tyrosine kinases associated with the regulation of Ras (as well as with PKCα). The C2 domains function as Ca^{2+} sensors in signaling proteins and therefore it is tempting to speculate that annexin 6 may be linked with a Ca^{2+} mediated regulation of p21 Ras activity (Gawler, 1998).

In addition, 14-3-3 proteins recruit inactive Raf-1 to the membrane (Fu et al., 2000) and have an annexin-like region. Although Raf does not bind to the annexin-like domain, this does not rule out the possibility that annexin 6 modifies the interaction of 14-3-3 (Aitken, 1996).

Here we show that overexpression of annexin 6 in CHO cells decreases activated Ras (Ras-GTP) and thus inhibits Raf-1 activity. The possible role of annexin 6 in a complex formed by Raf-1 and components of various signaling pathways is discussed.

III.3.3. Results and discussion

Raf-1 is a Ser/Thr kinase that plays a crucial role in the signal transduction pathway initiated by growth factors or 6a PKC (Morrison and Cutler, 1997). Upon activation, Raf-1 is recruited to the plasma membrane or to the early/sorting endocytic compartment (Leevers et al., 1994), (Stokoe et al., 1994), (Pol et al., 1998) where is activated by Ras-GTP, by mechanisms that are poorly understood. Down stream events involve the phosphorylation of the kinase Mek, leading to stimulation of the mitogen-activated protein kinase (MAPK). Where and how Raf-1 is regulated remains to be established.

At least three independent lines of evidence implicate annexin 6 in signal transduction pathways: first, it interacts directly with the C2 domain of the key Ras regulatory protein p120-GTPase ($p120^{GAP}$) (Chow et al., 2000), (Davis et al., 1996). GAP (Ras-GAP, $p120^{GAP}$) is a GTPase activating protein proposed to accelerate the inhibition of Ras. Second, an increase of intracellular Ca^{2+} activates Ras and MAPK, and in A431 cells EGF-dependent calcium influx was inhibited by annexin 6 (Fleet et al., 1999). And third, annexin 6 may play an important role in the recruitment (assembly) of protein complexes at the caveolae, including the constituents of the signal transduction machinery and calcium signaling.

Thus, to analyze the possible interaction of annexin 6 with the signal transduction machinery, extracts of CHO cells were incubated with immobilized

recombinant GST-annexin 6 (GST-anx6) protein. In agreement with data previously reported we identified GAP (also PKC, by immunoprecipitation with anti-annexin 6 in NRK cells, data not shown) in the GST-annexin 6 binding fraction. In addition, immunoblot analysis of pull down fractions showed the interaction of Raf-1 protein with GST-anx6. In contrast Western blots of GST-anx6 bound proteins did not reveal Mek or MAPK-P proteins (Fig. 1a). Besides, in a preliminary attempt to identify the domain of annexin 6 protein interacting with Raf-1, GST pull down experiments were performed with a truncated mutant fusion protein (lacking six of the eight repeats) GST-anx6$_{1\text{-}175}$ (Fig. 1b). In these experiments Raf-1 did not bind to GST-anx6$_{1\text{-}175}$, suggesting that the binding of Raf-1 to annexin 6 was not mediated through the N-terminal region of annexin 6. Binding of GAP to annexin 6 was located at the interlobular region of annexin 6, where the two lobules can rotate 90º, thus changing its conformation (Chow and Gawler, 1999).

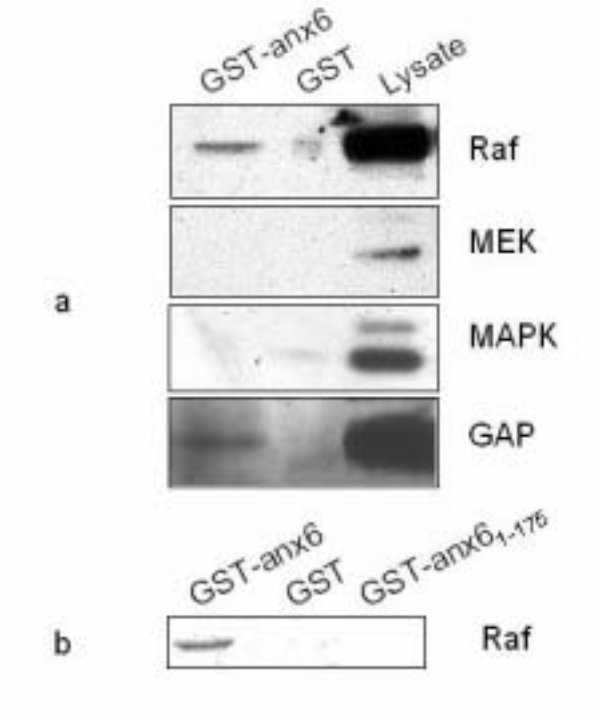


Figure 1. Interaction of annexin 6 with Raf in CHO cells.

Pull down experiments using GST-annexin 6 were performed to study the interactions of annexin 6 with proteins of the signal transduction machinery. CHO cell extracts were incubated with GST-annexin 6 for 2 hours at 4ºC. Bound proteins were collected, separated by polyacrylamide gel electrophoresis and analyzed by Western-blotting, using antibodies to Raf, Mek, MAPK and GAP (a). Controls with GST alone showed no binding. In (b) a mutated form of annexin 6 (annexin $6_{1\text{-}175}$, which lacks six of the eight repeats) did not bind to Raf.

To find out whether the interaction of Raf-1 with annexin 6 was dependent upon cellular activation, two approaches were followed: first, CHO cells were serum-deprived for 6 hours and then stimulated with EGF. Although these cells showed very low levels of EGF-R, the activation of MAPK (MAPK-P) was observed and it reached a peak after 2 minutes of EGF treatment, decreasing after 5 minutes (not shown). Extracts of CHO cells were then assayed for the binding to GST or GST-anx6 and subsequent Western blotting. In these experiments, interaction of GST-anx6 with Raf-1, and GAP, was independent of activation by EGF; the binding was also independent of the presence of Ca^{2+} or phosphatidylserine (not shown). Second, in CHO cells treated with TPA, which is more efficient than EGF in the MAPK-P pathway, there were no differences in the binding of Raf-1 to GST-anx6, compared to control untreated or EGF treated cells (Fig. 2).

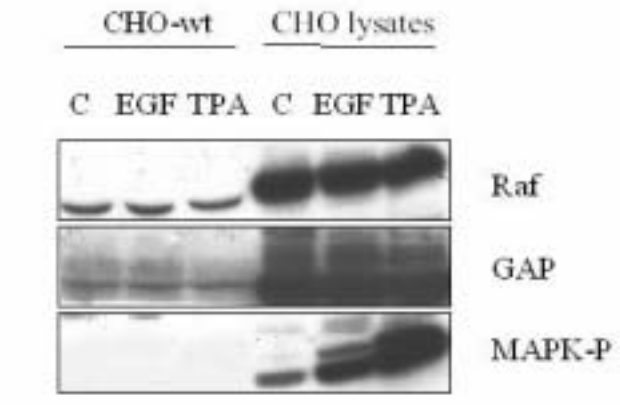


Figure 2. Interaction of annexin 6 with Raf is independent of cell activation.
CHO cells were deprived of serum for 24 hours and stimulated with EGF (10 ng/ml) or TPA (100 nM). Cell extracts were incubated with GST-anx6 as described above (pull down assay) and the bound material or lysates analyzed by Western-blotting with Raf, GAP or MAPK antibodies.

CHO cells have very low levels of annexin 6 (Grewal et al., 2000) so to understand the functional role of the annexin 6-Raf-1 complexes, we performed the same experiments but in CHO cells stably transfected with annexin 6 cDNA (CHO-anx6). The overexpression of annexin 6 had no effect on the MAPK-P activation by EGF or TPA, or on the interaction with Raf-1 or GAP (not shown). However, when the activity of Raf-1 was examined in CHO-anx6 cells, stimulated with EGF or with TPA, Raf-1 activity decreased, compared with CHO-wt, where Raf-1 activity increased 12% and 60% after EGF and TPA treatment, respectively (Fig. 3).

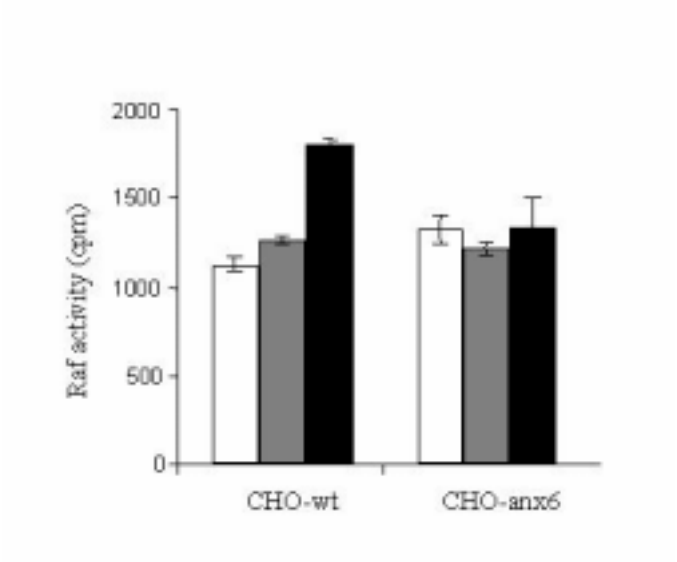


Figure 3. Activation of Raf in CHO wild type and CHO-anx6 cells.

Cells were deprived of serum for 24 hours and stimulated with EGF (10 ng/ml) or TPA (100 nM). The activity of Raf was measured from CHO cell extracts by immunoprecipitation with the anti-Raf antibody and kinase cascade assay, with GSTMek, GSTERK2 and MBP as substrates. The results show a representative experiment assayed in triplicate (n =3 ± SD). Open bars, control; grey bars, EGF; black bars, TPA.

Thus, to determine whether the decreased Raf-1 activity in CHO-anx6 cells was caused by interaction of annexin 6 and Raf-1 or due to an upstream event, the amount of Ras-GTP was monitored by its association with the GST-Ras-binding-domain (RBD) fusion protein. Cell extracts of non-activated or EGF/ TPA activated CHO cells and CHO-anx6 overexpressing cells were incubated with immobilized GST-RBD, and bound Ras-GTP protein was detected by Western-blotting. Fig. 4a shows the comparison of the total amount of Ras-GTP (anti-Pan-Ras antibody) in pull-down fractions from wild type or CHO-anx6 cells. There is significantly less Ras-GTP in EGF or TPA stimulated CHO-anx6 cells. However, MAPK-P was not affected, indicating that the activation MAPK was produced by a different mechanism (Fig.4b).

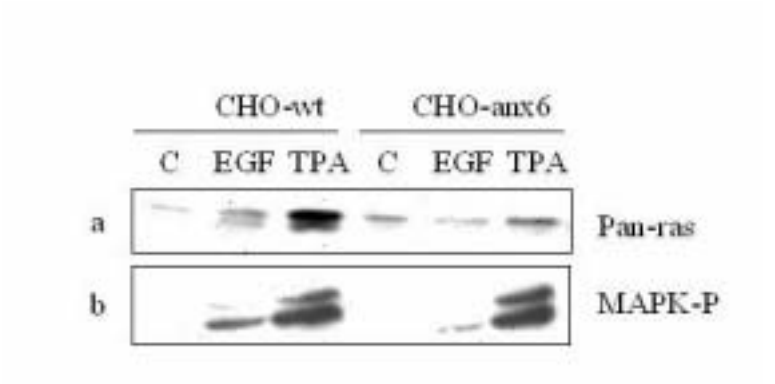


Figure 4. Amount of Ras-GTP in CHO wild type and in annexin 6 overexpressing cells (CHO-anx6). The expression of Ras-GTP in CHO wt or CHO-anx6 cells was assessed by GST-RBD pull-down and analyzed by Western-blotting with anti Pan-Ras antibody (a) in control, non-stimulated cells, or

activated with EGF or TPA. In both, the amount of Ras-GTP was decreased in the annexin 6 overexpressing cells. However, in lysates from the same experiment MAPK-P was not affected (b).

In comparison with recent studies in which a Ras mutant blocks activation of MAPK after receptor tyrosine kinase stimulation but not in response to activation by PKC (de Vries-Smits et al., 1992), we have demonstrated that in wild-type CHO cells Ras was activated after stimulation by either EGF or TPA in wild-type CHO cells. This is in agreement with recent studies showing the requirement of Ras-GTP-Raf-1 complexes to activate Raf-1 in response to TPA (Marais et al., 1998). Ras activation by phorbol esters is also required for MAPK phosphorylation in cardiac myocytes, but not in cardiac fibroblasts (Montessuit and Thorburn, 1999). Interestingly in CHO-anx6 cells Ras was activated after EGF or TPA treatment; however in both cell types MAPK was similarly activated. Thus, whereas in wild type CHO cells the MAP-K pathway can be activated 6a Ras/Raf or by a different (independent) pathway, in CHO-anx6 cells only this Ras/Raf-independent pathway seems to be operative, for the activation of MAPK.
Since GTP is more abundant than GDP and Ras molecules are filled spontaneously by GTP, the decreased Ras-GTP levels in CHO-anx6 cells suggest a possible annexin 6-mediated inhibition of Ras-GDP dissociation. Furthermore, the GTPase-activating protein (GAP) speeds up Ras deactivation binding to the Ras-GTP complex. Overexpression of annexin 6 could therefore facilitate/contribute to the inhibition of Ras by the GAP-Ras-GTP assembly.

Although it seems that the annexin-like region of 14-3-3 proteins was not involved in the interaction with Raf, overexpression of annexin 6 may displace 14-3-3 from Raf and therefore inhibit the translocation, mediated by 14-3-3 proteins, of inactive Raf from the cytosol to the Ras-GTP complex at the plasma membrane or to the endosomal compartment. In fact, it has been shown that the annexin-like structure of 14-3-3 is adjacent to a phosphorylation site, which inhibits Raf binding (Dubois et al., 1997). In rat liver we demonstrated that activated Raf-1 was present in early

endosomes (Davis et al., 1996) and signal transduction machinery was recruited, in response to EGF, from the caveolae to the endosomes where MAPK is eventually activated.

Finally, annexin 6 has been found in caveolae, clathrin coated pits and in endosomes but its location may depend on Ca^{2+} mobilization in the cell (Babiychuk and Draeger, 2000). In caveolae, interactions of proteins with PKCα, dynamin, Raf-1 or other kinases, may influence the regulation of signal transduction events downstream. Caveolin, through its scaffolding domain, interacts with molecules of the signal transduction machinery and annexins (e.g. annexin 2 and 6) might be involved in the assembly of those complexes and with the lipid bilayer.

Thus, as occurred (with TPA) for annexin 5 (Sato et al., 2000), in annexin 6 overexpressing cells the increase in Ras/Raf signaling caused by EGF or TPA was suppressed, and this inhibition occurs at the level or upstream of Ras-GTP; although, a downstream effect, directly on Raf activity, cannot be ruled out. The fact that MAPK activity was not affected indicates an alternative (independent) signaling pathway.

III.3.4. Resum capítol 3

Activation of Raf-1 and Ras is defective in annexin 6 overexpressing Chinese hamster ovary cells

Precedents

La implicació de l'anx6 en la transducció de senyal ja va ser suggerida quan es va mostrar que la seva interacció amb la PKC. Encara que aquesta unió era molt específica no va ser possible demostrar que l'anx6 era un substrat de la quinasa. Ja havia estat descrit que l'extrem amino de l'anx6 contenia dominis fosforilables per PKC i kinases tirosina però no s'ha pogut veure mai la seva fosforilació en residus tirosina. Més recentment es va demostrar la interacció directa de l'anx6 amb la proteïna activadora de GTPases GAP. Aquesta proteïna té una paper important en l'acceleració de l'activitat GTPasa de Ras-GTP, passant-lo a la forma inactiva Ras-GDP. Aquests mateixos autors van descriure que anx6 i GAP formaven part d'un complex amb dos quinases més associades amb la regulació de Ras, Fyn i Pyk2. En aquest estudi s'han utilitzat les cèl·lules CHO (CHOwt) degut a la seva baixa expressió d'anx6 i una línia cel·lular de CHO transfectades establement amb anx6 (CHOanx6). En aquestes cèl·lules, l'anx6 es troba majoritàriament a la membrana plasmàtica i en els endosomes primerencs, i és per això que vam estudiar el possible paper de l'anx6 en aquesta localització.

