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Translocation of Annexin I to the Nucleus by Epidermal Growth Factor in A549 Cells

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Annexin I (also called lipocortin 1), a 37-kDa member of the annexin family of proteins, has been implicated in the mitogenic signal transduction by epidermal growth factor (EGF). Annexin I is phosphorylated by the EGF signal, however, the role of annexin I in the EGF signal transduction is still unknown. To transduce extracellular signals into the intracellular targets, selective translocation of the signaling molecules to their targets would be necessary. In this study, we examined the subcellular locations of annexin I during EGF signal transduction. Treatment of A549 cells with EGF resulted in the translocation of cytoplasmic annexin I to the nucleus and perinuclear region as determined by Western blot and immunofluorescent staining. The nuclear translocation of annexin I was inhibited by tyrphostin AG 1478 and genistein, the inhibitors of EGF receptor kinase and downstream tyrosine kinases, respectively. Pretreatment of cells with cyclohexamide did not inhibit the nuclear translocation. The results suggest that nuclear translocation of annexin I is controlled by a series of kinase dependent events in the EGF receptor signaling pathway and may be important in tranducing the signals by EGF.

Keywords: Annexin I, Epidermal growth factor, Mitogenic signal transduction, Nuclear translocation.

Introduction

Annexins (also called lipocortins) are a family of structurally related, calcium-dependent phospholipid-binding proteins which have been implicated to play regulatory roles in diverse cellular processes including membrane trafficking, secretion, membrane-cytoskeleton interaction, signal transduction, proliferation, and differentiation (Raynal and Pollard, 1994). Differential expression of annexins during cell proliferation has been observed in human foreskin fibroblast and rat PC-12 cells (Schlaepfer and Haigler, 1990). The levels of annexins have been shown to vary with the cell cycle (Raynal *et al.*, 1997) and cell differentiation (William *et al.*, 1988; Sato *et al.*, 1995; Kang *et al.*, 1996). Despite these diverse activities, precise functions of annexins remain to be elucidated.

Among this family, annexins I and II have been proposed to be involved in mitogenic signal transduction. This hypothesis emerged from the observation that annexins I and II were major substrates of epidermal growth factor (EGF) receptor kinase (Haigler *et al.*, 1987; Pepinsky and Sinclair 1988) and $pp60^{v\text{-}src}$ (Glenney and Tack, 1985), respectively. Annexin I was also phosphorylated by hepatocyte growth factor (HGF) receptor kinase and the subsequent translocation might function in transducing the proliferating signals of HGF (Skoutis and Schroder, 1996). Furthermore, it was suggested that annexin I was a receptor for activated C-kinase which suggests that it might play a role in activation-induced translocation of PKC from the cytosol to the cytoskeletal elements (Mochly-Rosen *et al.*, 1991). Therefore, it is tempting to speculate that annexin I plays a regulatory role through phosphorylation-dephosphorylation in mitogenic signal transduction.

Annexins I and II are abundant intracellular proteins whose location is believed to be near the plasma membrane due to their ability to interact with phospholipids and actin filaments (Klee, 1988). Selective translocation of a number of signaling molecules to their targets would be essential for transducing extracellular signals to their intracellular targets. Herein, we examine the subcellular localization of annexin I during mitogenic signal transduction by EGF in A549 cells, a lung adenocarcinoma cell line.

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Materials and Methods

Antibodies and chemicals Rabbit antiserum was raised against human annexin I produced in *E. coli* as described previously (Huh *et al.*, 1990). FITC-labeled goat anti-rabbit antibody was from ICN Pharmaceuticals (Costa Mesa, USA). Biotinylated anti-rabbit IgG and streptavidin conjugated alkaline phosphatase were from Oncogene Research Products (USA). Tissue culture solutions were from Gibco BRL (Gaithersburg, USA). Tyrphostin AG 1478 and genistein were from Alexis (Switzerland).

