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Modulation of the actin cytoskeleton in the folliculo-stellate cell line TtT/GF by serum factors.

par

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en vue de l'obtention du grade de M.Sc.

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Identification du jury

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Modulation of the actin cytoskeleton in the folliculo-stellate cell line TtT/GF by serum factors

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Résumé

Nous avons choisi la cellule folliculo-stellaire (FS) de l'hypophyse antérieure comme modèle pour évaluer les mécanismes intracellulaires qui contrôlent la dynamique du cytosquelette d'actine. Les cellules FS sont localisées entre les cellules sécrétrices d'hormones de l'hypophyse antérieure et sont impliquées dans le contrôle paracrine de cette activité sécrétrice. La morphologie des cellules FS est affectée par l'activité endocrine de la glande pituitaire (Cardin et coll., 2000). Ces changements impliquent en particulier la disparition/réapparition des prolongements cellulaires. Le cytosquelette d'actine est le facteur principal qui détermine la forme de la cellule. Pour comprendre comment les facteurs systémiques et nerveux affectent la morphologie des cellules FS, nous avons étudié la dynamique du cytosquelette d'actine dans une lignée de cellules FS, les cellules TtT/GF. Ces cellules ont été largement utilisées comme modèle expérimental des cellules FS.

Dans la présente étude, nous avons démontré que deux protéines de liaison à l'actine, la cortactine et l'α-actinine sont impliquées dans la régulation de la dynamique du cytosquelette d'actine des cellules TtT/GF par des facteurs de croissance via les voies des tyrosines kinases et d'AMPc/PKA. Dans un premier temps, nous avons étudié comment l'absence de sérum affecte la morphologie des cellules TtT/GF, la dynamique du cytosquelette d'actine et la distribution sub-cellulaire de la cortactine et l'α-actinine. Deuxièmement, nous avons utilisé un inhibiteur des tyrosine kinases, la genisteine, pour examiner l'impact de la phosphorylation en tyrosine de la cortactine et de l' α-actinine dans les changements de morphologie induits par le sérum. Troisièmement, nous avons utilisé la forskoline et le KT5720 (activateur de l'adenylate cyclase et inhibiteur de PKA,

respectivement) pour explorer le rôle de la voie AMPc/PKA dans la régulation de la dynamique du cytosquelette d'actine dans les cellules TtT/GF par des facteurs sériques.

La privation du sérum était suivie de la perte du phénotype de fibroblaste typique des cellules TtT/GF cultivées en présence du sérum. En absence de sérum, les cellules présentaient des fibres d'actine plus épaisses (fibres de stress) comparées aux cellules contrôles cultivées en présence de sérum. En outre, en l'absence de sérum, la formation des pseudopodes et des lamellipodes était réduite, indiquant la perte de mobilité. La privation de sérum induisait aussi un déplacement important de la cortactine de la membrane vers le cytosol d'une part et du cytosquelette vers le non-cytosquelette d'autre part. L'absence de sérum réduisait l'association de l'α-actinine avec la membrane plasmique et avec les fractions du cytosquelette. Ces résultats étaient confirmés par les études d'immunofluorescence qui ont montré que ces deux protéines de liaison à l'actine étaient transférées de la périphérie de la cellule vers le cytoplasme suite à la privation de sérum. La genisteine, la forskoline, et le KT5720 affectaient l'expression et la localisation sub-cellulaire de la cortactine et de l'α-actinine, suggérant que le statut de phosphorylation de ces protéines est important dans le mécanisme de régulation de la morphologie des cellules TtT/GF.

Puis dans leur ensemble, nos résultats montrent que le cytosquelette d'actine, dans cellules de TtT/GF, est sensible à l'environnement extracellulaire et que la cortactine et l'α-actinine seraient des effecteurs de ces stimuli extracellulaires.

MOTS CLÉS : cellule folliculo-stellaire, cellules TtT/GF, cytosquelette d'actine, protéines de liaison à l'actine, cortactine, α-actinine, hypophyse antérieure, phosphorylation en tyrosine, AMPc/PKA, privation de sérum.