Objectius

Recerca d'interaccions de l'anx6 amb proteïnes de la via de senyalització.

2. Caracterització de la interacció Raf-1-Anx6.
3. Estudi de la via de les MAPK en cèl·lules que sobreexpressen l'anx6.

Resultats

Mitjançant assaigs de pull down amb la proteïna de fusió GST-anx6, vam trobar que Raf-1 interaccionava específicament amb GST-anx6 i no amb GST. A més aquesta interacció no es donava, com normalment ha estat acceptat, en l'extrem amino i era independent de Ca^{2+} i de PS. La interacció de Raf-1 per anx6 no variava després d'estimular les cèl·lules amb EGF o TPA, indicant que s'hi uneix tant la forma activa con la inactiva de Raf-1.

Per distingir si aquesta interacció afectava a la via de senyalització, vam fer estudis comparatius de les cèl·lules CHOwt i CHOanx6, estudiant l'activació de Raf. Els resultats ens mostraven una completa inhibició de Raf-1 en les cèl·lules que sobre-expressaven anx6, tant després d'estimular amb EGF com TPA, fent-se la inhibició més evident en les cèl·lules activades amb TPA degut al baix nivell de receptor d'EGF que tenen aquestes cèl·lules.

Una primera conclusió va ser que anx6 interaccionava amb Raf-1 inhibint la seva activació, però va ser ràpidament descartada al constatar que, upstream de Raf, concretament Ras, també estava inhibit. Extractes de cèl·lules CHO i CHOanx6 activades amb EGF i TPA, van ser incubats amb columnes de GST-RBD, on s'unia només la forma activa de Ras (Ras-GTP). Els nivells de Ras-GTP en les CHOwt augmentava lleugerament després d'una estimulació amb EGF i més fortament amb TPA. Però les CHOanx6 mostraven uns nivells basals de Ras-GTP, similars a les cèl·lules no activades.

Curiosament, els nivells de MAPK activada eren similars en els dos tipus cel·lulars, suggerint que en les cèl·lules CHOwt, MAPK pot ser activada via Ras/Raf-1 però en les CHOanx6 s'activa per una via totalment independent de Ras/Raf-1.

IV. Discussió

Les annexines han estat conegudes tradicionalment a través de la seva capacitat d'unir fosfolípids regulada per Ca^{2+}. Tanmateix però, no està clar perquè una família tan complex mostra unes característiques bioquímiques individuals tan similars. Això es podria explicar amb el fet que diferents annexines actuen en diferents membranes diana exercint activitats biològiques diferents.

L'extrem conservat de la proteïna media unions a membrana regulades per Ca^{2+} mentre que el domini divergent N-terminal està especialitzat en la funció de cada annexina a través d'interaccions amb lligands específics. Aquestes interaccions membrana-membrana i membrana-citoesquelet, impliquen l'organització de microdominis de membrana per que tingui lloc el tràfic de vesícules al llarg de les vies exocítiques i endocítiques.

IV.1. L'anx6 al final de la via endocítica en cèl·lules NRK

La localització intracel·lular de l'anx6 ha estat molt polèmica degut a la utilització de diferents tipus cel·lulars i anticossos. S'ha trobat a la membrana plasmàtica en hepatòcits (Tagoe et al., 1994) i associada amb el citoesquelet d'actina en fibroblasts (Hosoya et al., 1992). A més a més, molts dels estudis realitzats amb l'anx6 coincideixen amb la seva distribució endosomal en diferents tipus cel·lulars com els hepatòcits (Jackle et al., 1994), concretament en els endosomes de la regió peri-canalicular (Ortega et al., 1998) i també s'ha descrit en fagosomes tardans de macròfags aïllats (Desjardins et al., 1994).

La descripció estàndar de la via endocítica es divideix en endosomes primerencs, tardans i lisosomes. Malgrat tot els endosomes que participen en les darreres etapes

de l'endocitosi han estat classificats de maneres diferents. En el cas de les cèl·lules NRK, es diferèncien un tipus de lisosomes primerencs que anomenem pre-lisosomes. S'ha suggerit que els pre-lisosomes provenen de la fusió directa d'endosomes tardans i lisosomes (Bright et al., 1997) (Mullock et al., 1998). Aquest model concorda amb un compartiment pre-lisosomal (PLC) descrit amb anterioritat el qual és ric en proteases com els lisosomes però al contrari que aquest té una densitat baixa, i no presenta el M6PR característic dels endosomes tardans (Griffiths et al., 1990).

Es aquest estudi vam determinar la localització d'anx6 en cèl·lules NRK per microscòpia confocal, electrònica i per fraccionament cel·lular. La col·localització d'anx6 amb el marcador de lisosomes lgp120 ens va portar a realitzar experiments de pols i caça. El lligant internalitzat arribava al compartiment anx6 a partir de 45 minuts de caça, suggerint-nos una localització de l'anx6 en els últims estadis de l'endocitosi.

Per discernir el marcatge prelisosomal o lisosomal del marcatge dels endosomes tardans, es van realitzar experiments amb drogues com la cloroquina, brefeldina A i wortmannina, que modificaven la morfologia d'organels com els endosomes, separant així més les distribucions del M6PR i lgp120. Es va poder observar que després dels tractaments esmentats, la coincidència d'anx6 amb lgp120 era més forta que en condicions normals, excloent així una possible localització d'anx6 en els endosomes tardans.

També vam excloure la localització d'anx6 en endosomes primerencs degut a que el seu marcatge no coincidia amb cap estadi de l'internalització i reciclatge de la transferrina, encara que els experiments de fraccionament cel·lular ens indicaven una localització no exclusiva en vesícules lgp120 positives. Aproximadament un 50% de l'anx6 es trobava en un pic de densitat més lleugera que el dels pre-lisosomes i lisosomes, en el qual s'hi trobaven membrana plasmàtica, Golgi i endosomes primerencs. Aquesta aparent contradicció es podria explicar pels següents factors:

- La seva detecció per microscopia confocal va ser realitzada amb un anticòs produït contra una fracció d'endosomes crus de fetge de rata, on s'hi trobava unida l'anx6. Per tant, aquest anticòs ens reconeixia per immunocitoquímica tan sols l'anx6 dels endosomes i no l'anx6 d'altres parts de la cèl·lula. És per això que una part de l'anx6 podria estar associada, com en molts altres tipus cel·lulars, amb membrana plasmàtica, la qual és reconeguda per aquest anticòs només en els gradents sota condicions desnaturalitzants per Western blot.
- Per una altra banda, l'anx6 deu estar associada també a pre-lisosomes relativament lleugers que migren en el pic menys dens.

La microscopia electrònica també ens va revelar una localització de l'anx6 més pre-lisosomal que lisosomal, degut a la baixa col·localització de lgp120 i anx6 en lisosomes electro-densos madurs.

La localització de l'anx6 en els pre-lisosomes de cèl·lules NRK recolza estudis anteriors realitzats en macròfags. Encara que les annexines 1-6 es troben en els fagosomes, només l'anx4 i 6 es troben enriquides en els fagosomes tardans, la composició en dels quals és molt semblant a la dels lisosomes (Desjardins et al., 1994).

IV.2. Possible paper de l'annexina 6 en els pre-lisosomes

La localització majoritària d'anx6 en la membrana dels pre-lisosomes de cèl·lules NRK, ens fa pensar que podria ser un element important en la fusió de pre-lisosomes amb lisosomes, facilitant la seva interacció.

IV.2.1. Associació d'anx6 amb espectrina en els pre-lisosomes

L'espectrina és el component majoritari del citoesquelet associat a membrana implicat en el manteniment de la integritat estructural de les membranes. L'espectrina s'ha trobat també en el Golgi (Beck et al., 1994), vesícules citoplasmàtiques (De Matteis and Morrow, 1998) i macropinosomes (Xu et al., 2000).

L'espectrina és molt abundant en eritròcits i és aquí on es va demostrar que perquè es donés la fusió d'una vesícula exocítica amb la membrana plasmàtica o s'internalitzés una vesícula, era necessari el trencament del citoesquelet de la membrana (Hayashi et al., 1992). El trencament de l'espectrina és portat a terme per la proteasa cisteínica Calpaina I. Per això les calpaïnes han estat implicades en endocitosi i exocitosi, processos que són estimulats pel Ca^{2+} i involucren la fusió de membranes intracel·lulars. Recentment es van trobar les calpaïnes associades a la membrana de vesícules de clatrina i la seva activació implicava la digestió de components de les vesícules com la cadena lleugera de la clatrina, tubulina i adaptines (Sato et al., 1995). Aquests autors suggereixen que les calpaïnes estan implicades en la formació de les vesícules de clatrina i en la fusió d'aquestes als endosomes.

Altres autors van proposar a l'esquelet d'espectrina de la membrana com a complex de redistribució de proteïnes de la membrana (Beck and Nelson, 1996), basant-se en el seu ensamblatge en forma de xarxa bidimensional que segresta proteïnes específiques de membrana, i en la seva distribució en dominis especialitzats de membrana.

En aquest estudi, mitjançant experiments d'immunofluorescència i pull down en cèl·lules NRK, vam poder demostrar que l'anx6 està estretament unida a espectrina. La interacció d'anx6 i espectrina també va ser mostrada en la membrana plasmàtica,

concloent que aquesta unió inhibia la capacitat de l'espectrina per unir actina (Watanabe et al., 1994).

IV.2.2. Implicació d'annexina 6 en la degradació de LDL

Estudis anteriors van suggerir un paper conjunt de l'anx6 i espectrina en la membrana plasmàtica. Mitjançant assaigs in vitro, es va poder demostrar que l'anx6 era un element important pel trencament de l'espectrina de la membrana plasmàtica, i la posterior internalització de la vesícula de clatrina (Kamal et al., 1998). Aquests autors proposaven la intervenció de la unió de l'anx6 a espectrina facilitant la proteolisi de l'espectrina per la calpaïna I.

Donat que anx6 i espectrina col·localitzen i interaccionen en els pre-lisosomes, ens vam centrar en la idea de Kamal i col·laboradors però enfocat a nivell dels últims compartiments de la via endocítica, de tal manera que perquè es doni la fusió entre els pre-lisosomes i lisosomes, s'ha de trencar l'esquelet d'espectrina que envolta les vesícules.

Mitjançant assaigs in vitro, vam demostrar que l'anx6 dels pre-lisosomes podria actuar de forma similar que a la membrana plasmàtica, ja que la sobre-expressió d'un mutant de l'anx6 ($anx6_{1-175}$), el qual li falta les 6 darreres repeticions, provocava l'acumulació de LDL en els pre-lisosomes, efecte similar donat a les cèl·lules tractades amb l'inhibidor de la calpaïna I (ALLN). Aquesta acumulació de LDL es traduïa en una inhibició de la seva degradació. Això ens suggeria que les cèl·lules que sobre-expressaven el mutant, l'LDL no era capaç d'arribar als lisosomes per degradar-se i que l'anx6 podria tenir un paper important en l'eliminació de l'espectrina d'aquests compartiments, necessaria per la fusió amb altres vesícules.

La idea de que existeix un citoesquelet d'espectrina associat a organels cel·lulars ja va ser suggerit en l'any 1996, on es proposa la interacció del complex de dinactina

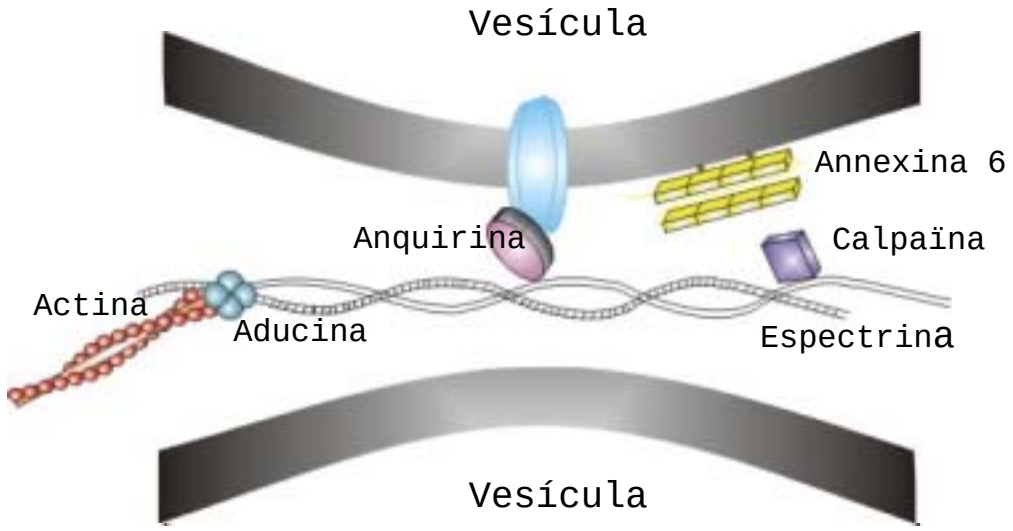
amb el citoesquelet d'espectrina del Golgi (Holleran et al., 1996). La dinactina es sembla a un filament curt d'actina (Schafer et al., 1994) i s'uneix a la dineïna (molècules motor associades als microtúbuls) (Karki and Holzbaur, 1995). Estudis genètics revelen que la dinactina i la dineïna citoplasmàtiques estan implicades en processos cel·lulars similars. La dineïna ha estat implicada en el manteniment de la localització perinuclear del Golgi (Corthesy-Theulaz et al., 1992), en el transport de lisosomes i vesícules endocítiques (Lin and Collins, 1992) i en el transport retrògrad d'organels al llarg de l'axó (Schnapp and Reese, 1989). D'aquesta manera es creu que la dineïna i la dinactina unida al citoesquelet d'espectrina podrien executar un paper en el tràfic i classificació d'organels.

Altres experiments de transport intracel·lular de LDL en cèl·lules CHO co-transfectades amb el receptor del LDL (LDLR) i l'anx6, demostraven un increment en la internalització i degradació de LDL. La internalització de transferrina també es veia augmentada quan es sobre-expressava l'anx6, però no així el seu reciclatge. En cèl·lules CHO transfectades de forma estable amb anx6 es va demostrar que quan s'internalitzava LDL, el patró de l'anx6 canviava distribuint-se juntament amb el LDL cap als pre-lisosomes (Grewal et al., 2000). Això ens indica que l'anx6, a part d'estimular el transport de lligands, té la capacitat de respondre a senyals d'aquests lligands i concentrar-se en endosomes amb direcció als prelisosomes.

Però el tràfic en les darreres etapes de la via endocítica no s'ha estudiat tant com en el sistema endosomal primarenc. Es creu que l'abocament del contingut dels endosomes en els lisosomes succeeix per una fusió directa entre els primers i lisosomes preexistents, per maduració dels endosomes tardans o bé per vesícules transportadores.

L'espectrina conté un domini SH3 (Src homology 3) el qual és present en moltes altres proteïnes. S'ha vist que tant la sinapsina com la dinamina són molt bons lligands pels dominis SH3 de la proteïna adaptadora Grb2 i al mateix temps són dos proteïnes que s'assòcien també a l'anx6. Grb2 és important per la transmisió del

senyal entre el EGFR i SOS, per tant, l'anx6 podria participar, a més a més de com a element estructural conjuntament amb el citoesquelet, en la via de senyalització formant complexes amb proteïnes estructurals i de senyalitzacio.



Esquema de fusió de vesícules

IV.3. Implicació d'anx6 en la transducció del senyal

La transducció del senyal comença a nivell de la membrana plasmàtica on els receptors s'activen. Per exemple està acceptat que el EGFR es troba en les caveoles i quan s'activa és dirigit a les invaginacions de clatrina per ser internalitzat. El primer destí de l'EGF i EGFR és la seva degradació en lisosomes però s'està proposant que l'endocitosi de receptors activats pot atenuar el seu senyal físicament o pot transportar el receptor al lloc apropiat per interaccionar amb molècules de

senyalització downstream i amplificar la resposta cel·lular als factors de creixement. De fet es van trobar proteïnes de senyalització com Shc, Grb2 i Sos que interaccionaven amb el EGFR internalitzat (Di Guglielmo et al., 1994).

