

Annexin I Is Phosphorylated in the Multivesicular Body During the Processing of the Epidermal Growth Factor Receptor

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Abstract. We have previously shown that an active epidermal growth factor receptor (EGF-R) kinase is necessary for efficient sorting of the EGF-R to the lysosome, and we have shown that this occurs in the multivesicular body (MVB), where EGF-R are sorted away from recycling receptors by being removed to the internal vesicles of the MVB. The aim of the present study was to identify substrates of the EGF-R kinase associated with MVBs which might play a role in this sorting process. We used a density shift technique to isolate MVBs and show that the major substrates phosphorylated in vitro within MVBs which contain an active EGF-R kinase are the EGF-R itself and annexin I. Annexin I is associated with both plasma membrane

and MVBs in a calcium-independent manner but can be phosphorylated in vitro only in MVBs. Phosphorylation of calcium-independent annexin I in isolated MVBs converts it to a form that requires calcium for membrane association. In cells with an active EGF-R kinase the amount of calcium-independent annexin I in MVBs is reduced, suggesting that a phosphorylation-induced conversion of the calcium independent to the calcium-dependent form also occurs in vivo. Our observations, together with the known properties of annexin I in mediating membrane fusion, suggest that inward vesiculation in MVBs is induced by the EGF-R and is mediated by phosphorylated annexin I.

WHEN EGF binds to its receptor the intrinsic tyrosine kinase of the receptor is activated, resulting in phosphorylation of the receptor itself and various other proteins (Ushiro and Cohen, 1980). During this time the EGF-EGF-receptor (EGF-R)¹ complex is rapidly internalized and processed within the endocytic pathway where it becomes degraded (Carpenter and Cohen, 1976). A mutant EGF-R lacking an active kinase is internalized in response to EGF at the same rate as the wild type EGF-R but the kinase-negative EGF-R is not efficiently degraded and a significant proportion of the mutant EGF-R recycle to the cell surface (Honegger et al., 1987; Felder et al., 1990). By EM we have shown that saturating concentrations of EGF stimulate internalization of both wild type and kinase negative EGF-R to multivesicular bodies but within this compartment they have distinctly different distributions (Felder et al., 1990). Since only wild type EGF-R are efficiently transferred to the internal vesicles of the multivesicular body (MVB) we have proposed that removal of EGF-R from the perimeter membrane of the endocytic pathway allows them to be degraded.

While it is possible that the activated EGF-R kinase initiates a series of events in the plasma membrane which results in sorting at the level of the MVB it is also possible that the

EGF-R kinase is active in the endosome where it triggers the events that lead to its degradation. The aim of the present study was to determine whether there are substrates of the EGF-R kinase specifically associated with MVBs. We have adapted a previously described density shift protocol (Futter and Hopkins, 1989) to isolate highly purified MVBs containing EGF-R and show that the major substrates of the EGF-R kinase in MVBs are the EGF-R itself and annexin I. Since annexin I is a protein which interacts with membranes and actin-containing cytoskeletal elements (Glenney et al., 1987) and can promote phospholipid vesicle fusion in vitro (Ernst et al., 1990), it could regulate the production of internal vesicles within MVBs.

Materials and Methods

Cell Lines

NIH 3T3 cells, strain 2.2, transfected with plasmid bearing either the full-length cDNA for the human EGF-R (HER14 cells), or the cDNA encoding the human EGF-R with lysine 721 replaced with alanine (K721A cells), and each expressing roughly 400,000 receptors per cell, were used (Honegger et al., 1987). Cells were maintained in DME supplemented with 10% FCS at 37°C in a 5% CO₂ atmosphere.

Colloidal Gold Complexes

Gold particles (10 nm) were prepared using the tannic acid method of Slot and Geuze, (1985), and were stabilized with the mAb 108, according to

1. *Abbreviations used in this paper:* EGF-R, epidermal growth factor receptor; MVB, multivesicular body.

standard procedures (DeMey, 1986). The complexes were stored in 0.02% azide at 4°C, and were washed by centrifugation in an airfuge (Beckman Instruments, Palo Alto, CA) at 150,000 g for 5 min immediately before use.

Iodination

EGF (mouse EGF; Sigma Chemical Co., Poole, UK) was iodinated according to the method of Hunter and Greenwood (1962) and protein A was iodinated using iodobeads (Pierce, Chester, UK).

Incubation Procedures

All incubations were performed in DME containing 20 mM Hepes, pH 7.4, 2 mg/ml BSA. EGF was used at a concentration of 20 nM. 108-gold was used at a concentration that gave OD₅₈₀ of 0.3–0.4. Incubations were performed at 37°C unless otherwise indicated and for the times indicated in Results. Where the proportion of cell-associated ¹²⁵I-EGF that was plasma membrane bound was to be determined, cells were incubated in acetic acid-saline, pH 2.5, for 5 min at 4°C to remove plasma membrane-bound EGF, before digestion of the cells in 5 M NaOH.