Cell culture A549, a lung adenocarcinoma cell line, was purchased from American Type Culture Collection (Rockville, USA). The cells were maintained in DMEM/F-12 media supplemented with 10% fetal bovine serum (FBS). For immunofluorescent staining study, cells were subcultured onto glass cover slides (22 × 22 mm). After 12–24 h of culture, they were further grown in the same medium supplemented with 0.1% FBS for 72 to 80 h and then treated with EGF (100 ng/ml).

Preparation of the nuclear, cytosolic, and membrane fractions After treatment with EGF for a given time, cells were washed once with ice-cold PBS and then collected by centrifugation at 1000 × *g* for 5 min at 4°C. The cells were resuspended in the 'lysis buffer' (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 50 mM sodium fluoride, 0.25 mM sodium vanadate) and then disrupted by sonication (3 × 20 s). The nuclei were collected by centrifugation at 6500 × *g* for 15 min at 4°C and washed three times with the 'lysis' buffer containing 3 mM EGTA. The cytosolic and the membrane fractions were separated by centrifugation at 100,000 × *g* for 1 h using a Beckman TL100 ultracentrifuge.

Western blot analysis Procedures for SDS-PAGE (polyacrylamide gel electrophoresis) and Western blotting were essentially as previously described (Kang *et al.*, 1996), and according to the instructions from Pierce (Rockford, USA). The protein concentration was determined using a protein assay kit (Bio-Rad Labs, Richmond, USA). For SDS-PAGE, 30 μg of protein lysate was loaded in each lane. The rabbit anti-human lipocortin I polyclonal antibody was prepared using recombinant lipocortin I produced in *E. coli* (Huh *et al.*, 1990). Immunodetection was performed using the anti-human lipocortin I antibody (1:1000 dilution), mouse biotinylated anti-rabbit immunoglobulin (1:10,000 dilution, Pierce), and strepavidine-conjugated alkaline phosphatase. 5-bromo-4-chloro-3′-indolylphosphate-toluidine salt and nitro-blue tetrazolium chloride were used as color substrates.

Immunofluorescent microscopy Immunofluorescent staining for annexin I was essentially as previously described (Park *et al.*, 1998). Cells on cover slides were washed once with ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and were fixed with 4% formaldehyde in PBS. After washing the fixed cells with PBS, they were permeabilized in PBS containing 0.1% Triton X-100 and 2% BSA for 1 h at room temperature and were incubated with anti-annexin I antibody (1:500 dilution), and then with FITC-labeled antibody (1:200 dilution), followed by three washes with PBS. The slides were mounted with Fluoromount-G (Southern Biotech Associates, Birmingham, USA) and were examined with a Leica DM RBE fluorescent microscope (Germany).

Results

Translocation of cytosolic annexin I to the nucleus by EGF EGF-induced variation of annexin I levels in the subcellular locations was analyzed by Western blot as described in Materials and Methods. The levels of annexin I in the cytosol, membrane, and nuclear fractions during a period of 120 min are presented in Fig. 1. In unstimulated cells, most of the annexin I was found in the cytosolic fraction (Fig. 1A, 0 min). Upon EGF stimulation, levels of annexin I in both the membrane and nuclear fractions