Una altre implicació de la senyalització amb l'endocitosi és l'associació de GAP amb components endocítics i amb l'EGFR internalitzat després de la seva activació (Wang et al., 1996). Aquests autors suggereixen que existeix un altre nivell de regulació de proteïnes de senyalització degut a la seva associació amb endosomes. Posteriorment es va demostrar la interacció de GAP amb Rab5 activant la seva activitat GTPasa, suggerint una possible regulació de la transducció del senyal de la fusió d'endosomes depenent de Rab5 via GAP (Liu and Li, 1998).

El cas contrari en que és la senyalització la que controla l'endocitosi ja s'havia presentat on proteïnes d'unió a GTP es requerien pel transport vesicular. Més tard es va demostrar que les proteïnes G heterotrimèriques regulaven la fusió entre endosomes senyalant que la transducció del senyal podria participar en la regulació del tràfic endocític (Colombo et al., 1994).

La sinapsina, que interacciona amb l'anx6, s'ha trobat que també interacciona amb proteïnes de la via de senyalització (Grb2 i cSrc) i amb components del citoesquelet com l'actina i l'espectrina. La fosforilació de la sinapsina impedia la seva unió a l'actina i a les vesícules sinàptiques, complicant les interaccions proteïna-proteïna a nivell de la terminal nerviosa (Lin et al., 1993).

IV.3.1. Annexina 6 s'uneix a Raf-1

Els resultats presentats en el capítol 3 dels resultats ens van fer pensar sobre la implicació de l'anx6 en la transducció del senyal, demostrant in vitro que Raf-1 s'uneix a anx6 i que aquesta unió té lloc en l'extrem C-terminal de l'anx6. A més a

més, la unió d'aquestes dos proteïnes no variava segons l'activació de la via de transducció de senyal que implica l'activació de Raf-1.

Ja s'havia demostrat la unió d'anx6 amb la proteïna activadora de GTPases GAP (Davis et al., 1996), la qual té la funció d'accelerar la inactivació de Ras. El lloc d'unió de GAP a anx6 es troba en l'extrem C-terminal, concretament en els 27 aa localitzats en la regió connectora dels 2 lòbuls de l'anx6. Aquesta regió es caracteritza per permetre una rotació de 90° dels lòbuls entre sí quan es troba en solució. Quan es troba unida a una monocapa de lípids, ambdós lòbuls es sitúen en el mateix pla.

IV.3.2. L'activitat Raf-1 és bloquejada per l'expressió d'annexina 6

Per determinar si aquesta unió Raf-anx6 tenia algun efecte sobre Raf-1, vam comparar l'activitat de Raf-1 en cèl·lules CHO control (amb molt poca quantitat d'anx6) i cèl·lules que estaven transfectades establement amb anx6. En les cèl·lules que sobre-expressaven anx6, no es va poder detectar activitat Raf-1 després d'activar les cèl·lules tan amb EGF com amb TPA, mentre que en les cèl·lules control hi havia un increment de l'activitat de Raf-1 d'un 12% i 60% després d'estimular les cèl·lules amb EGF i TPA respectivament.

Està descrit que Raf-1 té afinitat per les proteïnes 14-3-3 i també per PS (Campbell et al., 1998). Si s'impedeix aquesta unió, Raf-1 perdia la capacitat d' unir Ras i PS de tal manera que no es podia translocar a la membrana. Curiosament, les annexines són conegudes per la seva afinitat a fosfolípids, i l'anx6 s'uneix majoritàriament a PS.

També s'ha descrit que les proteïnes 14-3-3 tenen una seqüència molt similar a l'extrem C-terminal de les annexines. L'anx6, pel fet de tenir una duplicació en aquest extrem, presenta 2 regions similars. S'ha vist que aquesta seqüència era responsable de la funció d'activació de l'exocitosi que tenen l'anx2 i les proteïnes 14-3-3 (Roth et al., 1993).

L'existència del domini estructural annexina-like va ser confirmat per estudis de cristalografia i es trobava en una localització que tenia una elevada probabilitat d'estar implicada en les interaccions de les 14-3-3 amb proteïnes de senyalització (Xiao et al., 1995). No obstant, s'ha descrit que la unió de Raf-1 a 14-3-3 es donava en els 65 residus de l'extrem C-terminal (Luo et al., 1995) (Suen et al., 1995) que no contenien el domini annexina-like. Això no exclou la possibilitat de que aquest domini que tenen en comú les annexines i les proteïnes 14-3-3 estigui implicat en l'estabilització de la interacció de Raf-1 amb 14-3-3, i que la sobre-expressió d'anx6 interfereixi amb aquesta unió, inhibint així la translocació a la membrana i la posterior activació de Raf-1.

Altres estudis demostren que les proteïnes 14-3-3 també tenen una seqüència molt similar al domini del pseudo-substrat de PKC, la qual és la responsable de la inhibició de la seva activitat (Wheeler-Jones et al., 1996). També s'ha demostrat que tan els pèptids derivats del speudo-substrat de PKC com de la regió annexina-like, no afectaven la redistribució de PKC que es donava després d'activar les cèl·lules amb PMA. Altres estudis mostraven que membres de la família de les annexines podien actuar com a receptors de PKC activada i que un pèptid sintètic basat en l'extrem C-terminal de les annexines, o sigui en la regió annexina-like de les 14-3-3, inhibia la unió de PKC als seus receptors intracel·lulars (Mochly-Rosen et al., 1991). Posteriorment, mitjançant assaigs in vitro, va ser demostrat que l'anx5 era un inhibidor específic de la fosforilació portada a terme per la PKC d'anx1 i del substracte de la quinasa de la cadena lleugera de la miosina (Schlaepfer et al., 1992). Aquesta inhibició no implicava un segrest per part de l'anx5 de fosfolípids que requereix l'activació de la PKC, del tal manera que suggeria una inhibició mitjançant una interacció directa d'anx5 i PKC.

IV.3.3. L'annexina 6 inhibeix l'activació de Ras

La idea de que anx6 actués bloquejant la unió de 14-3-3 a Raf-1, inhibint així la seva activació per Ras, va ser descartada quan vam mesurar l'activació de Ras. Comparant la quantitat de Ras-GTP en cèl·lules CHO control (CHOwt) i cèl·lules CHO que sobre-expressaven anx6 (CHOanx6) després d'activar-les amb EGF i TPA, vam trobar nivells significativament més baixos en len CHOanx6 que en les cèl·lules CHOwt, suggerint-nos que anx6 actua upstream o a nivell de l'activació de Ras o inhibint la seva dissociació de GDP.

Sorprenentment, encara que Ras i Raf-1 es trobaven inactives en cèl·lules que sobreexpressaven anx6, el nivell de MAPK fosforilada no variava en comparació amb les cèl·lules CHO wt. Sembla ser que MAPK és activada bé via Ras-Raf o bé independentment de Ras i Raf. Així doncs en les cèl·lules CHOwt existirien els dos possibles tipus de vies d'activació de la MAPK i en les cèl·lules que sobreexpressen anx6, MAPK s'activaria per una via independent de Ras-Raf degut al seu estat inactiu en aquestes cèl·lules.

Aquesta inhibició de la transducció del senyal a nivell de Ras o Raf en les cèl·lules que sobre-expressaven anx6, ens va fer recordar uns estudis dels anys 80, en els quals demostraven la inhibició de la PLA_2 per les annexines. La PLA_2 és un substracte de MAPK, la qual regula la dinàmica del citoesquelet d'actina a través de la producció de derivats de l'àcid araquidònic (Lin et al., 1993); (Peppelenbosch et al., 1995; Peppelenbosch et al., 1993).

Estudis en els quals s'utilitzava la proteïna Ras mutada, demostraven que aquesta bloquejava l'activació de MAPK després d'una estimulació dels receptors tirosina quinasa però no en resposta a l'activació per PKC (de Vries-Smits et al., 1992), indicant que els senyals que provenen de la PKC no segueixen la via Ras. Els nostres resultats en cèl·lules CHOwt, en canvi, demostren que l'activació de MAPK per PKC es dóna també via Ras-Raf, i que aquesta activació és més forta quan

s'estimulen les cèl·lules amb TPA que amb EGF. En canvi en les cèl·lules CHOanx6, la MAPK tan sols s'activa per una via totalment independent de Ras-Raf, l'activitat dels quals és quasi nula. Estudis recents mostren també un requeriment de complexes formats per Ras-GTP i Raf-1 per activar Raf-1 tan en resposta a EGF com TPA (Marais et al., 1998). L'activació de Ras per ésters de forbol també era necessària per la fosforilació de MAPK en miòcits cardíacs, però no en els fibroblasts de cor (Montessuit and Thorburn, 1999).

IV.4. Possibles papers de l'annexina 6 en la transducció de senyal

La interacció d'anx6 amb GAP ens podria donar una possible explicació per la inhibició de Ras. GAP té l'habilitat d'unir fosfofípids de vesícules i membranes cel·lulars dependentment de Ca^{2+} i anx6 (Chow et al., 1999). La interacció d'anx6-GAP podria jugar un paper important en la capacitat de GAP per translocar i associar-se a la membrana en resposta a un increment del Ca^{2+} intracel·lular i així poder inactivar Ras, desplaçant l'equilibri cap a Ras-GDP. Ja es coneix la capacitat de l'anx6 de translocar-se de la seva localització citoplasmàtica a la membrana plasmàtica després d'un increment de Ca^{2+} (Barwise and Walker, 1996) (Babiychuk and Draeger, 2000). De la mateixa manera, GAP es transloca del citoplasma a la membrana plasmàtica per tenir accés a Ras. La inhibició de l'anx6 de la via de transducció del senyal via GAP podria donar una possible explicació a l'activitat supressora de tumors que tenia l'anx6 en les cèl·lules A431.

Per una altra banda, s'ha descrit la formació d'un complex format per anx6, GAP, Pick i Fyn2, i a més a més, anx6 s'uneix a PKC. Totes aquestes proteïnes són importants per la transducció del senyal i l'associació d'anx6 amb tals complexes podria influir en les seves funcions.

IV.5. Les annexines com a organitzadors de membrana

La descripció d'anx2, 5 i 6 en microdominis o rafts de la membrana plasmàtica en cèl·lules de la musculatura llisa i la seva elevada sensibilitat al Ca^{2+} (Babiychuk and Draeger, 2000) ens porten una nova configuració dels components units a membrana, amb conseqüències potencialment importants per processos de senyalització i de flux de Ca^{2+}.

Els rafts estan implicats en diferents processos de tràfic de membrana i de senyalització. Aquests rafts poden associar-se per formar complexes més grans i estables o moure's a invaginacions de membrana anomenades caveoles (Parton and Simons, 1995).

Aquests rafts estan enriquits en colesterol i esfingolípids (Parton and Simons, 1995) i actuen com a plataformes per concentrar receptors i components de la via de transducció de senyal. Estan associats amb proteïnes ancorades a glicosil fosfatidilinositol en la superfície cel·lular i amb membres de la família de les kinases src en la cara citoplasmàtica. Aquesta localització serveix per incrementar la concentració o estabilitat dels complexes afectant els nivells de senyalització (Kholodenko et al., 2000). S'ha postulat que la presència d'annexines 2, 5 i 6 en aquests rafts podrien regular l'ensamblatge dels rafts dependentment de Ca^{2+} (Babiychuk and Draeger, 2000). Aquests autors concretament van demostrar que l'anx2 agregava preparacions de rafts dependentment de Ca^{2+} i suggereixen que la senyalització depenent de rafts podria estar relacionada amb la transducció del senyal a través d'anx2.

Un exemple seria el complex format per l'anx6, GAP, la quinasa de la família src Fyn i Pyck2 (Chow et al., 2000). L'anx6 sembla que actua com a proteïna pont per la

formació del complex i potser també amb la membrana plasmàtica. Es sap que Fyn, Pyck2 i GAP estan associades amb la regulació de Ras, per això es pensa que el paper d'aquest complex ha d'estar íntimament unit a la regulació de l'activitat de Ras mediada per Ca^{2+}. La localització d'aquest complex podria estar situat a les caveoles on s'han trobat aquestes proteïnes a més a més de Raf-1, H-Ras i PKC.

S'ha demostrat que les caveoles són un centre de senyalització, però encara que siguin regions enriquides en proteïnes de senyalització, no es sap si aquestes estructures coordinen processos de senyalització espaialment o si controlen l'activitat d'aquests complexes per sí mateixes. Les proteïnes de senyalització que es troben en les caveoles es troben inactives i s'ha descrit a la caveolina com a regulador negatiu de la transducció del senyal. En canvi, en els endosomes primerencs s'ha vist que moltes d'aquestes proteines es trobaven activades .

IV.6. Relevància de l'expressió d'annexina 6

L'anx6 no s'expressa normalment en cèl·lules A431 però si es transfecten establement amb anx6, s'ha vist que es redueix la proliferació (Theobald et al., 1994). Altres estudis realitzats en miòcits aïllats de ratolins transgènics que sobre-expressen anx6, revelaven una concentració de Ca^{2+} menor i un pic de Ca^{2+} més atenuat després d'estimular les cèl·lules (Gunteski-Hamblin et al., 1996). D'acord amb aquestes obsevacions, l'anx6 també s'assòcia a organels segrestadors de Ca^{2+} i modula en certes neurones les conductàncies de Ca^{2+} i K^+ (Hazarika et al., 1991a), (Naciff et al., 1996).

El creixement de les cèl·lules tumorals A431 és depenent d'EGF (Kitagawa et al., 1995), (Tosi et al., 1995). Una resposta immediata de les cèl·lules A431 enfront una estimulació amb EGF és la mobilització de Ca^{2+}. Es va demostrar que, en cèl·lules

A431 que sobre-expressaven anx6, l'entrada de Ca^{2+} mediada per receptor en resposta a l'estimulació amb EGF era inhibida (Fleet et al., 1999). A més a més aquests autors van observar que només tenia aquesta propietat la forma de més alt pes molecular de l'anx6, generada per un processament diferent de l'exó 21 del gen de l'anx6. Sembla ser que l'anx6 pot modular la taxa de creixement cel·lular regulant l'entrada de Ca^{2+} a través d'una via sensible a factors de creixement. Per això, la capacitat de l'anx6 per actuar com a inhibidor de l'entrada de Ca^{2+}, podria ser responsable de la taxa tan baixa de proliferació d'aquestes cèl·lules.

De la mateixa manera, aquesta inhibició de l'entrada de Ca^{2+} per l'anx6 després de l'estimulació de les cèl·lules, podria ser la responsable per la incapacitat que tenen les cèl·lules CHO que sobre-expressen l'anx6 d'activar Ras i Raf-1. Tanmateix però, aquestes cèl·lules són viables degut a que el senyal és transmès activant MAPK independentment de Ras i Raf-1.

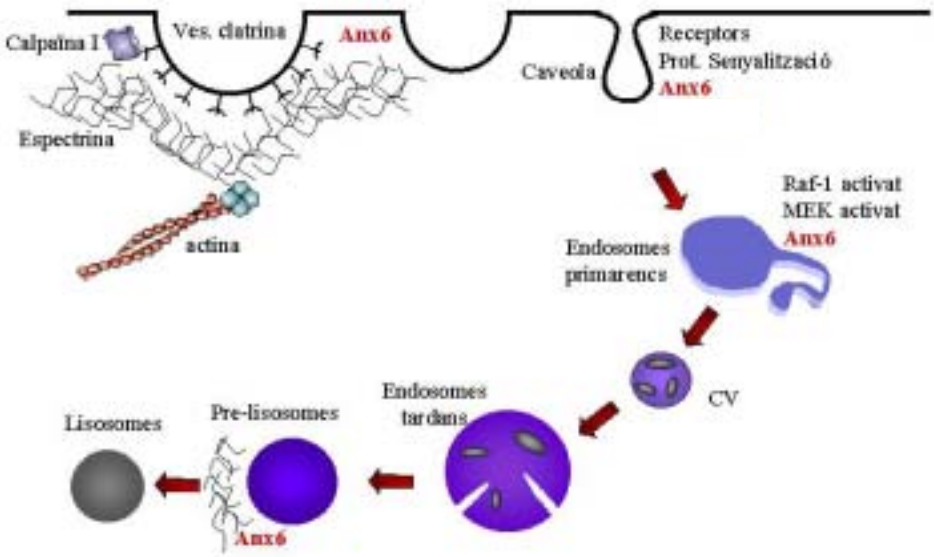
Malgrat tot, l'endocitosi tenia lloc independentment d'anx6 en les cèl·lules A431 (Smythe et al., 1994) i el gen de l'anx6 sembla no ser essencial per la viabilitat de ratolins knock outs per l'anx6 (Bandorowicz-Pikula et al., 1997).