Subcellular Fractionation Procedure

Cells were washed three times at 4°C with lysis buffer (10 mM triethanolamine, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 200 μM orthovanadate, 1 mM PMSF). Cells were scraped off the dish in the minimum volume of lysis buffer and lysed by eight strokes through a 21 g needle. Unbroken cells and nuclei were removed by centrifugation at 800 g for 5 min at 4°C. The resulting postnuclear supernatant was layered onto a 12 ml 26–52% sucrose gradient, containing triethanolamine, EDTA, orthovanadate, and PMSF at the same concentrations and pH as the lysis buffer. Gradients were then centrifuged in a Beckman SW40 rotor at 200,000 g for 15 h at 4°C in a Beckman L70 ultracentrifuge. Gradients were fractionated from the bottom into 15 × 0.9-ml fractions.

EM

Cells and fractions were fixed in dilute Karnovsky fixative, postfixed in osmium tetroxide, and embedded and sectioned so that the full thickness of the pellet could be examined. Sections were stained in aqueous uranyl acetate and lead citrate and examined in a CM12 Philips electron microscope.

SDS-PAGE and Western Blotting

SDS-PAGE was performed under reducing conditions. Proteins on SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose for 16 h in a transfer apparatus (Bio-Rad Laboratories, Cambridge, MA) at a constant current of 100 mA. After incubation with either rabbit anti-EGF-R antiserum, RK2 (Kris et al., 1985), rabbit anti-phosphotyrosine antiserum, or rabbit anti-annexin I antiserum (a gift from S. Moss, University College, London), blots were washed and incubated in ¹²⁵I-protein A. Blots were analyzed by autoradiography and, where quantitation was performed, radioactive bands were excised, and counted.

In Vitro Phosphorylation Studies

Cell fractions were resuspended in 20 mM Hepes, pH 7.4, 150 mM NaCl. Fractions were incubated for 15 min at 4°C in the presence of 5 mM MnCl₂, 20 μM orthovanadate, and [γ -³²P]ATP (5 μM). Reactions were stopped by the addition of 10% TCA, and TCA precipitates were examined by SDS-PAGE.

To examine the effects of phosphorylation on calcium dependence of membrane association of MVB proteins, phosphorylation reactions were performed as above. MVBs were then washed twice with 20 mM Hepes, pH 7.4, 150 mM NaCl, 200 μM orthovanadate, containing either 1.5 mM EDTA or 1.5 mM CaCl₂, by centrifugation in a Beckman TL100.4 rotor at 200,000 g for 1 h at 4°C in a Beckman TLX ultracentrifuge, and the pellets were examined by SDS-PAGE.

Annexin I Immunoprecipitation

To identify annexin I, *in vitro* phosphorylation assays were carried out as above, but were stopped by the addition of NDET (10 mM Tris, pH 7.4, 1% NP-40, 0.4% deoxycholate, 66 mM EDTA), containing 1 mM PMSF, 200 μM orthovanadate and 0.2% SDS. Samples were clarified by centrifugation (14,000 g for 5 min), and were then incubated with a rabbit polyclonal

antibody against annexin I. Antibody-bound proteins were immunoprecipitated using *Staphylococcus aureus*, and the washed immunoprecipitate was analyzed by SDS-PAGE.

Results

Isolation of Highly Purified Endosomes by Density-shift Using EGF-R Antibody-Gold Complexes

We have previously shown in H.Ep.2 cells that antibody to the transferrin receptor complexed to colloidal gold can be used to modify the density of endocytic compartments involved in the processing of the EGF-receptor complex, so that they can be purified by sucrose density centrifugation (Futter and Hopkins, 1989). In the present study we extended this technique to NIH-3T3 cells transfected with either the wild type human EGF receptor or a human EGF receptor with a point mutation in the putative ATP binding site, rendering the receptor kinase negative (Honegger et al., 1987). To isolate endocytic compartments containing EGF-R by density shift we employed a mAb (108) to EGF-R complexed to colloidal gold. The 108 antibody binds to the external domain of the EGF-R and has been shown, when saturating EGF concentrations are used, not to stimulate internalization or kinase activity of the EGF-R, or to interfere with EGF binding (Bellot et al., 1990). To induce a sufficient increase in density of endocytic compartments containing 108-gold, it was necessary to stimulate cells with saturating concentrations of EGF. Under these conditions both the kinase positive and negative EGF-R are internalized at similar rates (Felder et al., 1990). Our previous studies using gold complexes to density shift endocytic compartments showed that gold-loaded endosomes will pellet through 52% sucrose whereas gold-loaded plasma membranes will not (Futter and Hopkins, 1989). To begin we established the rate of EGF-R internalization by following ¹²⁵I-EGF uptake, and showed that >70% was internalized within 20 min of EGF stimulation (Fig. 1 a).