SUMMARY

To assess the intracellular mechanisms that modulate the actin cytoskeleton dynamics, we choose the anterior pituitary folliculo-stellate cell as model. Folliculo-stellate cells, which lie in between the hormone secreting cells of the anterior pituitary, are involved in the paracrine control of anterior pituitary hormone secretion. It is known that the morphology of anterior pituitary folliculo-stellate cells is affected by the endocrine activity of the pituitary gland (Cardin et al, 2000). These changes particularly involve the disappearance/reappearance of cellular processes, thus affecting the stellate morphology of folliculo-stellate cells. Because the actin cytoskeleton is the main factor determining the cell shape, to understand how systemic and nervous factors affect the morphology of folliculo-stellate cells, we decided to investigate the dynamics of the actin cytoskeleton in a folliculo-stellate cell line, the TtT/GF cells. These cells are have been widely used as an experimental model for folliculo-stellate cells.

In the present study, we demonstrated that two actin-binding proteins, cortactin and α-actinin are involved in the regulation of the actin cytoskeleton dynamics by serum factors in TtT/GF cells via the tyrosine kinases and cAMP/PKA pathways. First, we studied how serum starvation affected TtT/GF cells' morphology, the dynamics of the actin cytoskeleton, and cortactin and α-actinin subcellular distribution. Secondly, we used the tyrosine kinase inhibitor genistein to test the impact of tyrosine phosphorylation of these two actin-binding proteins in the serum-induced changes of TtT/GF cell morphology. Thirdly, we applied forskolin and KT5720 (adenylate cyclase activator and PKA inhibitor, respectively) to explore the role of cAMP/PKA pathway in the implication of these two actin-binding proteins in the regulation of the actin cytoskeleton dynamics in TtT/GF cells by serum factors.

Serum deprivation was followed by the loss of the fibroblast phenotype typical of TtT/GF cells cultured in the presence of serum. Serum-deprived cells possessed thicker actin stress fibers when compared to serum-cultured cells. In addition, in the absence of serum, the formation of membrane ruffles, pseudopodia and lamellipodia was reduced, indicating the loss of the mobile phenotype. We observed that serum starvation induced a marked redistribution of cortactin from the membrane into the cytosol and from the cytoskeleton into the non-cytoskeleton fractions. Serum withdrawal reduced α-actinin association with the membrane and the cytoskeleton fractions. These findings were confirmed by the immunofluoresence studies that showed that these two actin-binding proteins migrated from the periphery into the interior of the cells upon serum removal. Genistein, forskolin, and KT5720 affected the expression and the subcellular localization of cortactin and α-actinin, suggesting that the phosphorylation status of the proteins is important in mediating their participation in the serum-induced modulation of the TtT/GF cell morphology.

Collectively, our investigations show that the actin cytoskeleton in TtT/GF cells is sensitive to the extracellular environment and that the actin-binding proteins cortactin and α-actinin are effectors of these extracellular stimuli.

KEY WORDS: folliculo-stellate cell, TtT/GF, actin cytoskeleton, actin-binding proteins, cortactin, α-actinin, anterior pituitary, tyrosine phosphorylation, cAMP/PKA, serum starvation.

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LIST OF ABBREVIATIONS

ABD	actin-binding domain
AKAPs	A-kinase anchoring proteins
Arp	actin related proteins
BSA	bovine serum albumin
cAMP	cyclic AMP
C	cytosol or control
CK	cytoskeleton
CH	calponin homology
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EGF	epidermal growth factor
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FS	folliculo-stellate
G-actin	monomeric actin
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
H	hour
IL-6	interleukin-6
KDa	kilo Dalton

M	membrane
MLC	myosin light chain
MLCK	myosin light chain kinase
NCK	non-cytoskeleton
NTA	amino terminal acidic domain
P-cortactin	phospho-cortactin
PDGF	platelet-derived growth factor
PBS	phosphate buffered saline
PKA	cAMP-dependent protein kinase
PMSF	phenylmethylsulfonyl fluoride
PM	plasma membrane
PRL	prolactin
SDS-PAGE	dodecyl sulfate-poly-acrylamide gel electrophoresis
SH	Src homology
TBS	TRIS buffered saline
TRITC	tetramethylrhodamine isothiocyanate
WASP	Wiskott - Aldrich syndrome protein