També es va trobar que l'anx6 localitzada a la membrana plasmàtica dels macròfags es movia cap al citosol en estrès oxidatiu. Aquesta dissociació de la proteïna correlacionava amb un increment en Ca^{2+} lliure i una reducció d'ATP citosòlic (Lin and Cantiello, 1993). Aquests autors proposen que la dissociació de la proteïna de la membrana, permetria l'activació de la PLA_2 i, juntament amb l'increment de Ca^{2+}, alteraria la fagocitosi d'aquests macròfags.

Els nostres resultats suggereixen per una banda que l'anx6 forma un complex amb Raf i que, per una altra banda, després de l'estimulació de les cèl·lules amb EGF o TPA, l'anx6 bloqueja l'activació de Ras i Raf, ja sigui inhibint l'entrada de Ca^{2+} o bé retenint el complex GAP/Pyck/Fyn2/PKC en microdominis incapaços d'internalitzar-se.

La gran capacitat de les annexines d'unir Ca^{2+} i la seva prioritària localització en la cara interna de la membrana plasmàtica, ha suggerit un paper en la transducció de senyal. Tanmateix s'han identificat les annexines com a mediadors d'interaccions entre membranes i citoesquelet, com a inhibidors de l'activitat de la fosfolipasa A_2 i de la PKC, reguladors del fluxe d'ions, i com a reguladors de l'exocitosi, endocitosi i internalització de les vesícules de clatrina. Per tant queda molta investigació per davant per entendre les diferents funcions que té l'anx6 en els diferents teixits i per arribar a comprendre la importància que té la duplicació de les quatre repeticions de l'extrem C-terminal de l'annexina 6.

Els resultats d'aquest estudi ens relacionen l'anx6 en el transport de lligands dels prelisosomes als lisosomes i en la transducció del senyal. En el primer cas l'anx6 es troba en la membrana dels prelisosomes de cèl·lules NRK juntament amb l'espectrina que envolta els compartiments. La nostra hipòtesi és que anx6 estimula la calpaïna I per que obri l'espectrina i permeti així la fusió dels prelisosomes amb els lisosomes. En el segon resultat obtingut en cèl·lules CHO, s'ha descrit l'anx6 en la membrana plasmàtica i endosomes primerencs. Ja s'ha demostrat la interacció de l'anx6 amb diferents proteïnes de la transducció del senyal, els quals s'han trobat tant en caveoles a membrana plasmàtica com en endosomes primerencs. La funció de l'anx6 en aquests complexes podria ser estructural unint-los a membranes, o adaptadora, apropant proteïnes que han de ser activades entre si. El denominador comú d'aquests dos resultats és, com indica el seu propi nom, la capacitat d'annexionar diferents estructures o proteïnes amb membranes.



Diferents localitzacions de l'anx6 en les cèl·lules NRK i CHO

Conclusions

CONCLUSIONS

1 – En el present estudi s'ha indentificat per immunocitoquímica l'anx6 en la membrana dels prelisosomes de cèl·lules NRK. En canvi en cèl·lules polaritzades WIF-B només un 50% de l'anx6 s'ha trobat en aquest compartiment.

2 – L'anx6 interacciona amb l'espectrina associada als prelisosomes.

3 – La sobre-expressió de l'anx6$_{1\text{-}175}$ en les cèl·lules NRK provoca una acumulació de LDL en el compartiment prelisosomal similar a la que provoca la inhibició de la degradació de LDL després d'un tractament amb l'inhibidor de la calpaïna I.

4 – La sobre-expressió de l'anx6$_{1\text{-}175}$ en les cèl·lules CHO indueix la inhibició de la degradació de LDL.

5 – Mitjançant tècniques de pull down hem pogut demostrar la interacció d'anx6 amb Raf-1, independent de Ca^{2+} i fosfatidilcolina.

6 – La sobre-expressió d'anx6 en cèl·lules CHO disminueix la formació de Ras-GTP i inhibeix l'activació de Raf-1, mantenint intactes els nivell de MAPK activada, suggerint-nos que la seva activació en aquestes cèl·lules és independent de la via Ras/Raf-1.

V. Bibliografia

Bibliografia

Advani, R.J., Yang, B., Prekeris, R., Lee, K.C., Klumperman, J., and Scheller, R.H. (1999). VAMP-7 mediates vesicular transport from endosomes to lysosomes. J.Cell Biol *146*, 765-776.

Ahn, S., Maudsley, S., Luttrell, L.M., Lefkowitz, R.J., and Daaka, Y. (1999). Src-mediated tyrosine phosphorylation of dynamin is required for beta2-adrenergic receptor internalization and mitogen-activated protein kinase signaling. J Biol Chem *274*, 1185-1188.

Aitken, A. 14-3-3 and its possible role in co-ordinating multiple signaling pathways. Trends Cell Biol , 341-347. 1996.

Ali, S.M., Geisow, M.J., and Burgoyne, R.D. (1989). A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. Nature *340*, 313-315.

Ando, Y., Imamura, S., Owada, M.K., Kakunaga, T., and Kannagi, R. (1989). Cross-linking of lipocortin I and enhancement of its Ca2+ sensitivity by tissue transglutaminase. Biochem Biophys Res Commun *163*, 944-951.

Ando, Y., Imamura, S., Hong, Y.M., Owada, M.K., Kakunaga, T., and Kannagi, R. (1989). Enhancement of calcium sensitivity of lipocortin I in phospholipid binding induced by limited proteolysis and phosphorylation at the amino terminus as analyzed by phospholipid affinity column chromatography. J Biol Chem *264*, 6948-6955.

Aniento, F., Emans, N., Griffiths, G., and Gruenberg, J. (1993). Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. J Cell Biol *123*, 1373-1387.

Apodaca, G., Enrich, C., and Mostov, K.E. (1994). The calmodulin antagonist, W-13, alters transcytosis, recycling, and the morphology of the endocytic pathway in Madin-Darby canine kidney cells. J Biol Chem *269*, 19005-19013.

Apodaca, G., Enrich, C., and Mostov, K.E. (1994). The calmodulin antagonist, W-13, alters transcytosis, recycling, and the morphology of the endocytic pathway in Madin-Darby canine kidney cells. J.Biol Chem. *269*, 19005-19013.

Apodaca, G., Katz, L.A., and Mostov, K.E. (1994). Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. J.Cell Biol *125*, 67-86.

Babiychuk, E.B., Palstra, R.J., Schaller, J., Kampfer, U., and Draeger, A. (1999). Annexin VI participates in the formation of a reversible, membrane-cytoskeleton complex in smooth muscle cells. J Biol Chem *274*, 35191-35195.

Babiychuk, E.B. and Draeger, A. (2000). Annexins in cell membrane dynamics. Ca(2+)-regulated association of lipid microdomains. J Cell Biol *150*, 1113-1124.

Bandorowicz-Pikula, J., Wrzosek, A., Makowski, P., and Pikula, S. (1997). The relationship between the binding of ATP and calcium to annexin IV. Effect of nucleotide on the calcium-dependent interaction of annexin with phosphatidylserine. Mol Membr Biol *14*, 179-186.

Bandorowicz-Pikula, J. and Pikula, S. (1998). Annexins and ATP in membrane traffic: a comparison with membrane fusion machinery. Acta Biochim Pol *45*, 721-733.

Bandorowicz-Pikula, J. and Pikula, S. (1998). How do annexins, soluble calcium-binding proteins, form membrane ion channels?. Postepy Biochem *44*, 237-244.

Bandorowicz-Pikula, J. and Pikula, S. (1998). Modulation of annexin VI--driven aggregation of phosphatidylserine liposomes by ATP. Biochimie *80*, 613-620.

Bandorowicz-Pikula, J., Danieluk, M., Wrzosek, A., Bus, R., Buchet, R., and Pikula, S. (1999). Annexin VI: an intracellular target for ATP. Acta Biochim Pol *46*, 801-812.

Bandorowicz, J., Pikula, S., and Sobota, A. (1992). Annexins IV (p32) and VI (p68) interact with erythrocyte membrane in a calcium-dependent manner. Biochim Biophys Acta *1105*, 201-206.

Barr, V.A. and Hubbard, A.L. (1993). Newly synthesized hepatocyte plasma membrane proteins are transported in transcytotic vesicles in the bile duct-ligated rat. Gastroenterology *105*, 554-571.

Barr, V.A., Scott, L.J., and Hubbard, A.L. (1995). Immunoadsorption of hepatic vesicles carrying newly synthesized dipeptidyl peptidase IV and polymeric IgA receptor. J.Biol Chem. *270*, 27834-27844.

Barroso, M. and Sztul, E.S. (1994). Basolateral to apical transcytosis in polarized cells is indirect and involves BFA and trimeric G protein sensitive passage through the apical endosome. J Cell Biol *124*, 83-100.

Barwise, J.L. and Walker, J.H. (1996). Annexins II, IV, V and VI relocate in response to rises in intracellular calcium in human foreskin fibroblasts. J Cell Sci *109 (Pt 1)*, 247-255.

Beck, K.A., Buchanan, J.A., Malhotra, V., and Nelson, W.J. (1994). Golgi spectrin: identification of an erythroid beta-spectrin homolog associated with the Golgi complex. J Cell Biol *127*, 707-723.

Beck, K.A. and Nelson, W.J. (1996). The spectrin-based membrane skeleton as a membrane protein-sorting machine. Am J Physiol *270*, C1263-C1270

Benevolensky, D., Belikova, Y., Mohammadzadeh, R., Trouve, P., Marotte, F., Russo-Marie, F., Samuel, J.L., and Charlemagne, D. (2000). Expression and localization of the annexins II, V, and VI in myocardium from patients with end-stage heart failure. Lab Invest *80*, 123-133.

Benz, J., Bergner, A., Hofmann, A., Demange, P., Gottig, P., Liemann, S., Huber, R., and Voges, D. (1996). The structure of recombinant human annexin VI in crystals and membrane-bound. J Mol Biol *260*, 638-643.

Benz, J. and Hofmann, A. (1997). Annexins: from structure to function. Biol Chem *378*, 177-183.

Bianchi, R., Giambanco, I., Ceccarelli, P., Pula, G., and Donato, R. (1992). Membrane-bound annexin V isoforms (CaBP33 and CaBP37) and annexin VI in bovine tissues behave like integral membrane proteins. FEBS Lett *296*, 158-162.

Bianchi, R., Giambanco, I., and Donato, R. (1993). S-100 protein, but not calmodulin, binds to the glial fibrillary acidic protein and inhibits its polymerization in a Ca(2+)-dependent manner. J Biol Chem *268*, 12669-12674.

Bright, N.A., Reaves, B.J., Mullock, B.M., and Luzio, J.P. (1997). Dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. J Cell Sci *110 (Pt 17)*, 2027-2040.

Brown, W.J., Goodhouse, J., and Farquhar, M.G. (1986). Mannose-6-phosphate receptors for lysosomal enzymes cycle between the Golgi complex and endosomes. J Cell Biol *103*, 1235-1247.

Brownawell, A.M. and Creutz, C.E. (1996). Calcium-dependent binding of the plasma protein apolipoprotein A-I to two members of the annexin family. Biochemistry *35*, 6839-6845.

Brownawell, A.M. and Creutz, C.E. (1997). Calcium-dependent binding of sorcin to the N-terminal domain of synexin (annexin VII). J Biol Chem *272*, 22182-22190.

Burgoyne, R.D., Morgan, A., Robinson, I., Pender, N., and Cheek, T.R. (1993). Exocytosis in adrenal chromaffin cells. J Anat *183 (Pt 2)*, 309-314.

Callaghan, J., Nixon, S., Bucci, C., Toh, B.H., and Stenmark, H. (1999). Direct interaction of EEA1 with Rab5b . Eur.J.Biochem. *265*, 361-366.

Calvo, M., Pol, A., Lu, A., Ortega, D., Pons, M., Blasi, J., and Enrich, C. (2000). Cellubrevin is present in the basolateral endocytic compartment of hepatocytes and follows the transcytotic pathway after IgA internalization . J Biol Chem 2000. Mar.17;275 (11.): 7910-7917.

Campbell, S.L., Khosravi-Far, R., Rossman, K.L., Clark, G.J., and Der, C.J. (1998). Increasing complexity of Ras signaling. Oncogene *17*, 1395-1413.

Cassio, D., Hamon-Benais, C., Guerin, M., and Lecoq, O. (1991). Hybrid cell lines constitute a potential reservoir of polarized cells: isolation and study of highly differentiated hepatoma-derived hybrid cells able to form functional bile canaliculi in vitro. J Cell Biol *115*, 1397-1408.

Ceresa, B.P., Kao, A.W., Santeler, S.R., and Pessin, J.E. (1998). Inhibition of clathrin-mediated endocytosis selectively attenuates specific insulin receptor signal transduction pathways. Mol Cell Biol *18*, 3862-3870.

Ceresa, B.P. and Pessin, J.E. (1998). Insulin regulation of the Ras activation/inactivation cycle. Mol Cell Biochem *182*, 23-29.

Chan, H.C., Kaetzel, M.A., Gotter, A.L., Dedman, J.R., and Nelson, D.J. (1994). Annexin IV inhibits calmodulin-dependent protein kinase II-activated chloride conductance. A novel mechanism for ion channel regulation. J Biol Chem *269*, 32464-32468.

Chasserot-Golaz, S., Vitale, N., Sagot, I., Delouche, B., Dirrig, S., Pradel, L.A., Henry, J.P., Aunis, D., and Bader, M.F. (1996). Annexin II in exocytosis: catecholamine secretion requires the translocation of p36 to the subplasmalemmal region in chromaffin cells. J Cell Biol *133*, 1217-1236.

Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K., and Zerial, M. (1990). Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell *62*, 317-329.

Cheney, R.E. and Willard, M.B. (1989). Characterization of the interaction between calpactin I and fodrin (non-erythroid spectrin). J Biol Chem *264*, 18068-18075.

Chow, A. and Gawler, D. (1999). Mapping the site of interaction between annexin VI and the p120GAP C2 domain. FEBS Lett *460*, 166-172.

Chow, A., Davis, A.J., and Gawler, D.J. (1999). Investigating the role played by protein-lipid and protein-protein interactions in the membrane association of the p120GAP CaLB domain. Cell Signal *11*, 443-451.

Chow, A., Davis, A.J., and Gawler, D.J. (2000). Identification of a novel protein complex containing annexin VI, Fyn, Pyk2, and the p120(GAP) C2 domain. FEBS Lett *469*, 88-92.

Christmas, P., Callaway, J., Fallon, J., Jones, J., and Haigler, H.T. (1991). Selective secretion of annexin 1, a protein without a signal sequence, by the human prostate gland. J Biol Chem *266*, 2499-2507.

Chung, C.Y. and Erickson, H.P. (1994). Cell surface annexin II is a high affinity receptor for the alternatively spliced segment of tenascin-C. J Cell Biol *126*, 539-548.

Clark, D.M., Moss, S.E., Wright, N.A., and Crumpton, M.J. (1991). Expression of annexin VI (p68, 67 kDa-calelectrin) in normal human tissues: evidence for developmental regulation in B- and T-lymphocytes. Histochemistry *96*, 405-412.

