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

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Abstract Annexin 6 is a Ca^{2+}-dependent phospholipid-binding protein involved in membrane trafficking. In this study we demonstrate the association of Raf-1 with recombinant rat annexin 6. Raf–annexin 6 interaction was shown to be independent of cell activation by epidermal growth factor (EGF) or phorbol esters (12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)). A stable Chinese hamster ovary (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, was detected. In addition, the activity of Raf was completely inhibited, whereas the mitogen-activated protein kinase-P was unaffected.

Key words: Annexin 6; Raf-1; Epidermal growth factor; Ras; Signal transduction; 12-*O*-Tetradecanoyl-phorbol-13-acetate

1. Introduction

The annexins are a family of widely expressed calcium/phospholipid-binding proteins that are highly conserved [1]. In recent years, annexins 1, 2, 4, 6, 7 and 13 have been implicated in membrane trafficking ([1–5] and references therein).

Annexin 6 was first reported at the plasma membrane [6–8] and later in vesicles of the endocytic compartment [9–13]. More recently, annexins 2 and 6 have been described in isolated subcellular fractions enriched in caveolin, from endothelial cells [14], rat liver [15] and annexin 13a in MDCK cells [16]. The presence of annexins and other components of the budding and fusion machinery (i.e. SNAP receptors) has been associated with the dynamic internalization of caveolae and their subsequent interaction with the endocytic compartment [17,18]. Although annexin 6 does not contain the well conserved predicted caveolin-binding motif, shown by other molecules reported to interact with caveolin [19], it may interact indirectly through proteins such as PKCα [20], Fyn [21], filamin [22], dynamin [23], H-Ras [24] 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 (PS)) 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 conserved region 2 (C2) domain of key Ras regulatory p120-GTPase activating protein ($p120^{GAP}$) which forms part of a protein complex containing Fyn and Pyk2 [25]. 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. 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 [26].

Finally, the fact that GAP [27] and Raf-1 [28,29] were localized in endosomes may indicate that recruitment to the plasma membrane (caveolae), upon stimulation of cell surface receptor (e.g. by epidermal growth factor (EGF)), is transient and these proteins cycle to the endocytic compartment which might be their steady-state/functional location. Therefore, the assembling of signaling 'modules' – and their subsequent transport from the caveolae to the endocytic compartment – can be a key event which might be mediated by annexin 6.

2. Materials and methods

2.1. Reagents and antibodies

Nutrient mixture F-12 Ham medium, geneticin, glutathione, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and EGF were from Sigma Chemical Co. (Madrid, Spain). Fetal calf serum (FCS) was purchased from Biological Industries (Ben Haemeck, Israel). Glutathione-agarose beads and the pGEX expression system were from Amersham Pharmacia Biotech (Uppsala, Sweden). Peroxidase-labeled antibodies and SDS–PAGE molecular weight markers were from Bio-Rad. Anti-GAP monoclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA); anti-Raf monoclonal antibody was from Transduction Laboratories (Lexington, KY, USA); the rabbit antibody against phosphorylated mitogen-activated protein kinase (MAPK) was purchased from Cell Signaling (Beverly, MA, USA) and the monoclonal anti-H-Ras antibody was from Santa Cruz Technology, Inc. (Santa Cruz, CA, USA).

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Abbreviations: C2, conserved region 2; GAP, GTPase activating protein; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; GST, glutathione-*S*-transferase; CHO, Chinese hamster ovary cells; RBD, Ras-binding domain; MBP, myelin basic protein; PS, phosphatidylserine

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2.2. GST (glutathione-S-transferase)-anx6 pull-down

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 supplemented with 10% FCS. Annexin 6 overexpressing CHO cells were grown in the presence of 1 mg/ml G418. 75 μg of GST-anx6 and 21 μg of GST were incubated for 90 min with glutathione-Sepharose at 4°C in phosphate-buffered saline. After washing in 50 mM Tris, 100 mM NaCl, 1% Triton X-100, 0.1 mM $CaCl_2$ (lysing buffer), GST-anx6-Sepharose was then incubated with 600 μg cell extract for 2 h at 4°C and collected by a pulse of a centrifuge. Proteins bound to the column were washed and analyzed by electrophoresis and Western blot. Cell extracts for the affinity column were previously deprived of serum for 16 h (overnight), after which cells were either not treated or stimulated with 10 ng/ml EGF or 100 nM TPA. Cells were then placed in cold lysing buffer, scraped and centrifuged.