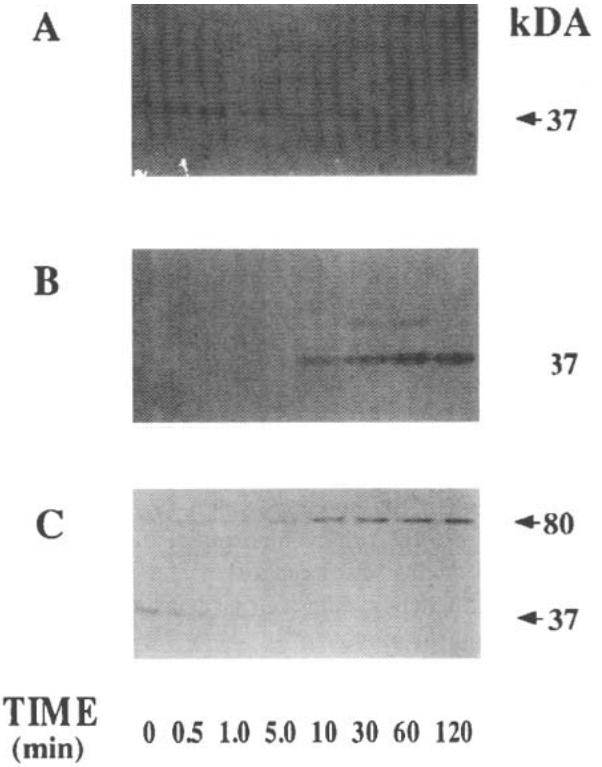

Fig. 1. Western blot analysis of EGF stimulated cells using anti-annexin I antibody. Translocation of cytosolic annexin I to the membrane and to the nucleus was examined by Western blot analysis. A549 cells were cultured in DMEM/F-12 medium supplemented with 10% FBS. After 24 h culture, the cells were starved in the same medium supplemented with 0.1% FBS for 80 h, and then stimulated with EGF (100 ng/ml) for the given times. After cell disruption, the cytosolic, membrane, and nuclear fractions were prepared by differential centrifugation as described in the Materials and Methods. Thirty μg protein samples of the cytosolic (A), membrane (B), and nuclear (C) fractions were loaded in each lane and separated by 10% SDS-PAGE. The resolved proteins were analyzed by Western blot using anti-annexin I antibody. Molecular mass (in kDa) estimated from pre-stained molecular weight markers (Novex, USA) is indicated on the right.

gradually increased with time, whereas the levels of annexin I in the cytosolic fraction decreased concomitantly. The increase in the nuclear fraction was detected within 0.5 min. Since the nuclear fractions were washed three times with 3 mM EGTA to remove annexin I from the contaminating membrane, the increase in nuclear annexin I was not due to contamination. Therefore, it is reasonable to conclude that translocation of cytosolic annexin I to the nucleus and to the membrane occurs rapidly (within 0.5 min) following EGF stimulation. Interestingly, the apparent molecular weight of annexin I (anti-annexin I immuno-reactive protein) in the nuclear fraction was 80 kDa compared to 37 kDa in both cytosolic and membrane fractions. The nature of the 80 kDa protein is not clear at the moment. The dimeric form of annexin I formed by tissue transglutaminase has been observed in a human epidermoid carcinoma cell line (Ando *et al.*, 1991). The 80 kDa protein might represent the dimeric form of annexin I or it might be formed by cross-linking of annexin I with another protein of similar size.

To further investigate the effects of EGF on the subcellular location of annexin I, EGF-stimulated A549 cells were immunostained and observed with a fluorescence microscope. EGF stimulation resulted in stronger nuclear and perinuclear staining and weaker cytoplasmic staining (Fig. 2B) when compared to unstimulated cells (Fig. 2A). A similar nuclear staining pattern was also observed in cells stimulated with 10% FBS (Fig. 2C). Background staining with the preimmune serum was negligible (data not shown). The specificity of the staining was further supported by the fact that staining disappeared upon prior incubation of the anti-annexin I antibody with an excess amount of pure annexin I (data not shown). Pretreatment of cells with cyclohexamide did not affect the EGF-induced nuclear translocation of annexin I (Fig. 2D) indicating that newly synthesized proteins are not required for nuclear translocation.

Regulation of nuclear translocation of annexin I by phosphorylation Although annexin I has been shown to be a major substrate of EGF receptor kinase, cellular roles of the phosphorylated annexin I are still unknown. To determine whether the phosphorylation of annexin I by EGF receptor kinase was involved in the nuclear translocation of annexin I, we examined the effect of tyrphostin AG 1478, a specific inhibitor of EGF receptor kinase (Levitzki and Gazit, 1995), on the translocation using immunofluorescent staining. Perturbation of EGF receptor kinase by tyrphostin AG 1478 inhibited the nuclear translocation of annexin I (Fig. 3, B *vs.* C). Inhibition of intracellular protein tyrosine kinases (PTK) by 25 μM genistein (Akiyama, 1987) also inhibited the nuclear translocation of annexin I (Fig. 3D). These results show that the nuclear translocation of annexin I is dependent on the EGF receptor kinase and PTK activities.