Colombo, M.I., Mayorga, L.S., Nishimoto, I., Ross, E.M., and Stahl, P.D. (1994). Gs regulation of endosome fusion suggests a role for signal transduction pathways in endocytosis. J Biol Chem *269*, 14919-14923.

Corthesy-Theulaz, I., Pauloin, A., and Rfeffer, S.R. (1992). Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. J Cell Biol *118*, 1333-1345.

Courtoy, PJ. Dissection of endosomes. In "Intracellular trafficking of proteins". C.J.Steer and Hanover, editors.Cambridge University Press, Cambridge , 103-156. 1991.

Creutz, C.E., Zaks, W.J., Hamman, H.C., Crane, S., Martin, W.H., Gould, K.L., Oddie, K.M., and Parsons, S.J. (1987). Identification of chromaffin granule-binding proteins. Relationship of the chromobindins to calelectrin, synhibin, and the tyrosine kinase substrates p35 and p36. J Biol Chem *262*, 1860-1868.

Creutz, C.E., Moss, S., Edwardson, J.M., Hide, I., and Gomperts, B. (1992). Differential recognition of secretory vesicles by annexins. European Molecular Biology Organization Course "Advanced Techniques for Studying Secretion". Biochem Biophys Res Commun *184*, 347-352.

Crompton, M.R., Moss, S.E., and Crumpton, M.J. (1988). Diversity in the lipocortin/calpactin family. Cell *55*, 1-3.

Crompton, M.R., Owens, R.J., Totty, N.F., Moss, S.E., Waterfield, M.D., and Crumpton, M.J. (1988). Primary structure of the human, membrane-associated Ca2+-binding protein p68 a novel member of a protein family. EMBO J. *7*, 21-27.

Croxtall, J.D. and Flower, R.J. (1992). Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. Proc Natl Acad Sci U S A *89*, 3571-3575.

Croxtall, J.D., Waheed, S., Choudhury, Q., Anand, R., and Flower, R.J. (1993). N-terminal peptide fragments of lipocortin-1 inhibit A549 cell growth and block EGF-induced stimulation of proliferation. Int J Cancer *54*, 153-158.

Croxtall, J.D., Choudhury, Q., Tokumoto, H., and Flower, R.J. (1995). Lipocortin-1 and the control of arachidonic acid release in cell signalling. Glucocorticoids (changed from glucorticoids) inhibit G protein-dependent activation of cPLA2 activity. Biochem Pharmacol *50*, 465-474.

Damke, H., Baba, T., Warnock, D.E., and Schmid, S.L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J Cell Biol *127*, 915-934.

Damke, H., Bouterfa, H., and Braulke, T. (1994). Effects of insulin-like growth factor II on the generation of inositol trisphosphate, diacylglycerol and cAMP in human fibroblasts. Mol Cell Endocrinol *99*, R25-R29

Davis, A.J., Butt, J.T., Walker, J.H., Moss, S.E., and Gawler, D.J. (1996). The Ca2+-dependent lipid binding domain of P120GAP mediates protein-protein interactions with Ca2+-dependent membrane-binding proteins. Evidence for a direct interaction between annexin VI and P120GAP. J Biol Chem *271*, 24333-24336.

Davis, P.D., Elliott, L.H., Harris, W., Hill, C.H., Hurst, S.A., Keech, E., Kumar, M.K., Lawton, G., Nixon, J.S., and Wilkinson, S.E. (1992). Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. J Med Chem *35*, 994-1001.

De Matteis, M.A. and Morrow, J.S. (1998). The role of ankyrin and spectrin in membrane transport and domain formation. Curr Opin Cell Biol *10*, 542-549.

de Rooij, J. and Bos, J.L. (1997). Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene *14*, 623-625.

de Vries-Smits, A.M., Burgering, B.M., Leevers, S.J., Marshall, C.J., and Bos, J.L. (1992). Involvement of p21ras in activation of extracellular signal-regulated kinase 2. Nature *357*, 602-604.

Demange, P., Voges, D., Benz, J., Liemann, S., Gottig, P., Berendes, R., Burger, A., and Huber, R. (1994). Annexin V: the key to understanding ion selectivity and voltage regulation?. Trends.Biochem Sci *19*, 272-276.

Desjardins, M., Celis, J.E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G., and Huber, L.A. (1994). Molecular characterization of phagosomes. J Biol Chem *269*, 32194-32200.

Devarajan, P., Stabach, P.R., Mann, A.S., Ardito, T., Kashgarian, M., and Morrow, J.S. (1996). Identification of a small cytoplasmic ankyrin (AnkG119) in the kidney and

muscle that binds beta I sigma spectrin and associates with the Golgi apparatus. J Cell Biol *133*, 819-830.

Di Guglielmo, G, Baass, P, Ou, W, Posner, B, and Bergeron, J. Compartmentalization of SHC, GRB2 and mSOS and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. EMBO J 13, 4269-4277. 1994.

Diakonova, M., Gerke, V., Ernst, J., Liautard, J.P., van, d., V, and Griffiths, G. (1997). Localization of five annexins in J774 macrophages and on isolated phagosomes. J Cell Sci *110 (Pt 10)*, 1199-1213.

Diaz-Munoz, M., Hamilton, S.L., Kaetzel, M.A., Hazarika, P., and Dedman, J.R. (1990). Modulation of Ca2+ release channel activity from sarcoplasmic reticulum by annexin VI (67-kDa calcimedin). J Biol Chem *265*, 15894-15899.

Donato, R. and Russo-Marie, F. (1999). The annexins: structure and functions. Cell Calcium *26*, 85-89.

Drust, D.S. and Creutz, C.E. (1988). Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature *331*, 88-91.

Dubois, T., Soula, M., Moss, S.E., Russo-Marie, F., and Rothhut, B. (1995). Potential interaction between annexin VI and a 56-kDa protein kinase in T cells. Biochem Biophys Res Commun *212*, 270-278.

Dubois, T., Bisagni-Faure, A., Coste, J., Mavoungou, E., Menkes, C.J., Russo-Marie, F., and Rothhut, B. (1995). High levels of antibodies to annexins V and VI in patients with rheumatoid arthritis. J Rheumatol *22*, 1230-1234.

Dubois, T., Rommel, C., Howell, S., Steinhussen, U., Soneji, Y., Morrice, N., Moelling, K., and Aitken, A. (1997). 14-3-3 is phosphorylated by casein kinase I on residue 233. Phosphorylation at this site in vivo regulates Raf/14-3-3 interaction. J.Biol.Chem. *272*, 28882-28888.

Edwards, H.C. and Booth, A.G. (1987). Calcium-sensitive, lipid-binding cytoskeletal proteins of the human placental microvillar region. J Cell Biol *105*, 303-311.

Edwards, H.C. and Moss, S.E. (1995). Functional and genetic analysis of annexin VI. Mol Cell Biochem *149-150*, 293-299.

Emans, N., Gorvel, J.P., Walter, C., Gerke, V., Kellner, R., Griffiths, G., and Gruenberg, J. (1993). Annexin II is a major component of fusogenic endosomal vesicles. J Cell Biol *120*, 1357-1369.

Engelman, J.A., Zhang, X.L., and Lisanti, M.P. (1998). Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known fragile site (FRA7G) that is frequently deleted in human cancers. FEBS Lett *436*, 403-410.

Engelman, J.A., Zhang, X.L., Galbiati, F., and Lisanti, M.P. (1998). Chromosomal localization, genomic organization, and developmental expression of the murine caveolin gene family (Cav-1, -2, and -3). Cav-1 and Cav-2 genes map to a known tumor suppressor locus (6-A2/7q31). FEBS Lett *429*, 330-336.

Engelman, J.A., Chu, C., Lin, A., Jo, H., Ikezu, T., Okamoto, T., Kohtz, D.S., and Lisanti, M.P. (1998). Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. FEBS Lett *428*, 205-211.

Enrich, C., Pol, A., Calvo, M., Pons, M., and Jackle, S. (1999). Dissection of the multifunctional "Receptor-Recycling" endocytic compartment of hepatocytes. Hepatology *30*, 1115-1120.

Erikson, E., Tomasiewicz, H.G., and Erikson, R.L. (1984). Biochemical characterization of a 34-kilodalton normal cellular substrate of pp60v-src and an associated 6-kilodalton protein. Mol Cell Biol *4*, 77-85.

Ernst, J.D., Hoye, E., Blackwood, R.A., and Mok, T.L. (1991). Identification of a domain that mediates vesicle aggregation reveals functional diversity of annexin repeats. J Biol Chem *266*, 6670-6673.

Ernst, J.D. (1991). Annexin III translocates to the periphagosomal region when neutrophils ingest opsonized yeast. J Immunol. *146*, 3110-3114.

Fan, H., Josic, D., Lim, Y.P., and Reutter, W. (1995). cDNA cloning and tissue-specific regulation of expression of rat calcium-binding protein 65/67. Identification as a homologue of annexin VI. Eur J Biochem *230*, 741-751.

Feig, L.A. and Cooper, G.M. (1988). Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol *8*, 3235-3243.

Fiedler, K., Lafont, F., Parton, R.G., and Simons, K. (1995). Annexin XIIIb: a novel epithelial specific annexin is implicated in vesicular traffic to the apical plasma membrane. J Cell Biol *128*, 1043-1053.

Fishman, J.B., Dickey, B.F., McCrory, M.F., and Fine, R.E. (1986). Reversible inactivation of vasopressin and angiotensin II binding to hepatocyte membranes by a calcium-dependent, cytosolic protein. J Biol Chem *261*, 5810-5816.

Fleet, A., Ashworth, R., Kubista, H., Edwards, H., Bolsover, S., Mobbs, P., and Moss, S.E. (1999). Inhibition of EGF-dependent calcium influx by annexin VI is splice form-specific. Biochem Biophys Res Commun *260*, 540-546.

Flower, R.J. (1988). Eleventh Gaddum memorial lecture. Lipocortin and the mechanism of action of the glucocorticoids. Br J Pharmacol *94*, 987-1015.

Fox, M.T., Prentice, D.A., and Hughes, J.P. (1991). Increases in p11 and annexin II proteins correlate with differentiation in the PC12 pheochromocytoma. Biochem Biophys Res Commun *177*, 1188-1193.

Francia, G., Mitchell, S.D., Moss, S.E., Hanby, A.M., Marshall, J.F., and Hart, I.R. (1996). Identification by differential display of annexin-VI, a gene differentially expressed during melanoma progression. Cancer Res *56*, 3855-3858.

Fu, H., Subramanian, R.R., and Masters, S.C. (2000). 14-3-3 proteins: structure, function, and regulation. Annu.Rev.Pharmacol.Toxicol.2000.;40.:617.-647.

Futter, C.E., Felder, S., Schlessinger, J., Ullrich, A., and Hopkins, C.R. (1993). Annexin I is phosphorylated in the multivesicular body during the processing of the epidermal growth factor receptor. J Cell Biol *120*, 77-83.

Futter, C.E., Pearse, A., Hewlett, L.J., and Hopkins, C.R. (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. J.Cell Biol *132*, 1011-1023.

Galbiati, F., Volonte, D., Engelman, J.A., Watanabe, G., Burk, R., Pestell, R.G., and Lisanti, M.P. (1998). Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. EMBO J *17*, 6633-6648.

Galli, T., Zahraoui, A., Vaidyanathan, V.V., Raposo, G., Tian, J.M., Karin, M., Niemann, H., and Louvard, D. (1998). A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. Mol.Biol Cell *9*, 1437-1448.

Garbuglia, M., Verzini, M., Giambanco, I., Spreca, A., and Donato, R. (1996). Effects of calcium-binding proteins (S-100a(o), S-100a, S-100b) on desmin assembly in vitro. FASEB J *10*, 317-324.

Garbuglia, M., Verzini, M., and Donato, R. (1998). Annexin VI binds S100A1 and S100B and blocks the ability of S100A1 and S100B to inhibit desmin and GFAP assemblies into intermediate filaments. Cell Calcium *24*, 177-191.

Gawler, D.J. (1998). Points of convergence between Ca2+ and Ras signalling pathways. Biochim.Biophys.Acta *1448*, 171-182.

Geisow, M.J. (1986). Common domain structure of Ca2+ and lipid-binding proteins. FEBS Lett *203*, 99-103.

Geisow, M.J., Fritsche, U., Hexham, J.M., Dash, B., and Johnson, T. (1986). A consensus amino-acid sequence repeat in Torpedo and mammalian Ca2+-dependent membrane-binding proteins. Nature *320*, 636-638.

Gerke, V. and Weber, K. (1984). Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin. EMBO J *3*, 227-233.

Gerke, V. and Weber, K. (1985). Calcium-dependent conformational changes in the 36-kDa subunit of intestinal protein I related to the cellular 36-kDa target of Rous sarcoma virus tyrosine kinase. J Biol Chem *260*, 1688-1695.

Gerke, V. and Moss, S.E. (1997). Annexins and membrane dynamics. Biochim Biophys Acta *1357*, 129-154.

Geuze, H.J., Stoorvogel, W., Strous, G.J., Slot, J.W., Bleekemolen, J.E., and Mellman, I. (1988). Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. J Cell Biol *107*, 2491-2501.

Giambanco, I., Verzini, M., and Donato, R. (1993). Annexins V and VI in rat tissues during post-natal development: immunochemical measurements. Biochem Biophys Res Commun *196*, 1221-1226.

Glenney, J.R.J., Boudreau, M., Galyean, R., Hunter, T., and Tack, B. (1986). Association of the S-100-related calpactin I light chain with the NH2-terminal tail of the 36-kDa heavy chain. J Biol Chem *261*, 10485-10488.

Glenney, J.R.J., Tack, B., and Powell, M.A. (1987). Calpactins: two distinct Ca++-regulated phospholipid- and actin-binding proteins isolated from lung and placenta. J Cell Biol *104*, 503-511.

Goldstein, J.L., Basu, S.K., and Brown, M.S. (1983). Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. Methods Enzymol *98*, 241-260.

Goulding, N.J. and Guyre, P.M. (1993). Lipocortin 1 binding to human leukocytes correlates with its ability to inhibit IgG interactions with Fc gamma receptors. Biochem Biophys Res Commun *192*, 351-358.

Grewal, T., Heeren, J., Mewawala, D., Schnitgerhans, T., Wendt, D., Salomon, G., Enrich, C., Beisiegel, U., and Jackle, S. (2000). Annexin VI stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the prelysosomal compartment. J.Biol.Chem.2000.Oct.27.;275.(43.):33806-33813.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell *52*, 329-341.

Griffiths, G., Matteoni, R., Back, R., and Hoflack, B. (1990). Characterization of the cation-independent mannose 6-phosphate receptor-enriched prelysosomal compartment in NRK cells. J Cell Sci *95 (Pt 3)*, 441-461.

Gruenberg, J. and Emans, N. Annexins in membrane traffic. Trends Cell Biol 3, 224-227. 1993.

Gruenberg, J. and Maxfield, F.R. (1995). Membrane transport in the endocytic pathway. Curr Opin Cell Biol *7*, 552-563.

Gu, F. and Gruenberg, J. (1999). Biogenesis of transport intermediates in the endocytic pathway. FEBS Lett. *452*, 61-66.

Gunteski-Hamblin, A.M., Song, G., Walsh, R.A., Frenzke, M., Boivin, G.P., Dorn, G.W., Kaetzel, M.A., Horseman, N.D., and Dedman, J.R. (1996). Annexin VI overexpression targeted to heart alters cardiomyocyte function in transgenic mice. Am J Physiol *270*, H1091-H1100

Gupta, S., Weiss, A., Kumar, G., Wang, S., and Nel, A. (1994). The T-cell antigen receptor utilizes Lck, Raf-1, and MEK-1 for activating mitogen-activated protein kinase. Evidence for the existence of a second protein kinase C-dependent pathway in an Lck-negative Jurkat cell mutant. J.Biol.Chem. *269* , 17349-17357.

Hajjar, K.A. and Krishnan, S. (1999). Annexin II: a mediator of the plasmin/plasminogen activator system. Trends Cardiovasc Med *9* , 128-138.

Harder, T., Thiel, C., and Gerke, V. (1993). Formation of the annexin II2p112 complex upon differentiation of F9 teratocarcinoma cells. J Cell Sci *104 (Pt 4)*, 1109-1117.