2.3. Measurement of Ras activation

The capacity of Ras-GTP to bind to RBD (Ras-binding domain of Raf-1) was used to analyze the amount of active Ras [30]. Cells (2×10^6) were lysed in the culture dish with buffer (20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM NaF, 10% (v/v) glycerol, 0.5% (v/v) β-mercaptoethanol, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM aprotinin and 20 μM leupeptin). Cleared ($10\,000\times g$) lysate was assayed for protein concentration by the Bradford method and protein-equalized supernatants were incubated for 2 h at 4°C with glutathione-Sepharose-4B beads pre-coupled with GST–RBD (1 h, 4°C). Beads were washed four times in the lysis buffer. Bound proteins were solubilized with Laemmli loading buffer and electrophoresed on 12.5% SDS–PAGE gels. Proteins were then transferred and immunoblotted using anti-(Pan)-Ras (Ab-3, OP-40, Oncogene Sciences, 1 μg/ml) monoclonal antibody.

2.4. Raf-1 kinase activity assays

To measure Raf-1 activity, kinase assays following immunoprecipitation were performed essentially as described [31]. Briefly, cells (2×10^6) were harvested on ice in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1% (v/v) Triton X-100, 5 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 mM aprotinin and 20 μM leupeptin) and clarified by centrifugation ($10\,000\times g$). Supernatants (equalized for protein concentration) were then immunoprecipitated for 2 h at 4°C with 2 μg of anti-Raf-1 (Transduction Laboratories, Lexington, KY, USA) pre-coupled with 20 μl of protein G-Sepharose (Pierce, Rockford, IL, USA) (2 h at room temperature). Immunoprecipitates were then washed three times in buffer (30 mM Tris, 0.1 mM EDTA, 0.3% β-mercaptoethanol, 10% glycerol, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4) with decreasing amounts of NaCl (high: 1 M, low: 0.1 M and salt-free). Washed immunoprecipitates were incubated for 30 min at 30°C in 20 μl of MEK buffer (30 mM Tris, 0.1 mM EDTA, 0.3% β-mercaptoethanol, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.8 mM ATP, 6.5 μg/ml GST-MEK, 100 μg/ml GST-ERK2), and the reaction was terminated by the addition of 20 μl of ice-cold stop buffer (30 mM Tris, 6 mM EDTA, 0.3% β-mercaptoethanol, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4). Following centrifugation, 6 μl of supernatants was incubated for 15 min at 30°C with 24 μl of MBP (myelin basic protein) buffer (50 mM Tris, 0.1 mM EDTA, 0.3% β-mercaptoethanol, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM Na_3VO_4, 0.1 mM ATP, 2.5 μl [^{32}P]ATP, 0.5 μg/μl MBP, 0.16 μg/μl bovine serum albumin), and then aliquots of 24 μl were loaded onto P81 sheets, and washed three times (20 min each) in 75 mM orthophosphoric acid and counted.

2.5. Recombinant DNA

The construction of the rat annexin VI expression vector pcDNAanx6 has been described recently [4]. Recombinant full length annexin 6 (GST-anx6) and truncated GST-anx6_{1-175} were obtained as GST fusion proteins [32]. Cloning of the 2.0 kb full length annexin 6 *Nco*I–*Xba*I fragment into pGEX-KG (Pharmacia) generated pGEX-anx6. Cloning of a 542 bp *Hin*dIII–*Asp*I annexin 6 cDNA fragment containing coding sequences from position +1 to position +527, which lack six of the eight annexin 6 repeats at the carboxy-terminal, generated pGEX-anx6_{1-175}. All cloning procedures were performed according to standard protocols [33]. GST-anx6 was expressed in *Escherichia coli* strain BL21 pLys and purified by glutathione-Sepharose chromatography.

2.6. Annexin 6 stable CHO cells

To generate stable annexin 6 overexpressing cells, 1×10^6 CHO cells were transfected with 10 μg pcDNAanx6 and the FUGENE™ 6 Transfection Reagent (Boehringer Mannheim). G418 (1 mg/ml) was added 24 h after transfection, and 13 days later G418-resistant colonies were isolated and examined for expression of annexin 6 by Western blotting and immunofluorescence [4].