DEDICATION

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PREFACE

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Zheng, G.F., Pelletier, R.M., and Vitale, M.L. (2003). Effet des facteurs sériques sur la dynamique du cytosquelette d'actine des cellules TtT/GF (poster presentation). *Médecine/Sciences 19 (Suppl 2): No.57, P 14*

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1 Introduction

1.1 The pituitary gland

1.1.1 General

The pituitary gland or hypophysis is an endocrine gland located in the sella turcica of the sphenoid, a depression in the bony floor of the cranial cavity. It is composed of two distinctive parts: the anterior pituitary and the posterior pituitary (Ball and Baker, 1969; Wingstrand, 1966). The anterior pituitary (adenohypophysis) is a classical gland composed of a mixed population of granular and agranular cells. The granular cell group comprises five kinds of endocrine cells that contain secretory granules in their cytoplasm and synthesize and secrete the pituitary hormones, growth hormone, prolactin, thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, and adrenocorticotropic hormone which control growth, sexual development and metabolism. The folliculo-stellate cells (FS cells) are the major cellular element of the agranular cells in the anterior pituitary gland (Matsumoto, H. et al., 1993). They do not possess secretory granules in their cytoplasm, thus differing from the glandular cells in their activities and their functions (Inoue et al., 1992). The posterior pituitary (neurohypophysis) is in fact an extension of the hypothalamus. It is composed largely of the axons of hypothalamic neurons that extend downward as a large bundle behind the anterior pituitary (Grandi and Chicca, 2004). Oxytocin and antidiuretin are the main hormones secreted by the neurohypophysis.

On the one hand, the pituitary gland controls the functions of the other endocrine glands in the body so it is sometimes called the "master" gland of the endocrine system (Grandi and Chicca, 2004). On the other hand, the secretion of pituitary hormones is modulated by systemic influences and by the hypothalamus. In this late sense, the

pituitary gland is an important link between the nervous system and the peripheral endocrine system.

1.1.2 The folliculo-stellate cells (FS cells)

The FS cells of the anterior pituitary gland were first reported by Rinhart and Farquhar (1953). FS cells represent about 5 to 10% of the anterior pituitary cell population (Rinehart & Farquhar 1953). These stellate-shaped cells cluster together into follicles with cytoplasmic processes extending outwards to connect other FS cells and endocrine cells, generating an extensive three-dimensional network within the anterior pituitary (Allaerts et al., 1990; Soji and Herbert, 1989; Inoue et al., 1999). Immunohistochemical studies at the light and electron microscopic levels revealed that FS cells are localized throughout the anterior pituitary, and form 2 to 10 cell clusters around a follicular cavity (Lloyd and Mailoux, 1988; Soji and Herbert, 1989). FS cell morphological features also include the presence of lysosomes, the expression of the protein S-100 and of the intermediate filament protein GFAP (glial fibrillary acidic protein) (Figure A).

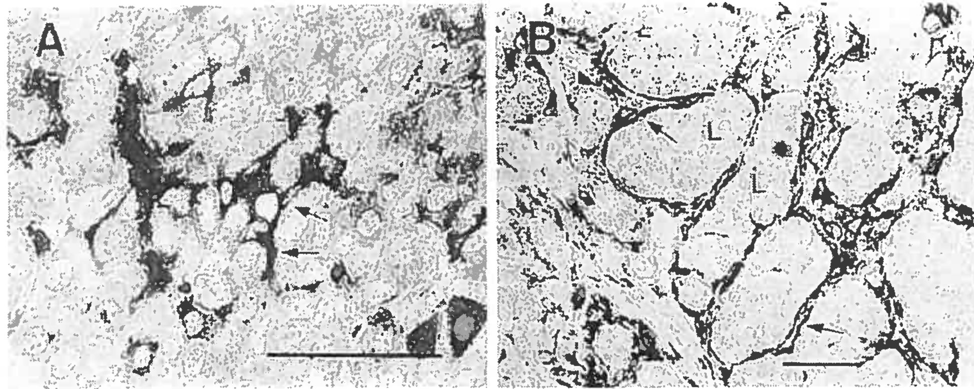


Figure A. Immunocytochemistry of GFAP in the anterior pituitary gland (A) and thyrotropic tumor (B). FS cells in the mouse anterior pituitary, surrounding neighboring glandular cells, are positively stained for GFAP (A, arrows). The GFAP-immunopositive cells are flat and uniformly surround the lobules (L) of parenchymal tissue in the thyrotropic pituitary tumor (B, arrows). Bars are 50 pm (Inoue et al., 1992).