Fig. 1 b shows the recovery of EGF and EGF-R in the pellet after sucrose density gradient fractionation of cells after similar stimulation with EGF. Recovery of EGF-R was assessed by Western blotting with anti-EGF-R antibody. The increase in recovery of EGF and EGF-R in the pellet with increasing lengths of incubation after EGF stimulation showed similar kinetics to that of EGF-stimulated internalization, showing that endocytic compartments rather than plasma membrane pellet under the fractionation conditions used.

Examination of the endosome pellet isolated by fractionation of 108-gold-loaded cells expressing the wild type EGF-R 20 min after EGF stimulation showed that all membranous elements labeled with gold were endosomal, many of which were clearly identifiable as MVBs (Fig. 2 a). Quantitation of the number of vesicles which contained gold by counting random sections showed that 34% of vesicle profiles found in the pellet contained gold. Vesicle profiles which lacked gold presumably contained gold particles in another section plane. Electron microscopic examination of the pellet from cells expressing the kinase negative EGF-R showed that 14% of the vesicle profiles contained gold. This lower figure compared to the wild type fraction is consistent

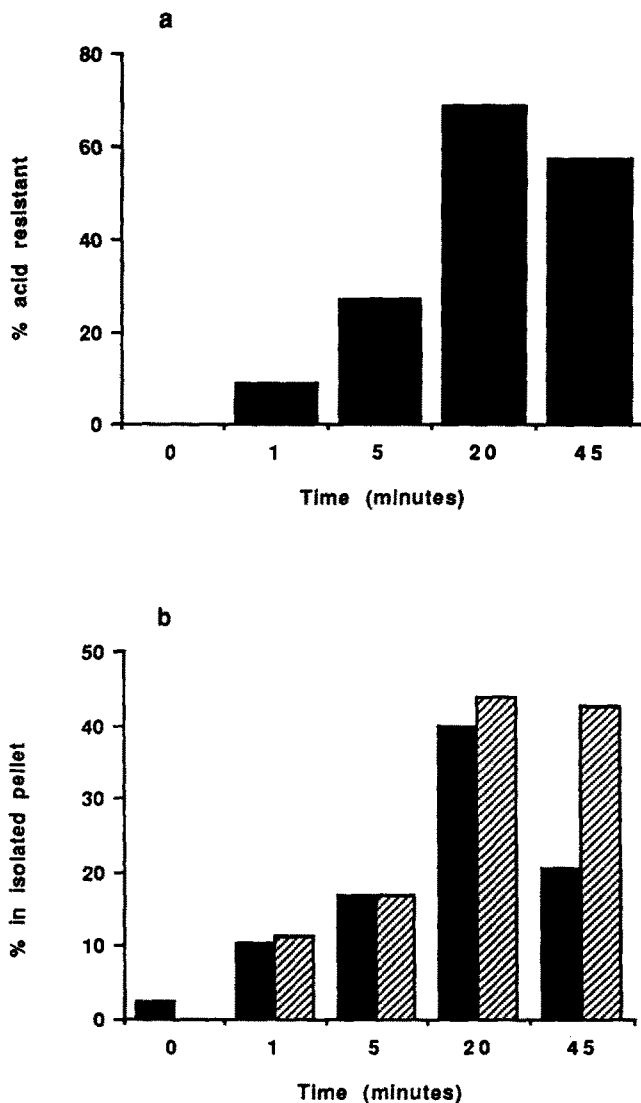


Figure 1. (a) Cells expressing wild type EGF-R were stimulated with ¹²⁵I-EGF for the indicated times. The percent of total cell-associated EGF that had been internalized was determined by acid stripping. (b) Cells expressing wild type EGF-R were incubated with 108-gold for 30 min and were then stimulated with ¹²⁵I-EGF for the indicated times. Cells were lysed and fractionated. The percent of postnuclear supernatant ¹²⁵I-EGF (◻) and EGF-R (■) that was recovered in the pellet after sucrose density gradient fractionation was determined. Quantitation of the EGF-R was performed by blotting gradient fractions with anti-EGF-R antibody. Iodinated protein A and autoradiography were used to visualize bands, which were excised and counted.