2.7. Other procedures

SDS–PAGE and Western blotting. CHO cell lysates or samples from pull-down experiments were separated by SDS–PAGE [34] and transferred to Immobilon-P (Millipore), followed by incubation with the appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

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 via PKC [35]. Upon activation, Raf-1 is recruited to the plasma membrane or to the early/sorting endocytic compartment [36–38] where is activated by Ras-GTP, by mechanisms that are poorly understood. Downstream events involve the phosphorylation of the kinase MEK, leading to stimulation of the 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 $p120^{GAP}$ [25,39]. GAP is a GTPase activating protein proposed to accelerate the inhibition of Ras. Second, in A431 cells EGF-dependent calcium influx was inhibited by annexin 6 [40]. And third, annexin 6 may play an important role in the

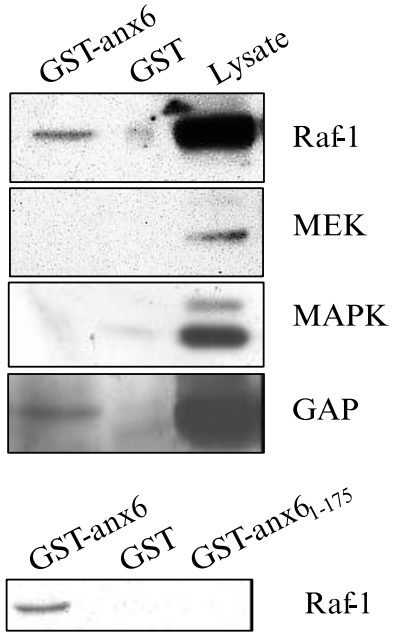

Fig. 1. Interaction of annexin 6 with Raf-1 in CHO cells. Pull-down experiments using GST-anx6 were performed to study the interactions of annexin 6 with proteins of the signal transduction machinery. CHO cell extracts were incubated with GST-anx6 for 2 h at 4°C. Bound proteins (GST-anx6) were collected, separated by PAGE and analyzed by Western blotting, using antibodies to Raf-1, MEK, MAPK and GAP. Controls with GST alone showed no binding. The mutated form of annexin 6 (annexin 6_{1-175}, which lacks six of the eight repeats) did not bind to Raf-1.

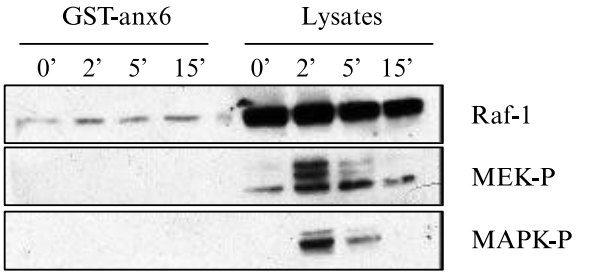

Fig. 2. Association of annexin 6 with Raf-1 is independent of EGF activation. Pull-down experiments using GST-anx6 were performed to study the interactions of annexin 6 with Raf-1, MEK-P and MAPK-P after stimulation of CHO cells with EGF for 2, 5 or 15 min. CHO cell extracts were incubated with GST-anx6 as described in Fig. 1. Bound proteins (GST-anx6) were collected, and analyzed by Western blotting, using antibodies to Raf-1, MEK-P and MAPK-P. The association of annexin 6 to Raf-1 is independent of cell activation by EGF. A peak of MEK-P and MAPK-P activity was restricted to 2 min.

recruitment (assembly) of protein complexes at the caveolae, including the constituents of the signal transduction machinery and calcium signaling.

3.1. GST-annexin 6 (GST-anx6) interacts with Raf-1 in CHO cells

Thus, to analyze the possible interaction of annexin 6 with the signal transduction machinery, extracts of CHO cells were incubated with immobilized recombinant GST-anx6 protein. In agreement with data previously reported we identified GAP in the GST-anx6-binding fraction. In addition, immunoblot analysis of pull-down fractions showed the interaction of Raf-1 with GST-anx6. In contrast Western blots of the GST-anx6 bound fraction did not reveal the presence of MEK or MAPK-P proteins (Fig. 1). 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$_{1-175}$ (Fig. 1). In these experiments Raf-1 did not bind to GST-anx6$_{1-175}$, 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 90°, thus changing its conformation [41].