The precise functions of the FS cells in the anterior pituitary gland are not yet truly understood. Several functions have been ascribed to FS cells including supportive and trophic effects, a role in ion transport, phagocytic and catabolic activities (Perryman, 1989), and many paracrine functions. With their long cytoplasmic processes rich in gap junctions extending between other endocrine cell types (Morand et al, 1996), the FS cells are thought to play a role in intercellular communication within the anterior pituitary. There is substantial evidence that FS cells modulate pituitary hormone secretion from surrounding endocrine cells through the release of several bioactive molecules, including follistatin, interleukin-6 (IL-6), nitric oxide, basic fibroblast growth factor, leptin (Allaerts et al, 1990). A paracrine mode of interaction was also suggested by the observation that FS cells contain and release the S-100 protein, and that this protein can stimulate PRL release from cultured clonal PRL cells (Ishikawa et al., 1983) and from normal dissociated anterior pituitary cells (Lloyd and Mailloux, 1988). Moreover, Fauquier et al. have demonstrated that the FS cell network forms an extensive functional intrapituitary circuitry in which information, Ca^{2+} and small diffusible

molecules can be transferred through gap junctions over long distances (Fauquier et al., 2001).

Work from our lab has shown that the morphology of anterior pituitary FS cells is affected by the secretory activity of the gland (Cardin et al, 2000). These changes particularly include the disappearance/reappearance of cellular processes, thus affecting the stellate morphology of FS cells. Because the actin cytoskeleton is the main factor determining the cell shape, to understand how systemic and nervous factors affect the morphology of FS cells, we decide to investigate first the dynamics of the actin cytoskeleton of FS cells.

1.2 The actin cytoskeleton

The cytoskeleton, the cell skeleton, is composed of three major types of filaments: the microtubules, the microfilaments, and the intermediate filaments. Microfilaments are polymers of actin that together with a large number of actin-binding and associated proteins constitutes the actin cytoskeleton (Stössel 1993; Botstein et al., 1997; Winsor and Schiebel 1997). Actin exists either in a monomeric (G-actin) or in a polymeric form (F-actin). Each actin molecule can bind ATP, which is hydrolyzed to ADP after incorporation of the actin molecule into the filament. Polymers assemble spontaneously via non-covalent interactions between the monomeric subunits and are highly dynamic structures with subunits turnover at both ends. The rate-limiting step in actin polymerization is nucleation, the assembly of the first subunits to generate a new filament. Actin filaments are structurally polarized and the kinetics of polymerization at each end is different, with the plus end growing more quickly than the minus one (Anja and Micheal 1998). Few filament properties are regulated by direct covalent

modification of the filament subunits, indeed most of the regulation is performed by accessory proteins that bind to either the filaments or their free subunits.

The assembly and reorganization of actin filaments are controlled by a host of actin-binding proteins under the influence of external and internal stimuli (Aspenstrom, 1999; Cooper, 1991; Hall, 1998; Hatano, 1994; Janmey, 1998; Tapon and Hall, 1997). Actin-binding proteins can bind to actin monomers and/or to actin filaments to form diverse structures and networks that vary in different cell types and in different parts of the same cell (Carlier, 1998; Condeelis, 1993; Furukawa and Fechheimer, 1997; McGough, 1998; Puius et al., 1998; Small et al., 1998; Van Troys et al., 1999). A large number of actin-binding proteins control assembly and length of actin filament, link actin filaments into bundles or networks, define the three-dimensional organization of actin filaments and link filaments to other cytoplasmic and membrane components (Stössel et al., 1985; Pollard and Cooper, 1986; Stössel, 1989), bringing the cytoskeletal structure under the control of extracellular and intracellular signals.