with observations on intact cells (Felder et al., 1990) which have shown that there is significantly less EGF-R in the MVB compartment of kinase negative cells. It should be noted nevertheless that the majority of gold-loaded elements in the pellets from the kinase negative cells are MVBs (Fig. 2 b). Presumably the other gold-containing elements of the endocytic pathway were too buoyant to pellet. Previous studies have shown, through measurement of marker enzyme activities and by EM, that in a number of different cell lines nonendosomal organelles do not pellet under the fractionation conditions used (Beardmore et al., 1988; Futter et al., 1989; Beaumelle and Hopkins, 1990). However amorphous mate-

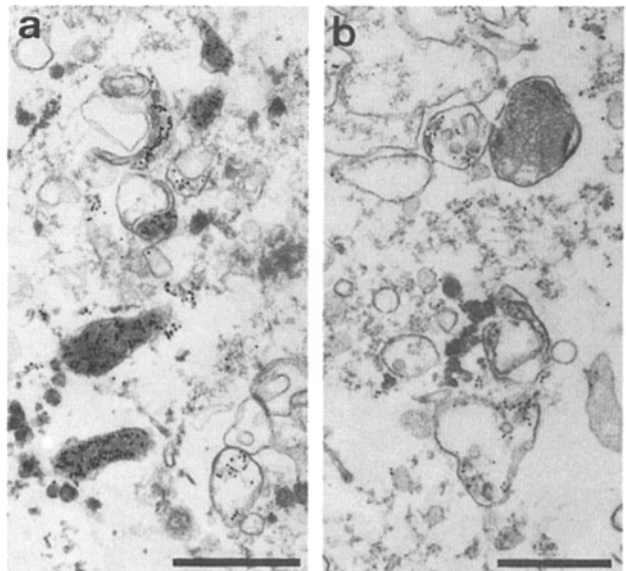


Figure 2. Cells expressing either wild type (a) or kinase negative (b) EGF-R were incubated with 108-gold for 30 min, and were then stimulated with EGF for 20 min. Cells were then lysed and fractionated, and the pellet after sucrose gradient fractionation was processed for microscopy. All membranous elements that contain gold are endosomal, many of which are MVBs. Amorphous material, presumably of cytoskeletal nature, consistently pellets with endosome fractions. Bar, 0.5 μm.

rial, presumably a component of the cytoskeleton, consistently pellets with endosome fractions.

The Fate of the Plasma Membrane during Density Shift Separation of Gold-loaded Endosomes

When cells were stimulated with EGF for 1 min after preincubation with 108-gold (when the majority of EGF-R are plasma membrane associated), the density of the plasma membrane fraction was increased. Thus, in the absence of 108-gold binding, plasma membrane was found in a peak in fractions 8-12 (Fig. 3 a) while binding of 108-gold to the plasma membrane caused a shift to fraction 6 (Fig. 3 b). After stimulation of cells with EGF for one minute EGF-R was still found in fraction 6 (Fig. 3 c). EM of this fraction showed that it was composed of large vesicle profiles bearing EGF-R-gold particles on their outer surfaces (results not shown), in contrast to MVBs where the gold particles were found only on the inner surfaces of the perimeter membrane.

Substrates Phosphorylated in Endosomes and Plasma Membrane Isolated from Cells Expressing Wild type EGF-R Kinase

To determine whether proteins are phosphorylated in MVBs as a result of activation of the EGF-R kinase, phosphorylation was examined in endosomes isolated from NIH 3T3 cells expressing either the wild type or the kinase negative EGF-R. Endosomes were isolated 20 min after EGF stimulation when the majority of both kinase negative and wild type EGF-R are in MVBs (Felder et al., 1990) and the density shifted fractions are composed primarily of MVBs (Fig. 2). When MVBs isolated from NIH 3T3 cells expressing the wild type EGF-R were incubated with [^γ-³²P]ATP in vitro

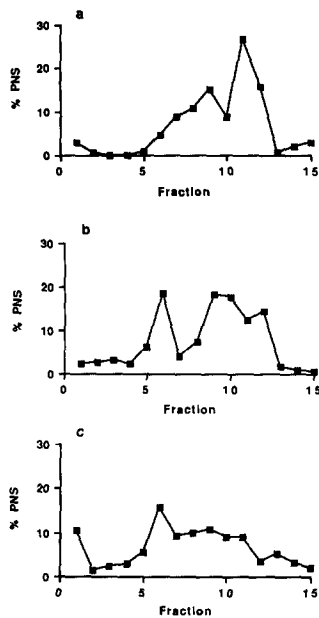


Figure 3. Cells expressing wild type EGF-R were incubated with no gold (a), 108-gold for 30 min (b), or 108-gold for 30 min, followed by stimulation with EGF for 1 min (c). Cells were lysed and fractionated and gradient fractions were analyzed by Western blotting with anti-EGF-R antibody. Iodinated protein A and autoradiography were used to visualize bands, which were excised and counted.

two major phosphorylated substrates were detected, the EGF-R itself and a protein of approximately 35 kD (Fig. 4 a). The 35-kD protein could be immunoprecipitated with anti-annexin I antibody (Fig. 4 b).