3.2. Interaction of Raf with annexin 6 is independent of cell activation

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 16 h and then stimulated with EGF. In a time course experiment (2–15 min) it can be observed that there were no changes in the binding to Raf-1. Although these cells showed very low levels of EGF-R [42], the activation of MAPK (MAPK-P) was observed and it reached a peak after 2 min of EGF treatment, decreasing after 5 min, shown in lysates (Fig. 2).

Second, CHO cells were treated with TPA (for 2 min) which is more efficient than EGF in the MAPK-P pathway, in this cell line. No differences in the binding of Raf-1 to GST-anx6, compared to control untreated cells, were observed (Fig. 3). Extracts of CHO cells were also assayed for binding of GST-anx6 with GAP and MAPK-P; the binding to Raf-1 was also independent of the presence of Ca^{2+} or PS (not shown). Opposite to EGF, in lysates of CHO cells treated with TPA, the MAPK-P activity was sustained for 30 min (not shown).

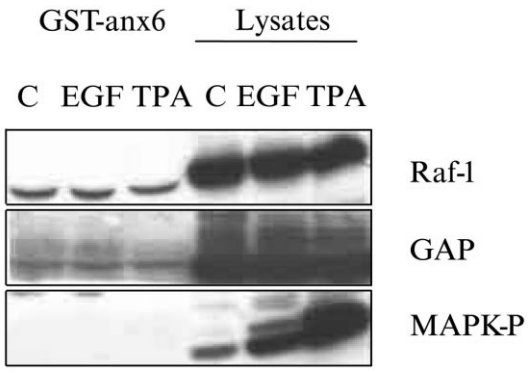

Fig. 3. Interaction of annexin 6 with Raf-1 and GAP is independent of cell activation. CHO cells were serum-deprived for 16 h and stimulated with EGF (10 ng/ml) or TPA (100 nM) for 2 min. Cell extracts were incubated with GST-anx6 as described above (pull-down assay) and the bound material or lysates analyzed by Western blotting with Raf-1, GAP or MAPK-P antibodies. Interaction of annexin 6 with Raf-1 and to GAP was independent of TPA activation.

3.3. Overexpression of annexin 6 inhibits Raf-1 activity

Since CHO cells have very low levels of annexin 6 [4], 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 wild-type (wt) CHO, where Raf-1 activity increased 12% and 60% after EGF and TPA treatment, respectively (Fig. 4). The low activation of Raf-1, by EGF, in the CHO cells

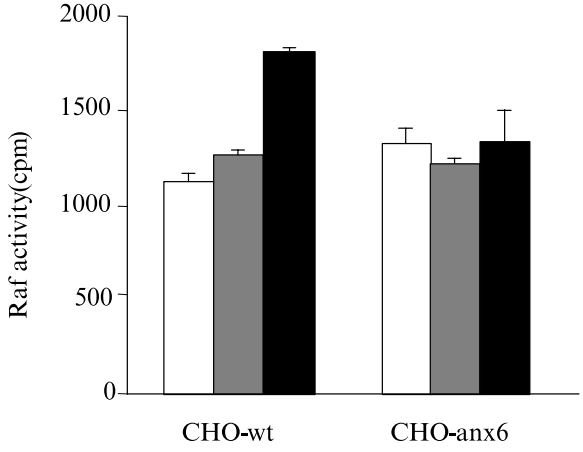

Fig. 4. Activation of Raf in CHO-wt and CHO-anx6 cells. Cells were deprived of serum for 16 h and stimulated with EGF (10 ng/ml) or TPA (100 nM) for 2 min. The activity of Raf was measured from CHO cell extracts by immunoprecipitation with the anti-Raf antibody and kinase cascade assay, with GST-MEK, GST-ERK2 and MBP as substrates. The results show a representative experiment assayed in triplicate ($n = 3 \pm$ S.D.). Open bars, control; gray bars, EGF; black bars, TPA.