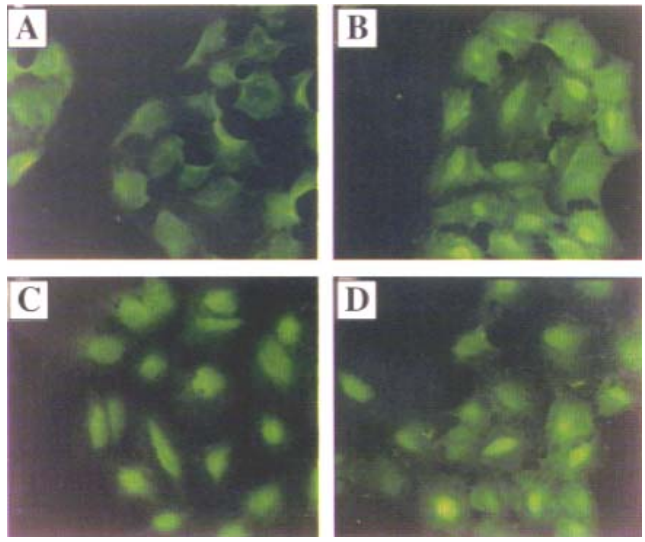

Fig. 2. Subcellular localization of annexin I in EGF stimulated A549 cells. A549 cells were subcultured onto glass cover slides in DMEM/F-12 medium supplemented with 10% FBS. After 24 h culture, the cells were starved in the same medium supplemented with 0.1% FBS for 80 h, and then stimulated with vehicle (A), 100 ng/ml EGF (B), 10% FBS (C), and 100 ng/ml EGF after pretreatment with cyclohexamide (100 μM, for 2 h) for 30 min (D), respectively. The cells were fixed and permeabilized with Triton X-100. They were stained using polyclonal anti-annexin I antibody and FITC-labeled anti-rabbit IgG antibody. The slides were examined with a fluorescent microscope (magnification, ×400).

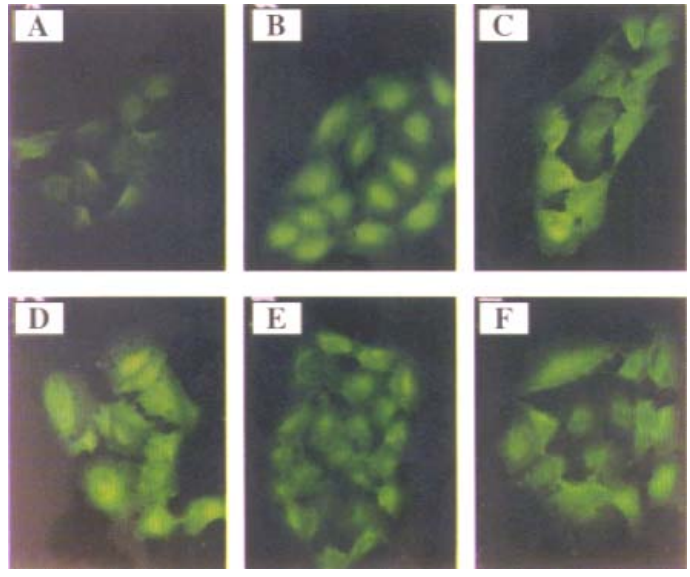

Fig. 3. Subcellular location of annexin I in the presence of kinase inhibitors. A549 cells were cultured and starved as in Fig. 2. The cells were treated with vehicle (B), 1 μM tyrphostin AG 1478 (C), 25 μM genistein (D), and 10 μM cystamine (E) for 2 h, and then stimulated with EGF (100 ng/ml) for 30 min. The serum-starved cells were treated with vehicle for 2 h (A), or 10 μM A23187 for 5 min (F). The cells were stained with anti-annexin I antibody and were observed as in Fig. 2.

It has been reported that intracellular calcium concentration increases upon EGF stimulation (Moolenaar, 1984). To test whether the increase in intracellular calcium concentration is a key regulatory factor in the EGF-induced nuclear translocation of annexin I, cells were treated with A23187. Treatment of the cells with A23187 did not induce the translocation of annexin I (Fig. 3, F *vs.* A).