1.2.1 The dynamics of the actin cytoskeleton in non-muscle cells

In nonmuscle cells, most actin filaments are highly dynamic structures that are being constantly assembled, disassembled, and reorganized under the influences of intra- and/or extra-cellular stimuli as the cell changes its shape, divides, crawls, and adheres to a substratum or to neighboring cells and adapts to the environment (Bershadsky and Vasiliev, 1988; Bray, 1992; Mitchison and Cramer, 1996; Small et al., 1996; 1999; Stössel, 1993; 1994; Theriot, 1994; Welch et al., 1997).

1.2.1.1 Extracellular and intracellular factors affecting the dynamics of the actin cytoskeleton in non-muscle cells

A variety of extracellular signals influence the actin cytoskeleton dynamics. For example, growth factors or extracellular matrix (ECM) proteins transmit signals through actin cytoskeleton to modulate intracellular trafficking, cell morphology, cell migration, and process extension (Zigmond, 1996). Epidermal growth factor (EGF) exerts its effects in the target cells by binding to the plasma memberane EGF receptor, resulting in the activation of its tyrosine kinase activity and subsequent receptor autophosphorylation, which is essential for the interaction of the receptor with its substrates (Boonstra et al., 1995). The EGF receptor is an actin-binding protein (Den Hartigh et al., 1992) which causes a rapid actin depolymerisation and the formation of membrane ruffles (Rijken et la., 1991). These membrane ruffles function as the primary site of signal transduction after EGF binding, and thus are thought to be signal transduction structures (Boonstra et al., 1995).

EGF-induced early signal transduction causes rapid remodeling of the actin microfilament system in a variety of cells (Rijken et al., 1991; Peppelenbosch et al., 1993). This is the case for other growth factors, such as nerve growth factor (Paves et al., 1990) and platelet-derived growth factor (PDGF) (Arvidsson et al., 1992; Kundra et al., 1994). The receptors for various growth factors, including PDGF (Frackelton et al., 1984; Ek and Heldin, 1984), insulin (Kasuga et al., 1982) and insulin-like growth factor I (Sasaki et al., 1985) are all protein tyrosine kinases.

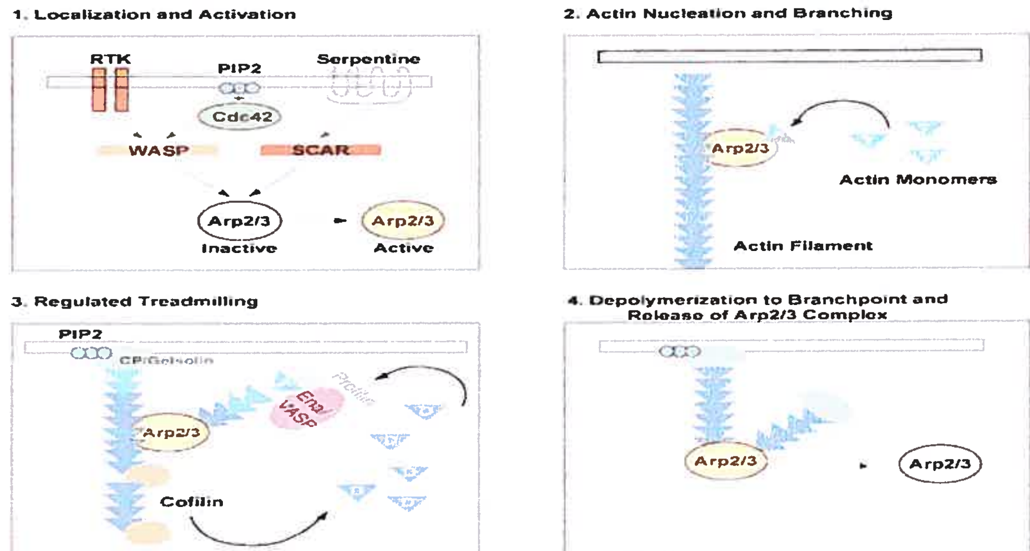
In addition, a number of key intracellular components had been found essential for control of actin polymerization and depolymerization including profilin, Arp2/3, WASp/Scar/WAVE, actin capping proteins, actin severing proteins, and Rho family