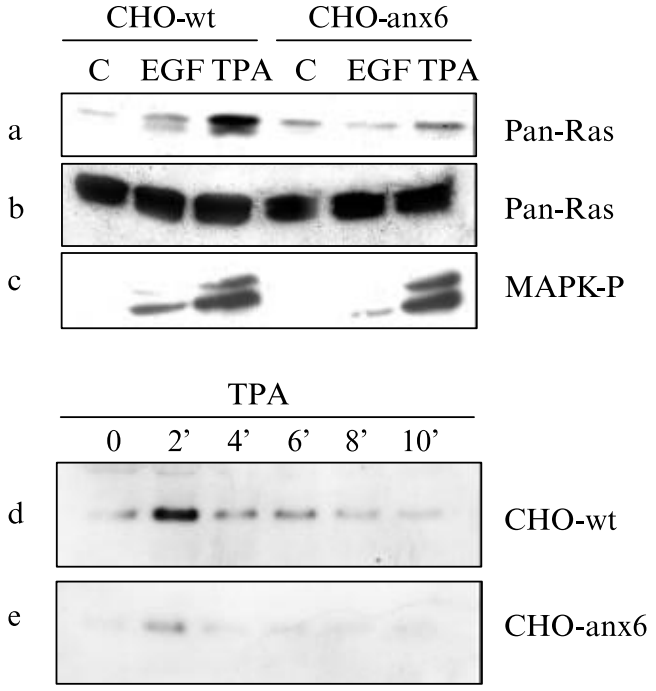

Fig. 5. Amount of Ras-GTP in CHO-wt type and in annexin 6 overexpressing cells (CHO-anx6). In (a), the amount of Ras-GTP in CHO-wt or CHO-anx6 cells was assessed by GST–RBD pull-down and analyzed by Western blotting with anti-Pan-Ras antibody in control (non-stimulated cells) or activated with EGF or TPA (for 2 min). 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 (c). In (b), the lysates of the same experiment are shown as a control of protein loading. In (d) and (e), a representative Western blot of a time course experiment (RBD-GST pull-down) to measure the activation of Ras (amount Ras-GTP) after TPA activation is shown. A significant decrease of Ras-GTP can be demonstrated at the 2 min point in the CHO-anx6 cells compared with control. A sustained activation (up to 10 min) of MAPK-P was observed in lysates from the same experiment (not shown).

may be explained, as mentioned before, by the low number of EGF-R.

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–RBD fusion protein. Cell extracts of non-activated or EGF/TPA-activated (for 2 min) CHO cells and CHO-anx6 overexpressing cells were incubated with immobilized GST–RBD, and bound Ras-GTP protein was detected by Western blotting. Fig. 5 shows the comparison of the total amount of Ras-GTP (anti-Pan-Ras antibody) in pull-down fractions from CHO-wt or CHO-anx6 cells. Fig. 5a shows that the amount of Ras-GTP was significantly reduced in EGF or TPA-stimulated CHO-anx6 cells; lysates showed MAPK-P was not affected (EGF or TPA), indicating that the activation of MAPK was produced by a different/independent mechanism (Fig. 5c).

Finally, in order to determine whether the annexin 6 overexpression inhibited Ras-GTP (and downstream Raf-1) or just delays it, we performed a time course study, from 2 to 10 min, in the CHO-wt and CHO-anx6 cells treated with TPA. Fig. 5d,e shows that there is a peak of Ras activation (Ras-GTP) at 2 min in the CHO-wt which was significantly inhibited in CHO-anx6 cells and no further activation was detected up to 10 min. Although a slightly increased amount of Ras-GTP was observed in the CHO-anx6 cells at the 2 min point, it was much less, and not significant compared with CHO-wt cells (the same variability was observed in the Raf assay, Fig. 4, also at 2 min). In all experiments the same amount of protein was loaded for the Western blotting analysis.

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 [43], we have demonstrated that in CHO-wt cells Ras was activated after stimulation by either EGF or TPA. This is in agreement with the requirement of Ras-GTP–Raf-1 complexes to activate Raf-1 in response to TPA [44]. Ras activation by phorbol esters is also required for MAPK phosphorylation in cardiac myocytes, but not in cardiac fibroblasts [45]. Thus, whereas in CHO-wt cells the MAPK pathway can be activated via 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 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.

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 [46]. In caveolae, interactions of proteins with PKCα, dynamin, Raf-1 or other kinases may influence the regulation of signal transduction downstream events. Caveolin, through its scaffolding domain, interacts with molecules of the signal transduction machinery and annexins (e.g. annexins 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 [47], 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.

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