Since the 80 kDa band was observed only in the nuclear fraction (Fig. 1), the possible involvement of tissue transglutaminase in the nuclear translocation was tested. The EGF-induced nuclear translocation of annexin I was inhibited by preincubation of A549 cells with cystamine, an inhibitor of transglutaminase (Fig. 3E). The results suggest that covalent cross-linking of annexin I may be important in the EGF-induced nuclear translocation.

Discussion

Annexin I has been proposed to be involved in the mitogenic signal transduction by EGF and HGF in A549 cells, although its role in this process has remained unknown. In this study, we have demonstrated that (1) translocation of cytosolic annexin I to the nucleus occurs upon EGF stimulation in A549 cells, (2) the translocation depends on EGF receptor kinase activity and PTK activity, (3) protein synthesis is not necessary for the translocation, and (4) transglutaminase is involved in the translocation.

In unstimulated cells, annexin I was partially localized in the nucleus (Figs. 1C and 2A) which is consistent with previous results (Raynal *et al.*, 1994; Vervoordeldonk *et al.*, 1994). The nuclear translocation of annexin I occurred within 0.5 min after EGF treatment. In addition to EGF, PDGF also induced translocation of cytosolic annexin I to the nucleus of Hela cells within a few minutes (unpublished observation). This rapid change in the subcellular distribution of annexin I by the mitogens suggests that annexin I may play a role in transmitting the mitogenic signals to the nucleus. Annexin I was phosphorylated within 1 min after EGF stimulation (unpublished observation). Therefore, phosphorylation of annexin I may be concurrent with the nuclear translocation. This speculation is further supported by results showing that translocation was inhibited by the inhibition of EGF receptor kinase (Fig. 3C).

Recently, nuclear localization of annexin V (Mohiti *et al.*, 1997) and XI (Mizutani *et al.*, 1995) was reported. However, annexins have no sequence similarity to nuclear localization signals (NLSs) of the SV40 large T (Chelsky *et al.*, 1989) type or nucleoplasmin bipartite type (Robbins *et al.*, 1991). Therefore, the mechanism by which annexins localize to the nuclei was intriguing. In contrast to the other annexins, annexin I contains a putative NLS (Fig. 4) which is a unique sequence among the 12 annexin family

211 231

---RRKGTDVNVFNTILTTRSYPQ LRR---

Fig. 4. Putative nuclear localization signal (NLS) in human annexin I. The underlined amino acids represent the two arms of basic residues in the bipartite NLS (Robbins *et al.*, 1991). The numbers above the sequence indicate the amino acid number in human annexin I.

proteins. The putative NLS might mediate the nuclear localization of annexin I. However, the possibility that the putative NLS of annexin I does not function in nuclear targeting cannot be excluded because the two series of basic residues are separated by a longer amino acid spacer (19 residues) than is typical (10 to 12 residues; Robbins *et al.*, 1991). In this case, nuclear localization of annexin I might occur via association with NLS-bearing proteins (Shaulsky *et al.*, 1990; Kang *et al.*, 1994). Anti-annexin I immuno-reactive 80 kDa protein was observed only in the nuclear fraction (Fig. 1C), which might be formed by covalent cross-linking of annexin I by transglutaminase as described in A431 cells (Ando *et al.*, 1991). This cross-linking in the nucleus can be advantageous in nuclear localization of annexin I because soluble proteins less than 40 kDa can diffuse through the nuclear pore complex. Although the function of covalent cross-linking of annexin I by transglutaminase is still obscure, in this context, there is evidence for the association of increased activity of transglutaminase in the nucleus with proliferation of cells (Remington *et al.*, 1982; el Alaoui *et al.*, 1991).

Previously, it has been reported that translocation of annexin IV and V is induced upon increased intracellular calcium concentration (Raynal *et al.*, 1996). In contrast, treatment of A549 cells with A23187 did not induce the tranlocation of annexin I in our experiments (Fig. 3F). The reason for this difference is unclear at the moment and elucidation of the mechanism will require detailed investigation.

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