Harder, T. and Gerke, V. (1993). The subcellular distribution of early endosomes is affected by the annexin II2p11(2) complex. J Cell Biol *123*, 1119-1132.

Harder, T., Kellner, R., Parton, R.G., and Gruenberg, J. (1997). Specific release of membrane-bound annexin II and cortical cytoskeletal elements by sequestration of membrane cholesterol. Mol Biol Cell *8*, 533-545.

Hawes, B.E., van Biesen, T., Koch, W.J., Luttrell, L.M., and Lefkowitz, R.J. (1995). Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. J Biol Chem *270*, 17148-17153.

Hawkins, T.E., Roes, J., Rees, D., Monkhouse, J., and Moss, S.E. (1999). Immunological development and cardiovascular function are normal in annexin VI null mutant mice. Mol.Cell Biol *19*, 8028-8032.

Hayashi, M., Imai, Y., Naraba, H., Tomoda, H., Omura, S., and Oh-ishi, S. (1992). Enhanced production of platelet-activating factor in stimulated rat leukocytes pretreated with triacsin C, A novel acyl-coA synthetase inhibitor. Biochem Biophys Res Commun *188*, 1280-1285.

Hazarika, P., Kaetzel, M.A., Sheldon, A., Karin, N.J., Fleischer, S., Nelson, T.E., and Dedman, J.R. (1991). Annexin VI is associated with calcium-sequestering organelles. J Cell Biochem *46*, 78-85.

Hazarika, P., Sheldon, A., Kaetzel, M.A., Diaz-Munoz, M., Hamilton, S.L., and Dedman, J.R. (1991). Regulation of the sarcoplasmic reticulum Ca(2+)-release channel requires intact annexin VI. J Cell Biochem *46*, 86-93.

Henley, J.R., Krueger, E.W., Oswald, B.J., and McNiven, M.A. (1998). Dynamin-mediated internalization of caveolae. J.Cell Biol. *141*, 85-99.

Hirst, J., Futter, C.E., and Hopkins, C.R. (1998). The kinetics of mannose 6-phosphate receptor trafficking in the endocytic pathway in HEp-2 cells: the receptor enters and rapidly leaves multivesicular endosomes without accumulating in a prelysosomal compartment. Mol.Biol Cell *9*, 809-816.

Holleran, E.A., Tokito, M.K., Karki, S., and Holzbaur, E.L. (1996). Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. J Cell Biol *135*, 1815-1829.

Holtzman, E. Lysosomes. Plenum Press . 1989.

Hosoya, H., Kobayashi, R., Tsukita, S., and Matsumura, F. (1992). Ca(2+)-regulated actin and phospholipid binding protein (68 kD-protein) from bovine liver: identification as a homologue for annexin VI and intracellular localization. Cell Motil Cytoskeleton *22*, 200-210.

Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C.J. (1992). Activation of the MAP kinase pathway by the protein kinase raf. Cell *71*, 335-342.

Huber, R., Romisch, J., and Paques, E.P. (1990). The crystal and molecular structure of human annexin V, an anticoagulant protein that binds to calcium and membranes. EMBO J *9*, 3867-3874.

Ihrke, G., Neufeld, E.B., Meads, T., Shanks, M.R., Cassio, D., Laurent, M., Schroer, T.A., Pagano, R.E., and Hubbard, A.L. (1993). WIF-B cells: an in vitro model for studies of hepatocyte polarity. J Cell Biol *123*, 1761-1775.

Ihrke, G., Martin, G.V., Shanks, M.R., Schrader, M., Schroer, T.A., and Hubbard, A.L. (1998). Apical plasma membrane proteins and endolyn-78 travel through a subapical compartment in polarized WIF-B hepatocytes. J Cell Biol *141*, 115-133.

Inui, M., Watanabe, T., and Sobue, K. (1994). Annexin VI binds to a synaptic vesicle protein, synapsin I. J Neurochem *63*, 1917-1923.

Isacke, C.M., Lindberg, R.A., and Hunter, T. (1989). Synthesis of p36 and p35 is increased when U-937 cells differentiate in culture but expression is not inducible by glucocorticoids. Mol Cell Biol *9*, 232-240.

Jackle, S., Beisiegel, U., Rinninger, F., Buck, F., Grigoleit, A., Block, A., Groger, I. , Greten, H., and Windler, E. (1994). Annexin VI, a marker protein of hepatocytic endosomes. J Biol Chem *269*, 1026-1032.

Jahraus, A., Storrie, B., Griffiths, G., and Desjardins, M. (1994). Evidence for retrograde traffic between terminal lysosomes and the prelysosomal/late endosome compartment. J.Cell Sci. *107 (Pt 1)*, 145-157.

Jost, M., Zeuschner, D., Seemann, J., Weber, K., and Gerke, V. (1997). Identification and characterization of a novel type of annexin-membrane interaction: Ca2+ is not required for the association of annexin II with early endosomes. J Cell Sci *110 (Pt 2)*, 221-228.

Junker, M. and Creutz, C.E. (1993). Endonexin (annexin IV)-mediated lateral segregation of phosphatidylglycerol in phosphatidylglycerol/phosphatidylcholine membranes. Biochemistry *32*, 9968-9974.

Kaelin, W.G.J., Pallas, D.C., DeCaprio, J.A., Kaye, F.J., and Livingston, D.M. (1991). Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell *64*, 521-532.

Kaetzel, M.A., Pula, G., Campos, B., Uhrin, P., Horseman, N., and Dedman, J.R. (1994). Annexin VI isoforms are differentially expressed in mammalian tissues. Biochim Biophys Acta *1223*, 368-374.

Kaetzel, M.A., Chan, H.C., Dubinsky, W.P., Dedman, J.R., and Nelson, D.J. (1994). A role for annexin IV in epithelial cell function. Inhibition of calcium-activated chloride conductance. J Biol Chem *269*, 5297-5302.

Kamal, A., Ying, Y., and Anderson, R.G. (1998). Annexin VI-mediated loss of spectrin during coated pit budding is coupled to delivery of LDL to lysosomes. J Cell Biol *142*, 937-947.

Karki, S. and Holzbaur, E.L. (1995). Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. J Biol Chem *270*, 28806-28811.

Kawasaki, H., Avila-Sakar, A., Creutz, C.E., and Kretsinger, R.H. (1996). The crystal structure of annexin VI indicates relative rotation of the two lobes upon membrane binding. Biochim Biophys Acta *1313*, 277-282.

Kawasaki, H. and Kawashima, S. (1996). Regulation of the calpain-calpastatin system by membranes (review). Mol Membr.Biol *13*, 217-224.

Kholodenko, B.N., Hoek, J.B., and Westerhoff, H.V. (2000). Why cytoplasmic signalling proteins should be recruited to cell membranes. Trends Cell Biol *10*, 173-178.

Kim, M.J., Dawes, J., and Jessup, W. (1994). Transendothelial transport of modified low-density lipoproteins. Atherosclerosis *108*, 5-17.

Kim, S., Ko, J., Kim, J.H., Choi, E.C., and Na, D.S. (2001). Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model. FEBS Lett 2001.Feb.2.;489.(2.-3.):243.-8. *489*, 243-248.

Kitagawa, Y., Ueda, M., Ando, N., Ozawa, S., and Kitajima, M. (1995). Effect of endogenous and exogenous EGF on the growth of EGF receptor-hyperproducing human squamous cell carcinoma implanted in nude mice. Br J Cancer *72*, 865-868.

Kojima, K., Utsumi, H., Ogawa, H., and Matsumoto, I. (1994). Highly polarized expression of carbohydrate-binding protein p33/41 (annexin IV) on the apical plasma membrane of epithelial cells in renal proximal tubules. FEBS Lett *342*, 313-318.

Kornfeld, S. and Mellman, I. (1989). The biogenesis of lysosomes. Annu.Rev.Cell Biol *5*, 483-525.

Koumanov, D, Wolf, C. And Bereziat, G. (1997). Modulation of human type II secretory phospholipase A2 by sphingomyelin and annexin VI. Biochem J Aug 15;326: 227-33.

Kuijpers, G.A., Lee, G., and Pollard, H.B. (1992). Immunolocalization of synexin (annexin VII) in adrenal chromaffin granules and chromaffin cells: evidence for a dynamic role in the secretory process. Cell Tissue Res *269*, 323-330.

Lamaze, C., Fujimoto, L.M., Yin, H.L., and Schmid, S.L. (1997). The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. J Biol Chem *272*, 20332-20335.

Lambert, O., Gerke, V., Bader, M.F., Porte, F., and Brisson, A. (1997). Structural analysis of junctions formed between lipid membranes and several annexins by cryo-electron microscopy. J Mol Biol *272*, 42-55.

Langanger, G., de Mey, J., Moeremans, M., Daneels, G., de Brabander, M., and Small, J.V. (1984). Ultrastructural localization of alpha-actinin and filamin in cultured cells with the immunogold staining (IGS) method. J.Cell Biol. *99*, 1324-1334.

Lavialle, F., Rainteau, D., Massey-Harroche, D., and Metz, F. (2000). Establishment of plasma membrane polarity in mammary epithelial cells correlates with changes in prolactin trafficking and in annexin VI recruitment to membranes. Biochim Biophys Acta 2000.Mar.15.;1464.(1.):83.-94.

Le, C., V and Maridonneau-Parini, I. (1994). Annexin 3 is associated with cytoplasmic granules in neutrophils and monocytes and translocates to the plasma membrane in activated cells. Biochem J *303 (Pt 2)*, 481-487.

Leevers SJ, Marshall CJ. (1992). Activation of extracellular signal-regulated kinase,ERK2, by p21ras oncoprotein. EMBO J Feb, 11(2):569-574 2000.

Leevers, S.J., Paterson, H.F., and Marshall, C.J. (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature *369*, 411-414.

Liemann, S. and Lewit-Bentley, A. (1995). Annexins: a novel family of calcium- and membrane-binding proteins in search of a function. Structure *3*, 233-237.

Lin, C.H. and Forscher, P. (1993). Cytoskeletal remodeling during growth cone-target interactions. J Cell Biol *121*, 1369-1383.

Lin, C.S., Chen, Z.P., Park, T., Ghosh, K., and Leavitt, J. (1993). Characterization of the human L-plastin gene promoter in normal and neoplastic cells. J Biol Chem *268*, 2793-2801.

Lin, C.S., Park, T., Chen, Z.P., and Leavitt, J. (1993). Human plastin genes. Comparative gene structure, chromosome location, and differential expression in normal and neoplastic cells. J Biol Chem *268*, 2781-2792.

Lin, E.C. and Cantiello, H.F. (1993). A novel method to study the electrodynamic behavior of actin filaments. Evidence for cable-like properties of actin. Biophys J *65*, 1371-1378.

Lin, H.C., Sudhof, T.C., and Anderson, R.G. (1992). Annexin VI is required for budding of clathrin-coated pits. Cell *70*, 283-291.

Lin, M.C., Mullady, E., and Wilson, F.A. (1993). Timed photoaffinity labeling and characterization of bile acid binding and transport proteins in rat ileum. Am J Physiol *265*, G56-G62

Lin, P.S., Ho, K.C., and Sung, S.J. (1993). Combined treatments of heat, radiation, or cytokines with flavone acetic acid on the growth of cultured endothelial cells. Int J Hyperthermia *9* , 517-528.

Lin, S.H., Harzelrig, J.B., and Cheung, H.C. (1993). Transient kinetics of the interaction of actin with myosin subfragment-1 in the absence of nucleotide . Biophys J *65*, 1433-1444.

Lin, S.X. and Collins, C.A. (1992). Immunolocalization of cytoplasmic dynein to lysosomes in cultured cells. J Cell Sci *101 (Pt 1)*, 125-137.

Lin, Y., Ishikawa, R., and Kohama, K. (1993). Role of myosin in the stimulatory effect of caldesmon on the interaction between actin, myosin, and ATP. J Biochem (Tokyo) *114*, 279-283.

Lin, Y., Ye, L.H., Ishikawa, R., Fujita, K., and Kohama, K. (1993). Stimulatory effect of calponin on myosin ATPase activity. J Biochem (Tokyo) *113*, 643-645.

Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R.D. (1991). Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell *67*, 601-616.

Liu, K. and Li, G. (1998). Catalytic domain of the p120 Ras GAP binds to RAb5 and stimulates its GTPase activity. J.Biol.Chem. 273, 10087-90.

Liu, L., Fisher, A.B., and Zimmerman, U.J. (1995). Lung annexin II promotes fusion of isolated lamellar bodies with liposomes. Biochim Biophys Acta *1259*, 166-172.

Lombardi, D., Soldati, T., Riederer, M.A., Goda, Y., Zerial, M., and Pfeffer, S.R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. EMBO J. *12*, 677-682.

Luckcuck, T., Trotter, P.J., and Walker, J.H. (1998). Localization of annexin VI in the adult and neonatal heart. Cell Biol Int *22*, 199-205.

Lukowski, S., Lecomte, M.C., Mira, J.P., Marin, P., Gautero, H., Russo-Marie, F., and Geny, B. (1996). Inhibition of phospholipase D activity by fodrin. An active role for the cytoskeleton. J Biol Chem *271*, 24164-24171.

Luo, Z.J., Zhang, X.F., Rapp, U., and Avruch, J. (1995). Identification of the 14.3.3 zeta domains important for self-association and Raf binding. J Biol Chem *270*, 23681-23687.

Luzio, J.P., Rous, B.A., Bright, N.A., Pryor, P.R., Mullock, B.M., and Piper, R.C. (2000). Lysosome-endosome fusion and lysosome biogenesis. J.Cell Sci.2000.May.;113.(Pt.9.):1515.-24.

Mailliard, W.S., Haigler, H.T., and Schlaepfer, D.D. (1996). Calcium-dependent binding of S100C to the N-terminal domain of annexin I. J Biol Chem *271*, 719-725.

Malarkey, K., Belham, C.M., Paul, A., Graham, A., McLees, A., Scott, P.H., and Plevin, R. (1995). The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. Biochem J *309 (Pt 2)*, 361-375.

Mancuso, F., Flower, R.J., and Perretti, M. (1995). Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule. Involvement of endogenous lipocortin 1. J Immunol. *155*, 377-386.

Marais, R., Light, Y., Paterson, H.F., and Marshall, C.J. (1995). Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J *14*, 3136-3145.

Marais, R. and Marshall, C.J. (1996). Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv *27*, 101-125.

Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M.F., and Marshall, C.J. (1998). Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C Science *280*, 109-112.

Marshall, M.S. (1995). Ras target proteins in eukaryotic cells. FASEB J *9*, 1311-1318.

Masiakowski, P. and Shooter, E.M. (1990). Changes in PC12 cell morphology induced by transfection with 42C cDNA, coding for a member of the S-100 protein family. J Neurosci.Res *27*, 264-269.

Massey-Harroche, D., Mayran, N., and Maroux, S. (1998). Polarized localizations of annexins I, II, VI and XIII in epithelial cells of intestinal, hepatic and pancreatic tissues. J Cell Sci *111 (Pt 20)*, 3007-3015.

Massey, D., Traverso, V., and Maroux, S. (1991). Lipocortin IV is a basolateral cytoskeleton constituent of rabbit enterocytes. J Biol Chem *266*, 3125-3130.

Matteo, R.G. and Moravec, C.S. (2000). Immunolocalization of annexins IV, V and VI in the failing and non-failing human heart. Cardiovasc Res *45*, 961-970.

May, M.J., Wheeler-Jones, C.P., and Pearson, J.D. (1996). Effects of protein tyrosine kinase inhibitors on cytokine-induced adhesion molecule expression by human umbilical vein endothelial cells. Br J Pharmacol *118*, 1761-1771.

Mayorga, L.S., Beron, W., Sarrouf, M.N., Colombo, M.I., Creutz, C., and Stahl, P.D. (1994). Calcium-dependent fusion among endosomes. J Biol Chem *269*, 30927-30934.