To determine whether annexin I is phosphorylated in plasma membrane fractions in addition to MVBs, plasma membrane fractions were isolated from cells stimulated with EGF for one minute. When these fractions were incubated with [γ - 32 P]ATP in vitro several substrates were phosphorylated including the EGF-R (Fig. 4 a), but immunoprecipitation with annexin I antibody showed they did not include annexin I (Fig. 4 b).

MVB and plasma membrane fractions from cells expressing the kinase negative EGF-R, used as a control, showed that neither the EGF-R nor the 35-kD protein were phosphorylated (Fig. 4, a and b).

To confirm that the phosphorylation of annexin I in the MVB was due to the presence of an active EGF-R kinase we carried out a parallel study using H.Ep.2 cells in which we have shown previously that EGF-R containing MVBs can be isolated by density shift when loaded with anti-transferrin receptor-gold complexes and stimulated with EGF (Futter and Hopkins, 1989). Western blotting the isolated MVBs showed that EGF stimulation of transferrin receptor-gold-loaded cells resulted in a fivefold increase in the amount of EGF-R that co-localized with transferrin receptor in MVBs (results not shown). In vitro phosphorylation assays showed that a number of proteins became phosphorylated in MVBs isolated from H.Ep.2 cells, but only in MVBs that contained an active EGF-R kinase did the EGF-R and a 35-kD protein become phosphorylated (Fig. 5 a). Immunoprecipitation confirmed this protein was annexin I (Fig. 5 b).

We conclude that annexin I is present in isolated MVBs and that it can be phosphorylated in vitro in the presence of an active EGF-R kinase.

Phosphorylation and the Calcium Dependence of Annexin I Binding to MVBs and Plasma Membrane

Annexin I has been isolated as an EDTA eluate of mem-

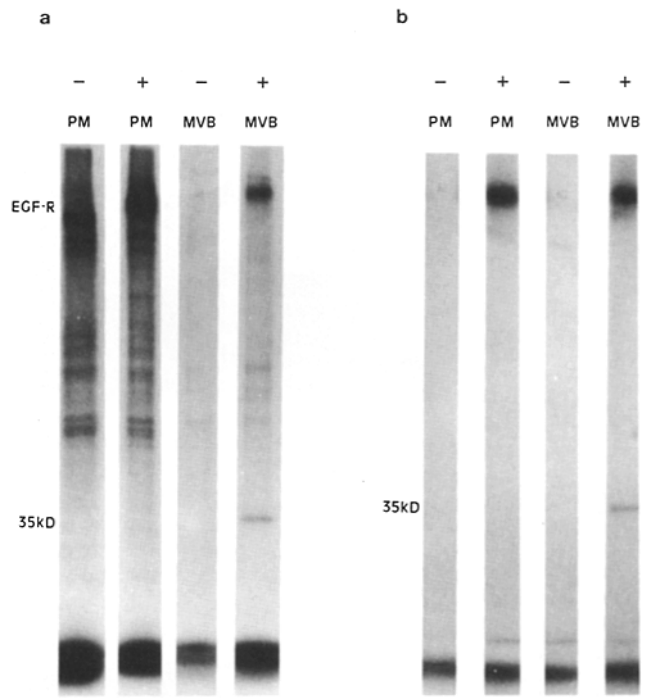


Figure 4. NIH 3T3 cells expressing either the wild type (+) or the kinase negative (-) EGF-R were incubated with 108-gold for 30 min. Cells were then stimulated with EGF for 1 min to isolate a plasma membrane fraction (PM) or for 20 min to isolate an MVB fraction (MVB). Cells were then lysed and fractionated. The PM fraction was taken from fraction 6 and the MVB fraction was taken from the pellet after sucrose gradient fractionation. In vitro phosphorylation reactions were performed on the isolated fractions as described in Materials and Methods. Either the whole fraction (a) or an anti-annexin I immunoprecipitation of the fraction (b) were analyzed by SDS-PAGE on a 10% gel. The EGF-R is present in anti-annexin I immunoprecipitations because the fractions contain anti-EGF-R gold, but in control immunoprecipitations that do not include anti-annexin I antibody, annexin I is not immunoprecipitated. Gel loadings with respect to the EGF-R are 1:2:1:1.4 for -PM: +PM: -MVB: +MVB.