GTPases (Pollard et al., 2000; Bear et al., 2001). It is now clear that the Arp2/3 complex regulates the assembly of new actin filament networks at the leading edges of cells. Proteins of the WASP (Wiskott - Aldrich syndrome protein) family bind directly to the Arp2/3 complex and stimulate its ability to promote the nucleation of new actin filaments. Upstream of WASP-family proteins, receptor tyrosine kinases, the Rho-family GTPase Cdc42, and likely G protein-coupled receptors, receive and transmit the signals leading to WASP-Arp2/3 complex-mediated actin nucleation. Together, these data suggest that signaling pathways from outside the cell induce actin polymerization with the resultant polymerization-driven cell motility. Scar proteins might be involved in pathways through serpentine receptors and heterotrimeric G-proteins (Machesky and Insall, 1999).

Cdc42 and PIP_2 also cooperate with N-WASP to activate Arp2/3 complex-induced actin nucleation activity (Rohatgi et al., 1999). This indicates a signaling pathway from inositol phospholipids to Cdc42 and WASP/N-WASP. In addition to Arp2/3 complex-mediated actin nucleation, both mammalian cells and *Listeria* also use Ena/VASP proteins to promote actin filament assembly (Machesky and Insall, 1999).

A regulated treadmilling model was suggested for actin dynamics in motility. In this model, the Arp2/3 complex becomes activated through one of the WASP family proteins depending on the nature of the signal for the actin polymerization. Activated Arp2/3 complex binds to the sides of actin filaments and nucleates new branches with free barbed ends. Once these dendritic structures assemble, their elongation are controlled by capping proteins, which dynamically associate and dissociate with the barbed ends of the filaments, with the relative rates of capping and uncapping being controlled by signaling intermediates such as plasma membrane phosphoinositides.

Additionally, the rate at which new filaments elongate is accelerated by regulated association with Ena/VASP proteins and profilin. Established filaments are severed and depolymerized by cofilin (Maciver, 1998). Finally, when the filament depolymerises, the Arp2/3 complex falls off and may be recycled in new filaments (Schema 1).



Schema 1. Regulated treadmilling model for actin dynamics. (1) Various signals trigger localization and/or activation of WASP/Scar proteins, activating and localizing the Arp2/3 complex to nucleate actin filaments. (2) Arp2/3 complex binds to the sides of actin filaments and nucleates branches. (3) The growing barbed ends of the filaments are regulated by capping protein and gelsolin. Ena/ VASP proteins catalyze the elongation of newly nucleated filaments. The pointed end of the filaments is depolymerizing with the help of cofilin. (4) If the filaments depolymerize down to a branchpoint, Arp2/3 complex may fall off and is recycled to participate in nucleation (Macheskey and Insall, 1999).

1.2.1.2 Regulation of actin cytoskeleton dynamics through cAMP/PKA pathway

Cyclic AMP (cAMP) is a key second messenger mediating the biological effects of several hormones which activate the adenylate cyclase catalyzing the production of intracellular cAMP. The mode of action of cAMP is via the activation of the cAMP-dependent protein kinase (PKA or A-kinase) by promoting the dissociation of the inactive holozyme into regulatory subunits and active catalytic subunits (Glass and

Krebs, 1980), which catalyze the phosphorylation of certain proteins which serve to regulate their subsequent biological activity (Cohen, 1982 and 1985; Glass and Krebs, 1980; Krebs and Beavo, 1980; Shackter et al., 1984). PKA is implicated in mediating a number of intracellular events including the regulation of cytoskeletal structure (Albertini and Herman, 1984; Porter et al., 1974).

A variety of signaling substances utilize the PKA pathway to regulate actin cytoskeletal dynamics and cell migration (Howe, 2004). While some of these intracellular events require PKA activity, others are inhibited by it. Also, cell migration and invasion can be impeded by either inhibition or hyper-activation of PKA. In addition, a number of A-kinase anchoring proteins (AKAPs) serve to associate PKA with various components of the actin cytoskeleton, thereby enhancing and/or specifying cAMP/PKA signaling in those regions (Howe, 2004).