McPherson, P.S., Czernik, A.J., Chilcote, T.J., Onofri, F., Benfenati, F., Greengard, P., Schlessinger, J., and De Camilli, P. (1994). Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin I. Proc Natl Acad Sci U S A *91*, 6486-6490.

McPherson, R.A. (1998). Platelet antibody testing for evaluating immune thrombocytopenias [editorial; comment]. Am J Clin Pathol *109*, 123-126.

McPherson, R.A., Harding, A., Roy, S., Lane, A., and Hancock, J.F. (1999). Interactions of c-Raf-1 with phosphatidylserine and 14-3-3. Oncogene *18*, 3862-3869.

Medema, R.H., Burgering, B.M., and Bos, J.L. (1991). Insulin-induced p21ras activation does not require protein kinase C, but a protein sensitive to phenylarsine oxide. J Biol Chem *266*, 21186-21189.

Mellman, I. (1996). Endocytosis and molecular sorting. Annu.Rev.Cell Dev.Biol *12*, 575-625.

Menell, J.S., Cesarman, G.M., Jacovina, A.T., McLaughlin, M.A., Lev, E.A., and Hajjar, K.A. (1999). Annexin II and bleeding in acute promyelocytic leukemia [see comments]. N Engl J Med *340*, 994-1004.

Menell, J.S., Cesarman, G.M., Jacovina, A.T., McLaughlin, M.A., Lev, E.A., and Hajjar, K.A. (1999). Annexin II and bleeding in acute promyelocytic leukemia. N Engl J Med *340*, 994-1004.

Michaely, P., Kamal, A., Anderson, R.G., and Bennett, V. (1999). A requirement for ankyrin binding to clathrin during coated pit budding. J Biol Chem *274*, 35908-35913.

Mizutani, A., Usuda, N., Tokumitsu, H., Minami, H., Yasui, K., Kobayashi, R., and Hidaka, H. (1992). CAP-50, a newly identified annexin, localizes in nuclei of cultured fibroblast 3Y1 cells. J Biol Chem *267*, 13498-13504.

Mizutani, A., Tokumitsu, H., Kobayashi, R., and Hidaka, H. (1993). Phosphorylation of annexin XI (CAP-50) in SR-3Y1 cells. J Biol Chem *268*, 15517-15522.

Mochly-Rosen, D., Khaner, H., Lopez, J., and Smith, B.L. (1991). Intracellular receptors for activated protein kinase C. Identification of a binding site for the enzyme. J Biol Chem *266*, 14866-14868.

Montessuit, C. and Thorburn, A. (1999). Activation of Ras by phorbol esters in cardiac myocytes. Role of guanine nucleotide exchange factors. FEBS Lett *460*, 57-60.

Morrison, D.K. and Cutler, R.E. (1997). The complexity of Raf-1 regulation. Curr.Opin.Cell Biol. *9*, 174-179.

Moss, S.E., Crompton, M.R., and Crumpton, M.J. (1988). Molecular cloning of murine p68, a Ca2+-binding protein of the lipocortin family. Eur J Biochem *177*, 21-27.

Moss, S.E. and Crumpton, M.J. (1990). Alternative splicing gives rise to two forms of the p68 Ca2(+)-binding protein. FEBS Lett *261*, 299-302.

Moss, S.E. and Crumpton, M.J. (1990). Structural and functional investigation of p68--a protein of the lipocortin/calpactin family. Adv Exp Med Biol *269*, 79-83.

Moss, S.E. and Crumpton, M.J. (1990). The lipocortins and the EF hand proteins: Ca2(+)-binding sites and evolution. Trends Biochem Sci *15*, 11-12.

Mostov, K.E., Verges, M., and Altschuler, Y. (2000). Membrane traffic in polarized epithelial cells. Curr.Opin.Cell Biol 2000.Aug.;12.(4.):483.-90.

Mullock, B.M., Bright, N.A., Fearon, C.W., Gray, S.R., and Luzio, J.P. (1998). Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. J Cell Biol *140*, 591-601.

Mullock, B.M., Smith, C.W., Ihrke, G., Bright, N.A., Lindsay, M., Parkinson, E.J., Brooks, D.A., Parton, R.G., James, D.E., Luzio, J.P., and Piper, R.C. (2000). Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and Is required for late endosome-lysosome fusion. Mol.Biol Cell 2000.Sep.;11.(9.):3137.-53.

Naciff, J.M., Behbehani, M.M., Kaetzel, M.A., and Dedman, J.R. (1996). Annexin VI modulates Ca2+ and K+ conductances of spinal cord and dorsal root ganglion neurons. Am J Physiol *271*, C2004-C2015

Naciff, J.M., Kaetzel, M.A., Behbehani, M.M., and Dedman, J.R. (1996). Differential expression of annexins I-VI in the rat dorsal root ganglia and spinal cord. J Comp Neurol *368*, 356-370.

Naka, M., Qing, Z.X., Sasaki, T., Kise, H., Tawara, I., Hamaguchi, S., and Tanaka, T. (1994). Purification and characterization of a novel calcium-binding protein, S100C, from porcine heart. Biochim Biophys Acta *1223*, 348-353.

Nakata, T., Sobue, K., and Hirokawa, N. (1990). Conformational change and localization of calpactin I complex involved in exocytosis as revealed by quick-freeze, deep-etch electron microscopy and immunocytochemistry. J Cell Biol *110*, 13-25.

Nori, M., L'Allemain, G., and Weber, M.J. (1992). Regulation of tetradecanoyl phorbol acetate-induced responses in NIH 3T3 cells by GAP, the GTPase-activating protein associated with p21c-ras. Mol Cell Biol *12*, 936-945.

Novick, P. and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. Curr.Opin.Cell Biol *9*, 496-504.

Ohnishi, M., Tokuda, M., Masaki, T., Fujimura, T., Tai, Y., Itano, T., Matsui, H., Ishida, T., Konishi, R., and Takahara, J. (1995). Involvement of annexin-I in glucose-induced insulin secretion in rat pancreatic islets. Endocrinology *136*, 2421-2426.

Okamoto, T., Schlegel, A., Scherer, P.E., and Lisanti, M.P. (1998). Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J.Biol.Chem. *273*, 5419-5422.

Oliani, S.M., Christian, H.C., Manston, J., Flower, R.J., and Perretti, M. (2000). An immunocytochemical and in situ hybridization analysis of annexin 1 expression in rat mast cells: modulation by inflammation and dexamethasone. Lab Invest *80*, 1429-1438.

Ortega, D., Pol, A., Biermer, M., Jackle, S., and Enrich, C. (1998). Annexin VI defines an apical endocytic compartment in rat liver hepatocytes. J Cell Sci *111 (Pt 2)*, 261-269.

Owens, R.J. and Crumpton, M.J. (1984). Isolation and characterization of a novel 68,000-Mr Ca2+-binding protein of lymphocyte plasma membrane. Biochem J *219*, 309-316.

Parton, R.G. and Simons, K. (1995). Digging into caveolae. Science *269*, 1398-1399.

Peppelenbosch, M.P., Tertoolen, L.G., den Hertog, J., and de Laat, S.W. (1992). Epidermal growth factor activates calcium channels by phospholipase A2/5-lipoxygenase-mediated leukotriene C4 production. Cell *69*, 295-303.

Peppelenbosch, M.P., Tertoolen, L.G., Hage, W.J., and de Laat, S.W. (1993). Epidermal growth factor-induced actin remodeling is regulated by 5-lipoxygenase and cyclooxygenase products. Cell *74*, 565-575.

Peppelenbosch, M.P., Qiu, R.G., de Vries-Smits, A.M., Tertoolen, L.G., de Laat, S.W., McCormick, F., Hall, A., Symons, M.H., and Bos, J.L. (1995). Rac mediates growth factor-induced arachidonic acid release. Cell *81*, 849-856.

Peppelenbosch, M.P., Tertoolen, L.G., de Laat, S.W., and Zivkovic, D. (1995). Ionic responses to epidermal growth factor in zebrafish cells. Exp Cell Res *218*, 183-188.

Peppelenbosch, M.P., Tertoolen, L.G., Van der Flier, A., and de Laat, S.W. (1995). Evaluation of single-channel gating kinetics produced after amplitude-based separation of unitary currents. J Neurosci Methods *58*, 49-59.

Persaud, S.J., Wheeler-Jones, C.P., and Jones, P.M. (1996). The mitogen-activated protein kinase pathway in rat islets of Langerhans: studies on the regulation of insulin secretion. Biochem J *313 (Pt 1)*, 119-124.

Pfaffle, M., Ruggiero, F., Hofmann, H., Fernandez, M.P., Selmin, O., Yamada, Y., Garrone, R., and von der, M. (1988). Biosynthesis, secretion and extracellular localization of anchorin CII, a collagen-binding protein of the calpactin family. EMBO J *7*, 2335-2342.

Pitas, R.E., Boyles, J., Mahley, R.W., and Bissell, D.M. (1985). Uptake of chemically modified low density lipoproteins in vivo is mediated by specific endothelial cells. J Cell Biol *100*, 103-117.

Pol, A., Ortega, D., and Enrich, C. (1997). Identification of cytoskeleton-associated proteins in isolated rat liver endosomes. Biochem J *327 (Pt 3)*, 741-746.

Pol, A. and Enrich, C. (1997). Membrane transport in rat liver endocytic pathways: preparation, biochemical properties and functional roles of hepatic endosomes. Electrophoresis *18*, 2548-2557.

Pol, A., Ortega, D., and Enrich, C. (1997). Identification and distribution of proteins in isolated endosomal fractions of rat liver: involvement in endocytosis, recycling and transcytosis. Biochem J *323 (Pt 2)*, 435-443.

Pol, A., Calvo, M., and Enrich, C. (1998). Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment. FEBS Lett *441*, 34-38.

Pol, A., Calvo, M., Lu, A., and Enrich, C. (1999). The "early-sorting" endocytic compartment of rat hepatocytes is involved in the intracellular pathway of caveolin-1 (VIP-21). Hepatology *29*, 1848-1857.

Pol, A., Lu, A., Pons, M., Peiro, S., and Enrich, C. (2000). EGF-mediated caveolin recruitment to early endosomes and MAPK activation. Role of cholesterol and actin-cytoskeleton. J Biol Chem 2000.Jul.10.

Pol, A., Calvo, M., Lu, A., and Enrich, C. (2000). EGF triggers caveolin redistribution from the plasma membrane to the early/sorting endocytic compartment of hepatocytes. Cell Signal.2000.Aug.;12.(8.):537.-40.

Pollard, H.B. and Scott, J.H. (1982). Synhibin: a new calcium-dependent membrane-binding protein that inhibits synexin-induced chromaffin granule aggregation and fusion. FEBS Lett *150*, 201-206.

Pollard, H.B. and Rojas, E. (1988). Ca2+-activated synexin forms highly selective, voltage-gated Ca2+ channels in phosphatidylserine bilayer membranes. Proc Natl Acad Sci U S A *85*, 2974-2978.

Pons, M., Ihrke, G., Koch, S., Biermer, M., Pol, A., Grewal, T., Jackle, S., and Enrich, C. (2000). Late endocytic compartments are major sites of annexin VI localization in NRK fibroblasts and polarized WIF-B hepatoma cells. Exp Cell Res *257*, 33-47.

Powell, M.A. and Glenney, J.R. (1987). Regulation of calpactin I phospholipid binding by calpactin I light-chain binding and phosphorylation by p60v-src. Biochem J *247*, 321-328.

Pryor, P.R., Mullock, B.M., Bright, N.A., Gray, S.R., and Luzio, J.P. (2000). The role of intraorganellar Ca(2+) in late endosome-lysosome heterotypic fusion and in the reformation of lysosomes from hybrid organelles. J.Cell Biol 2000, May 29;149 (5.): 1053.-62.

Puisieux, A., Ji, J., and Ozturk, M. (1996). Annexin II up-regulates cellular levels of p11 protein by a post-translational mechanisms. Biochem J *313 (Pt 1)*, 51-55.

Raeymaekers, L., Wuytack, F., and Casteels, R. (1985). Isolation of calelectrin-like proteins associated with smooth muscle plasma membranes. Biochem Biophys Res Commun *132*, 526-532.

Rainteau, D., Mansuelle, P., Rochat, H., and Weinman, S. (1995). Characterization and ultrastructural localization of annexin VI from mitochondria. FEBS Lett *360*, 80-84.

Rand, J.H., Wu, X.X., Guller, S., Gil, J., Guha, A., Scher, J., and Lockwood, C.J. (1994). Reduction of annexin-V (placental anticoagulant protein-I) on placental villi of women with antiphospholipid antibodies and recurrent spontaneous abortion. Am J Obstet Gynecol *171*, 1566-1572.

Raynal, P. and Pollard, H.B. (1994). Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta *1197*, 63-93.

Reaves, B.J., Bright, N.A., Mullock, B.M., and Luzio, J.P. (1996). The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins suggests a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway. J Cell Sci *109 (Pt 4)*, 749-762.

Regnouf, F., Sagot, I., Delouche, B., Devilliers, G., Cartaud, J., Henry, J.P., and Pradel, L.A. (1995). "In vitro" phosphorylation of annexin 2 heterotetramer by protein kinase C. Comparative properties of the unphosphorylated and phosphorylated annexin 2 on the aggregation and fusion of chromaffin granule membranes. J Biol Chem *270*, 27143-27150.

Reutelingsperger, C.P. and van Heerde, W.L. (1997). Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. Cell Mol Life Sci *53*, 527-532.

Rojas, E. and Pollard, H.B. (1987). Membrane capacity measurements suggest a calcium-dependent insertion of synexin into phosphatidylserine bilayers. FEBS Lett *217*, 25-31.

Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C., and Burns, A.L. (1990). Calcium-activated endonexin II forms calcium channels across acidic phospholipid bilayer membranes. J Biol Chem *265*, 21207-21215.

Roth, D., Morgan, A., and Burgoyne, R.D. (1993). Identification of a key domain in annexin and 14-3-3 proteins that stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. FEBS Lett *320*, 207-210.

Roy, S., Luetterforst, R., Harding, A., Apolloni, A., Etheridge, M., Stang, E., Rolls, B., Hancock, J.F., and Parton, R.G. (1999). Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. Nat Cell Biol *1*, 98-105.

Saris, C.J., Kristensen, T., D'Eustachio, P., Hicks, L.J., Noonan, D.J., Hunter, T., and Tack, B.F. (1987). cDNA sequence and tissue distribution of the mRNA for bovine and murine p11, the S100-related light chain of the protein-tyrosine kinase substrate p36 (calpactin I). J Biol Chem *262*, 10663-10671.

Sato, H., Ogata, H., and De Luca, L.M. (2000). Annexin V inhibits the 12-O-tetradecanoylphorbol-13-acetate-induced activation of Ras/extracellular signal-regulated kinase (ERK) signaling pathway upstream of Shc in MCF-7 cells. Oncogene 2000.Jun.8.;19.(25.):2904.-12.

Sato, K., Saito, Y., and Kawashima, S. (1995). Identification and characterization of membrane-bound calpains in clathrin-coated vesicles from bovine brain. Eur J Biochem *230*, 25-31.

Sawyer, S.T. and Cohen, S. (1985). Epidermal growth factor stimulates the phosphorylation of the calcium-dependent 35,000-dalton substrate in intact A-431 cells. J Biol Chem *260*, 8233-8236.

Schafer, D.A., Gill, S.R., Cooper, J.A., Heuser, J.E., and Schroer, T.A. (1994). Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin. J Cell Biol *126*, 403-412.

Schlaepfer, D.D. and Haigler, H.T. (1990). Expression of annexins as a function of cellular growth state. J Cell Biol *111*, 229-238.

Schlaepfer, D.D., Fisher, D.A., Brandt, M.E., Bode, H.R., Jones, J.M., and Haigler, H.T. (1992). Identification of a novel annexin in Hydra vulgaris. Characterization, cDNA cloning, and protein kinase C phosphorylation of annexin XII. J Biol Chem *267*, 9529-9539.

Schlaepfer, D.D., Jones, J., and Haigler, H.T. (1992). Inhibition of protein kinase C by annexin V. Biochemistry *31*, 1886-1891.