branes and shown to associate with phospholipids in a calcium dependent manner (Glenney et al., 1987). Haigler et al., (1987) have shown that in addition to the calcium-dependent form there is a form of annexin I in human placenta which associates with membranes independently of calcium and can be phosphorylated by the EGF-R kinase. Phosphorylation of this form of annexin I converts the protein into a form that associates with membranes only in the presence of calcium. The MVBs used in the present study are isolated in the presence of EDTA and so the annexin I that is phosphorylated in vitro must remain associated with membranes in the absence of calcium. In order to determine the effect of phosphorylation on the calcium dependence of annexin I in MVBs, isolated fractions were incubated with [γ - 32 P]ATP and then washed two times in lysis buffer containing either 1.5 mM EDTA or 1.5 mM Ca^{2+} . The membrane pellet was examined by SDS-PAGE. After washing in EDTA phosphorylated annexin I was no longer associated with the MVB pellet. However in the presence of calcium the protein remained associated with MVBs (Fig. 6).

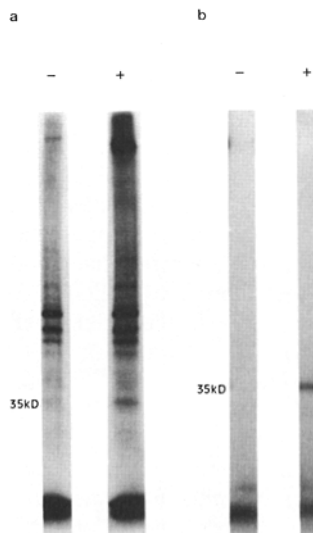


Figure 5. H.Ep.2 cells were incubated with B3/25 (anti-transferrin receptor)-gold for 60 min and were then incubated in the presence (+) or absence (-) of EGF for 20 min (in the continued presence of B3/25-gold). Cells were then lysed and fractionated. In vitro phosphorylation reactions were performed on the MVB fractions as described in Materials and Methods. Either the whole fraction (a) or an anti-annexin I immunoprecipitation of the fraction (b) were analyzed by SDS-PAGE on a 10% gel.

The Influence of EGF-R Kinase on the Amount of Annexin I Associated with Plasma Membrane and MVBs

To determine whether an active EGF-R kinase is necessary for association of annexin I with MVBs isolated MVBs were Western blotted with anti-annexin I antibody and the amount of annexin I relative to EGF-R was quantitated. Annexin I was present in MVB fractions from cells expressing the wild type EGF-R and also those expressing the kinase negative EGF-R. However MVBs from NIH 3T3 cells containing wild type EGF-R had three- to fourfold less annexin I (relative to EGF-R) than kinase negative MVBs (Fig. 7). Similarly annexin I was present in MVBs from H.Ep.2 cells iso-

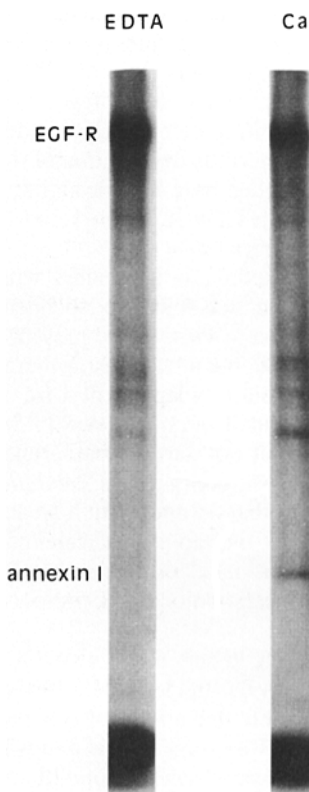


Figure 6. NIH 3T3 cells expressing wild type EGF-R were incubated with 108-gold for 30 min and were then stimulated with EGF for 20 min. Cells were then lysed and fractionated. In vitro phosphorylation reactions were performed on the MVB fractions as described in Materials and Methods. MVBs were then washed in the presence of EDTA (EDTA) or calcium (Ca). Washed pellets were analyzed by SDS-PAGE on a 10% gel.

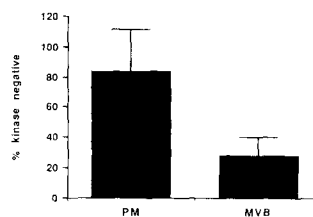


Figure 7. (a) NIH 3T3 cells expressing wild type (+) or kinase negative (-) EGF-R were incubated with 108 gold for 30 min. Cells were then stimulated with EGF for 1 min and a plasma membrane fraction isolated (PM) or for 20 min and an MVB fraction iso-

lated (MVB). Fractions were Western blotted with anti-EGF-R antibody and anti-annexin-I antibody. Iodinated protein A and autoradiography were used to visualize bands, which were excised and counted. The amount of annexin I (relative to the EGF-R) in fractions from wild type cells is expressed as a % of that in the corresponding fractions from kinase negative cells. Results are mean \pm SEM of four observations.

lated using transferrin receptor gold with or without prior EGF stimulation, but MVBs containing an active EGF-R kinase contained approximately twofold less annexin I (relative to transferrin receptor) than MVBs lacking an active EGF-R kinase (results not shown). We conclude therefore that an active EGF-R kinase is not necessary for association of annexin I with MVBs. In addition, the reduction in the amount of calcium-independent annexin I in MVBs containing an active EGF-R kinase is consistent with the phosphorylation of annexin I in vivo, as we have shown phosphorylation of annexin I in vitro to cause its release from MVBs in the presence of calcium chelator. To confirm, as our in vitro results would suggest, that the loss of calcium-independent annexin I through phosphorylation occurs in MVBs rather than on the plasma membrane, the amount of annexin I associated with plasma membrane fractions containing wild type and those containing kinase negative EGF-R was compared. Plasma membrane fractions containing wild type EGF-R and those containing kinase negative EGF-R contained similar levels of annexin I relative to EGF-R (Fig. 7), suggesting that the release of calcium independent annexin I through phosphorylation does not occur on the plasma membrane.

Discussion

We have shown in previous studies that an active EGF-R kinase is necessary for lysosomal sorting (Honegger et al., 1987; Felder et al., 1990). Other investigators have also shown a reduction in EGF-stimulated degradation of EGF in cells expressing a kinase negative EGF-R but they have concluded that this is due to a reduced internalization rate, rather than to defective lysosomal sorting (Glenny et al., 1988; Chen et al., 1989; Wiley et al., 1991). Most recently Felder et al. (1992) obtained data to suggest that the role of the EGF-R kinase in internalization concerns primarily high affinity EGF-R. At the saturating concentrations of EGF used in the present study our previous biochemical and morphological studies (Felder et al., 1990) clearly show that there is a similar rate of internalization of both the wild type and the kinase negative EGF-R and it is in the MVB that the pathways of the wild type and kinase negative EGF-R diverge. A recent study of another growth factor receptor kinase, the receptor for colony stimulating factor 1, has shown that an active kinase is not necessary for internalization but is a requirement for lysosomal sorting (Carlberg et al., 1991).

The requirement for an active EGF-R kinase for efficient degradation indicates that either the EGF-R must be autophosphorylated to be efficiently targeted to lysosomes, or the kinase phosphorylates a substrate necessary for lysosomal targeting. When kinase positive and kinase negative EGF-R are expressed in the same cell, heterodimers form in response to EGF and the kinase negative EGF-R becomes tyrosine phosphorylated (Honegger et al., 1990). However, in this system kinase negative EGF-R are still predominantly recycled. It is therefore likely that phosphorylation of a substrate accessible only in the MVB is necessary for degradation. This would require the EGF-R kinase to be active in the MVB. There is considerable evidence that the EGF-R kinase is active in the endosome. Autophosphorylation activity of the EGF-R kinase has been demonstrated *in vitro* in endosome fractions from A431 cells (Cohen and Fava, 1985) and rat liver (Kay et al., 1986; Lai et al., 1989). We and others (Carpentier et al., 1987; Nesterov et al., 1990) have shown that EGF stimulation results in prolonged autophosphorylation of the EGF-R, suggesting that the EGF-R kinase is still active after internalization *in vivo*. Wada et al. (1992) have shown that the EGF-R kinase is more highly phosphorylated in the endosome than on the plasma membrane. Moreover the internalized EGF-R kinase can phosphorylate a synthetic substrate introduced into permeabilized cells (Nesterov et al., 1990).

In this study we searched for substrates of the EGF-R kinase associated with MVBs. Western blotting isolated MVBs with anti-phosphotyrosine antibody failed to reveal any major substrates of the EGF-R kinase apart from the EGF-R itself. However, when highly purified MVBs containing the wild type EGF-R are incubated with [γ - 32 P]ATP the major proteins phosphorylated are the EGF-R itself and annexin I. Neither proteins are phosphorylated in MVBs containing the kinase negative EGF-R. Similarly when MVBs from H.Ep.2 cells are isolated using transferrin receptor gold in the presence or absence of EGF, only in endosomes isolated from cells that have been stimulated with EGF does annexin I become phosphorylated. In contrast, annexin I is not phosphorylated in plasma membrane fractions whether or not an active EGF-R kinase is present. We have demonstrated therefore that in the presence of an active EGF-R kinase annexin I can be phosphorylated in MVBs, but not plasma membrane, *in vitro*. It is likely that annexin I is phosphorylated directly by the EGF-R kinase, rather than through activation of an intermediate kinase, as annexin I is efficiently phosphorylated by purified EGF-R kinase (De et al., 1986; Huang et al., 1986) and there is no evidence of an intermediate substrate in the MVB preparations.