Schmitz-Peiffer, C., Browne, C.L., Walker, J.H., and Biden, T.J. (1998). Activated protein kinase C alpha associates with annexin VI from skeletal muscle. Biochem J *330 (Pt 2)*, 675-681.

Schnapp, B.J. and Reese, T.S. (1989). Dynein is the motor for retrograde axonal transport of organelles. Proc Natl Acad Sci U S A *86*, 1548-1552.

Schnitzer, J.E., Liu, J., and Oh, P. (1995). Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases. J.Biol.Chem. *270*, 14399-14404.

Seemann, J., Weber, K., Osborn, M., Parton, R.G., and Gerke, V. (1996). The association of annexin I with early endosomes is regulated by Ca2+ and requires an intact N-terminal domain. Mol Biol Cell *7*, 1359-1374.

Seemann, J., Weber, K., and Gerke, V. (1996). Structural requirements for annexin I-S100C complex-formation. Biochem J *319 (Pt 1)*, 123-129.

Seemann, J., Weber, K., and Gerke, V. (1997). Annexin I targets S100C to early endosomes. FEBS Lett *413* , 185-190.

Selbert, S., Fischer, P., Pongratz, D., Stewart, M., and Noegel, A.A. (1995). Expression and localization of annexin VII (synexin) in muscle cells. J Cell Sci *108 (Pt 1)*, 85-95.

Selbert, S., Fischer, P., Menke, A., Jockusch, H., Pongratz, D., and Noegel, A.A. (1996). Annexin VII relocalization as a result of dystrophin deficiency. Exp Cell Res *222*, 199-208.

Senda, T., Okabe, T., Matsuda, M., and Fujita, H. (1994). Quick-freeze, deep-etch visualization of exocytosis in anterior pituitary secretory cells: localization and possible roles of actin and annexin II. Cell Tissue Res *277*, 51-60.

Shadle, P.J., Gerke, V., and Weber, K. (1985). Three Ca2+-binding proteins from porcine liver and intestine differ immunologically and physicochemically and are distinct in Ca2+ affinities. J Biol Chem *260*, 16354-16360.

Smart, E.J., Ying, Y.S., Conrad, P.A., and Anderson, R.G. (1994). Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. J.Cell Biol. *127*, 1185-1197.

Smart, E.J., Foster, D.C., Ying, Y.S., Kamen, B.A., and Anderson, R.G. (1994). Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalization of caveolae. J.Cell Biol. *124*, 307-313.

Smythe, E., Smith, P.D., Jacob, S.M., Theobald, J., and Moss, S.E. (1994). Endocytosis occurs independently of annexin VI in human A431 cells. J Cell Biol *124*, 301-306.

Solito, E., Raguenes-Nicol, C., de Coupade, C., Bisagni-Faure, A., and Russo-Marie, F. (1998). U937 cells deprived of endogenous annexin 1 demonstrate an increased PLA2 activity. Br J Pharmacol *124*, 1675-1683.

Song, K.S., Li, S., Okamoto, T., Quilliam, L.A., Sargiacomo, M., and Lisanti, M.P. (1996). Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J.Biol.Chem. *271*, 9690-9697.

Spreca, A., Rambotti, M.G., Giambanco, I., Pula, G., Bianchi, R., Ceccarelli, P., and Donato, R. (1992). Immunocytochemical localization of annexin V (CaBP33), a Ca(2+)-dependent phospholipid- and membrane-binding protein, in the rat nervous system and skeletal muscles and in the porcine heart. J Cell Physiol *152*, 587-598.

Srivastava, M., Atwater, I., Glasman, M., Leighton, X., Goping, G., Caohuy, H., Miller, G., Pichel, J., Westphal, H., Mears, D., Rojas, E., and Pollard, H.B. (1999). Defects in inositol 1,4,5-trisphosphate receptor expression, Ca(2+) signaling, and insulin secretion in the anx7(+/-) knockout mouse. Proc Natl Acad Sci U S A *96*, 13783-13788.

Stankewich, M.C., Tse, W.T., Peters, L.L., Ch'ng, Y., John, K.M., Stabach, P.R., Devarajan, P., Morrow, J.S., and Lux, S.E. (1998). A widely expressed betaIII spectrin associated with Golgi and cytoplasmic vesicles. Proc Natl Acad Sci U S A *95*, 14158-14163.

Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M., and Hancock, J.F. (1994). Activation of Raf as a result of recruitment to the plasma membrane. Science *264*, 1463-1467.

Storrie, B. and Desjardins, M. (1996). The biogenesis of lysosomes: is it a kiss and run, continuous fusion and fission process? Bioessays *18*, 895-903.

Sudhof, T.C., Ebbecke, M., Walker, J.H., Fritsche, U., and Boustead, C. (1984). Isolation of mammalian calelectrins: a new class of ubiquitous Ca2+-regulated proteins. Biochemistry *23*, 1103-1109.

Suen, K.L., Bustelo, X.R., and Barbacid, M. (1995). Lack of evidence for the activation of the Ras/Raf mitogenic pathway by 14-3-3 proteins in mammalian cells. Oncogene *11*, 825-831.

Swairjo, M.A. and Seaton, B.A. (1994). Annexin structure and membrane interactions: a molecular perspective. Annu Rev Biophys Biomol Struct *23*, 193-213.

Tagoe, C.E., Boustead, C.M., Higgins, S.J., and Walker, J.H. (1994). Characterization and immunolocalization of rat liver annexin VI. Biochim Biophys Acta *1192*, 272-280.

Taylor, S.J. and Shalloway, D. (1996). Cell cycle-dependent activation of Ras. Curr Biol *6*, 1621-1627.

Theobald, J., Smith, P.D., Jacob, S.M., and Moss, S.E. (1994). Expression of annexin VI in A431 carcinoma cells suppresses proliferation: a possible role for annexin VI in cell growth regulation. Biochim Biophys Acta *1223*, 383-390.

Theobald, J., Hanby, A., Patel, K., and Moss, S.E. (1995). Annexin VI has tumour-suppressor activity in human A431 squamous epithelial carcinoma cells. Br.J Cancer *71*, 786-788.

Thiel, C., Osborn, M., and Gerke, V. (1992). The tight association of the tyrosine kinase substrate annexin II with the submembranous cytoskeleton depends on intact p11- and Ca(2+)-binding sites. J Cell Sci *103 (Pt 3)*, 733-742.

Thorin, B., Gache, G., Dubois, T., Grataroli, R., Domingo, N., Russo-Marie, F., and Lafont, H. (1995). Annexin VI is secreted in human bile. Biochem Biophys Res Commun *209*, 1039-1045.

Tokumitsu, H., Mizutani, A., Minami, H., Kobayashi, R., and Hidaka, H. (1992). A calcyclin-associated protein is a newly identified member of the Ca2+/phospholipid-binding proteins, annexin family. J Biol Chem *267*, 8919-8924.

Tosi, E., Valota, O., Negri, D.R., Adobati, E., Mazzoni, A., Meazza, R., Ferrini, S., Colnaghi, M.I., and Canevari, S. (1995). Anti-tumor efficacy of an anti-epidermal-growth-factor-receptor monoclonal antibody and its F(ab')2 fragment against high- and low-EGFR-expressing carcinomas in nude mice. Int J Cancer *62*, 643-650.

Tressler, R.J., Updyke, T.V., Yeatman, T., and Nicolson, G.L. (1993). Extracellular annexin II is associated with divalent cation-dependent tumor cell-endothelial cell adhesion of metastatic RAW117 large-cell lymphoma cells. J Cell Biochem *53*, 265-276.

Tressler, R.J., Yeatman, T., and Nicolson, G.L. (1994). Extracellular annexin VI expression is associated with divalent cation-dependent endothelial cell adhesion of metastatic RAW117 large-cell lymphoma cells. Exp Cell Res *215*, 395-400.

Trouve, P., Legot, S., Belikova, I., Marotte, F., Benevolensky, D., Russo-Marie, F., Samuel, J.L., and Charlemagne, D. (1999). Localization and quantitation of cardiac annexins II, V, and VI in hypertensive guinea pigs. Am J Physiol *276*, H1159-H1166

Tuma, P.L., Finnegan, C.M., Yi, J.H., and Hubbard, A.L. (1999). Evidence for apical endocytosis in polarized hepatic cells: phosphoinositide 3-kinase inhibitors lead to the lysosomal accumulation of resident apical plasma membrane proteins. J Cell Biol *145*, 1089-1102.

Turpin, E., Russo-Marie, F., Dubois, T., de Paillerets, C., Alfsen, A., and Bomsel, M. (1998). In adrenocortical tissue, annexins II and VI are attached to clathrin coated vesicles in a calcium-independent manner. Biochim Biophys Acta *1402*, 115-130.

Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K., and Ohno, S. (1996). Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem *271*, 23512-23519.

Vaaraniemi, J., Huotari, V., Lehto, V.P., and Eskelinen, S. (1994). The effects of PMA and TFP and alterations in intracellular pH and calcium concentration on the membrane associations of phospholipid-binding proteins fodrin, protein kinase C and annexin II in cultured MDCK cells. Biochim Biophys Acta *1189*, 21-30.

van Biesen, T., Hawes, B.E., Raymond, J.R., Luttrell, L.M., Koch, W.J., and Lefkowitz, R.J. (1996). G(o)-protein alpha-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. J Biol Chem *271*, 1266-1269.

van Engeland, M., Ramaekers, F.C., Schutte, B., and Reutelingsperger, C.P. (1996). A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry *24*, 131-139.

Wang, S., Dibenedetto, A.J., and Pittman, R.N. (1997). Genes induced in programmed cell death of neuronal PC12 cells and developing sympathetic neurons in vivo. Dev Biol *188*, 322-336.

Wang, X., Campos, B., Kaetzel, M.A., and Dedman, J.R. (1999). Annexin V is critical in the maintenance of murine placental integrity . Am J Obstet Gynecol *180*, 1008-1016.

Wang, Z., Tung, P.S., and Moran, M.F. (1996). Association of p120 ras GAP with endocytic components and colocalization with epidermal growth factor (EGF) receptor in response to EGF stimulation. Cell Growth Differ *7*, 123-133.

Wary, K.K., Mariotti, A., Zurzolo, C., and Giancotti, F.G. (1998). A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell *94*, 625-634.

Watanabe, T., Inui, M., Chen, B.Y., Iga, M., and Sobue, K. (1994). Annexin VI-binding proteins in brain. Interaction of annexin VI with a membrane skeletal protein, calspectin (brain spectrin or fodrin). J Biol Chem *269*, 17656-17662.

Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999). The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. J Biol Chem *274*, 22151-22154.

Weinman, J.S., Feinberg, J.M., Rainteau, D.P., Gaspera, B.D., and Weinman, S.J. (1994). Annexins in rat enterocyte and hepatocyte: an immunogold electron-microscope study. Cell Tissue Res *278*, 389-397.

Wheeler-Jones, C.P., May, M.J., Houliston, R.A., and Pearson, J.D. (1996). Inhibition of MAP kinase kinase (MEK) blocks endothelial PGI2 release but has no effect on von Willebrand factor secretion or E-selectin expression. FEBS Lett *388*, 180-184.

Wheeler-Jones, C.P., Learmonth, M.P., Martin, H., and Aitken, A. (1996). Identification of 14-3-3 proteins in human platelets: effects of synthetic peptides on protein kinase C activation. Biochem J *315 (Pt 1)*, 41-47.

Wice, B.M. and Gordon, J.I. (1992). A strategy for isolation of cDNAs encoding proteins affecting human intestinal epithelial cell growth and differentiation: characterization of a novel gut-specific N-myristoylated annexin. J Cell Biol *116*, 405-422.

Wilde, A., Beattie, E.C., Lem, L., Riethof, D.A., Liu, S.H., Mobley, W.C., Soriano, P., and Brodsky, F.M. (1999). EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. Cell *96*, 677-687.

William, F., Mroczkowski, B., Cohen, S., and Kraft, A.S. (1988). Differentiation of HL-60 cells is associated with an increase in the 35-kDa protein lipocortin I. J Cell Physiol *137*, 402-410.

Wood, K.W., Sarnecki, C., Roberts, T.M., and Blenis, J. (1992). ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell *68*, 1041-1050.

Wood, S.A. and Brown, W.J. (1992). The morphology but not the function of endosomes and lysosomes is altered by brefeldin A. J Cell Biol *119*, 273-285.

Xiao, B., Smerdon, S.J., Jones, D.H., Dodson, G.G., Soneji, Y., Aitken, A., and Gamblin, S.J. (1995). Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. Nature *376*, 188-191.

Xu, J., Ziemnicka, D., Merz, G.S., and Kotula, L. (2000). Human spectrin Src homology 3 domain binding protein 1 regulates macropinocytosis in NIH 3T3 cells. J Cell Sci *113*, 3805-3814.

Zaks, W.J. and Creutz, C.E. (1990). Annexin-chromaffin granule membrane interactions: a comparative study of synexin, p32 and p67. Biochim.Biophys.Acta *1029*, 149-160.

Zaks, W.J. and Creutz, C.E. (1990). Evaluation of the annexins as potential mediators of membrane fusion in exocytosis. J.Bioenerg.Biomembr. *22*, 97-120.

Zeng, F.Y., Gerke, V., and Gabius, H.J. (1993). Identification of annexin II, annexin VI and glyceraldehyde-3-phosphate dehydrogenase as calcyclin-binding proteins in bovine heart. Int J Biochem *25*, 1019-1027.

Zokas, L. and Glenney, J.R.J. (1987). The calpactin light chain is tightly linked to the cytoskeletal form of calpactin I: studies using monoclonal antibodies to calpactin subunits. J Cell Biol *105*, 2111-2121.

Zou, Y., Komuro, I., Yamazaki, T., Aikawa, R., Kudoh, S., Shiojima, I., Hiroi, Y., Mizuno, T., and Yazaki, Y. (1996). Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Raf-1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. J Biol Chem *271*, 33592-33597.

Relació de publicacions obtingudes durant la realització d'aquesta tesi doctoral.

- **Mònica Pons**, Gudrun Ihrke, Stefanie Koch, Michael Biermer, Albert Pol, Thomas Grewal, Stefan Jäckle i Carlos Enrich
  Late endocytic compartments are major sites of annexin VI localization in NRK fibroblasts and polarized WIF-B hepatoma cells

  EXPERIMENTAL CELL RESEARCH, 257(1), 33-47 (2000)

- **Mònica Pons**, Thomas Grewal, Eulalia Rius, Stefan Jäckle i Carlos Enrich
  The intracellular trafficking at the pre-lysosomal compartment is modulated by annexin VI

  EXPERIMENTAL CELL RESEARCH. (Acceptat en revisió)

- **Mònica Pons**, Francesc Tebar, Sandra Peiró, Thomas Grewal i Carlos Enrich
  Activation of Raf-1 and Ras is defective in annexin 6 overexpressing Chinese hamster ovary cells

  FEBS letter. (enviat)

- Carlos Enrich, Albert Pol, Maria Calvo, **Mònica Pons** i Stefan Jäckle.
  Dissection of the multifunctional "Receptor-Recycling endocytic compartment of hepatocytes.

  HEPATOLOGY, vol. 30: 1115-1120 (1999)

- Maria Calvo, Albert Pol, Albert Lu, David Ortega, **Mònica Pons,** Joan Blasi i Carles Enrich.
  Cellubrevin is present in the basolateral endocytic compartment of hepatocytes and follows the transcytotic pathway after IgA internalization.

  JOURNAL OF BIOLOGICAL CHEMISTRY,275: 7910-7917 (2000).

- Albert Pol, Maria Calvo, **Mònica Pons**, Sandra Peiró, Noemí Andrés, Francesc Tebar i Carlos Enrich.
  Role of the hepatic endocytic compartment in signal transduction.

  ELECTRONIC JOURNAL OF PATHOLOGY I HISTOLOGY. March 2000, number 6.1

- Albert Pol, Albert Lu, **Mònica Pons**, Sandra Peiró, i Carlos Enrich.
  EGF-mediated caveolin recruitment to early endosomes and MAPK activation. Role of cholesterol and actin-cytoskeleton.

  JOURNAL OF BIOLOGICAL CHEMISTRY. Jul. 2000