Our *in vitro* results are consistent with the kinetics of phosphorylation of annexin I *in vivo*. Sawyer and Cohen (1985) showed that annexin I is not phosphorylated until the majority of EGF-R have been internalized and suggested that annexin is phosphorylated by the endosomal EGF-R kinase. Cohen and Fava (1985) showed that purified annexin I added to a partially purified intracellular vesicle fraction could be phosphorylated by the EGF-R kinase, and that this reaction was dependent upon the presence of calcium. In the present study we show an association of endogenous annexin I with endosomes that is independent of calcium but this form of annexin I can be phosphorylated by the EGF-R kinase and upon phosphorylation requires calcium for membrane as-

sociation. This is in agreement with the studies of Haigler et al. (1987) who showed that phosphorylation of the calcium-independent form of annexin I in human placental membranes converts it into a form that requires calcium for membrane association. The nature of the calcium-independent interaction of annexin I with membranes is unknown but we have shown here that the association is independent of the density of EGF-R within the membrane. The interaction therefore presumably does not involve a direct interaction with the EGF-R such as has been described for the EGF-R kinase substrates, Phospholipase C γ (Miesenhelder et al., 1989), GAP (Bouton et al., 1991), Raf-1 (App et al., 1991), and Vav (Bustelo et al., 1992; Margolis et al., 1992) which interact with EGF-R via SH2 domains. Quantitation of the total amount of annexin I in plasma membrane and MVB fractions has shown that the presence or absence of an active EGF-R kinase has little effect on the amount of annexin I in plasma membrane fractions but the presence of an active EGF-R kinase causes a reduction in the amount of annexin I in MVB fractions. These results are consistent with annexin I being phosphorylated in MVBs and thus converted to a form that is eluted during fractionation in the presence of calcium chelator.

Wada et al. (1992) in a study of phosphoproteins associated with endosomes of rat liver did not report annexin I associated with endosomes. Wada et al. (1992) did however detect a 55-kD phosphoprotein associated with endosomes. We were not able to detect this substrate in either NIH 3T3 or H.Ep.2 cells, either after *in vitro* phosphorylation or by Western blotting isolated fractions with anti-phosphotyrosine antibody (results not shown). These differences may arise because different stages of processing of the EGF-R were examined. Wada et al. (1992) studied endosomes isolated a maximum of 15 min after EGF stimulation. At this time point the authors were unable to demonstrate sequestration of EGF-R within intra-luminal vesicles in the liver. That sequestration of EGF-R in intra-luminal vesicles does occur in the liver has been shown by Renfrew and Hubbard (1992). In agreement with our proposal the latter authors suggest that movement of EGF-R from the limiting membrane of endosomes to the lumen of lysosomes permits degradation of the EGF-R. Wada et al. (1992) may thus have been examining early events in the processing of the EGF-R, before removal of EGF-R from the limiting membrane.

Thus we have shown that annexin I is associated with plasma membrane and MVBs in a calcium-independent manner and can be phosphorylated *in vitro* in the presence of an active EGF-R kinase in MVBs, but not in plasma membrane. Phosphorylation of the calcium-independent form of annexin I converts it into a form that requires calcium for membrane association. MVBs, but not plasma membrane, containing an active EGF-R kinase contain less calcium-independent annexin I than those that do not contain an active EGF-R kinase. We believe therefore that calcium-independent annexin I is phosphorylated *in vivo* in MVBs whereupon it is released during fractionation in the presence of calcium chelator.

The requirement for the EGF-R kinase to be active for transfer to the inner vesicles of MVBs (Felder et al., 1990), together with the demonstration here that annexin I is a major phosphorylated substrate in MVBs, suggest that annexin I may play a role in mediating inward vesiculation. Mem-

brane invagination to form free vesicles is a well-established process at other membrane boundaries but it is important to note that in these situations the evaginating face of the membrane is cytoplasmic. In contrast, during inward vesiculation in MVBs the evaginating face of the membrane is luminal. Annexin I has been shown to mediate vesicle aggregation and a model has been proposed whereby annexin molecules bind to phospholipid vesicles and fusion between neighboring membranes is mediated by interaction of annexin molecules (Blackwood and Ernst, 1990; Ernst et al., 1991). In the MVB interaction of neighbouring molecules of annexin I on the perimeter membrane may have a role in driving inward vesiculation. Release of annexin I through phosphorylation may then be required for the inward release of vesicles from the perimeter membrane. Subfractionation of the MVB to analyse the protein composition of the perimeter membrane and inner vesicles and the development of in vitro systems to study inward vesiculation may allow the molecular dissection of the events leading to the formation of internal vesicles in the MVB.

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