Agonists of cAMP/PKA signaling cause significant changes in cellular architecture, such as dissolution of stress fibers and induction of stellate morphology in neurons and other cells (Dong et al., 1998; Ramakers and Moolenaar, 1998; Edwards et al., 1993). Negative effects of PKA on migration were reported for endothelial cell migration on vitronectin (Kim et al., 2000). Also, matrix-specific down-regulation of cAMP/PKA signaling is required for collagen-induced F-actin synthesis and stress fiber formation in endothelial cells (Whelan and Senger, 2003). Conversely, elevation of cAMP and activation of PKA have been shown to be required for efficient cell migration in several systems. These include: formation of filopodia and lamellipodia in response to follicle stimulating hormone (Grieshaber et al., 2000), mammary epithelial cell migration on laminin (Plopper et al., 2000), microfilament assembly (Whittard and Akiyama, 2001), and activation of Cdc42 and Rac (Feoktistov et al., 2000; O'Connor

and Mercurio, 2001). It is clear that in most cell types, it is not simply a matter of cAMP/PKA signaling exerting a negative or positive effect on the cytoskeleton. Rather, a balance of cAMP/PKA activity in extent, space, and time is crucial for successful cytoskeletal organization (Edin et al., 2001; Ydrenius, et al., 1997; O'Connor and Mercurio, 2001).

The control of nonmuscle microfilament contraction via phosphorylation of myosin light chain kinase (MLCK) by PKA has been suggested (Conti and Adelstein, 1981). In contrast to skeletal muscle, actin-myosine interaction in nonmuscle cells is controlled by the phosphorylation of the regulatory myosin light chain (Sellers and Adelstein, 1987), which is catalyzed at least partly, by the enzyme myosin light chain kinase (MLCK), the activity of which is dependent upon calcium and calmodulin (Conti and Adelstein, 1981). The activity of MLCK can be inhibited in vitro and in vivo by phosphorylation of the enzyme catalyzed by PKA (Conti and Adelstein, 1981; Silver and DiSalvo, 1979). This phosphorylation interferes with Ca^{2+}/calmodulin binding to MLCK and thereby results in the inhibition of MLCK activity in vitro (Adelstein, 1982). In nonmuscle cells PKA regulates microfilament structure through the phosphorylation and inhibition of MLCK activity (Lamb et al., 1988).

It is shown that PKA directly phosphorylates monomeric actin at serine residues, a modification that significantly decreases monomer 'polymerizibility' in vitro (Ohta et al., 1987). Although this event is not yet well elucidated, at least the data indicate a pathway for regulation of actin dynamics by PKA (Howe, 2004).

1.2.1.3 The roles of the actin cytoskeleton in non-muscle cells

The actin cytoskeleton of an eukaryotic cell is central to locomotion, phagocytosis, contractility, shape changes, cytokinesis, maintenance of polarity, exocytosis and endocytosis. Actin filament nucleation most frequently occurs at the plasma membrane, which is regulated by external signals, allowing the cell to change its shape and stiffness rapidly in response to changes in its external environment. Therefore, the highest density of actin filaments in most cells is at the periphery. These actin filaments in the layer underlying the plasma membrane, called the cell cortex, determine the shape and movement of the cell surface. Actin structures can form many different types of cell surface projections, including microvilli or filopodia, flat protrusions called lamellipodia that help move cells over solid substrates. Regulation of cell morphology is essential for cell division and cell function in organisms. Small GTPbinding protein Rho family plays a key role in such regulations by controlling the actin cytoskeleton (Hall, 1998; Etienne-Manneville and Hall, 2002).

Research in fibroblasts on the molecular mechanisms mediating morphological transformations has identified a signaling cascade via small GTPases that link membrane receptors to the cytoskeleton (Ridley and Hall, 1992). RhoA is activated by extracellular signals such as lysophosphatic acid (LPA) leading to the assembly of stress fibers and focal adhesions (Hall et al., 1993; Ridley and Hall, 1994; Hall, 1998). Assembly of microfilaments is facilitated by the localization of RhoA and Rac1 in caveolae (Michaely et al., 1999), plasma membrane domains associated with actin-rich regions. Rac1 induces membrane ruffling and lamellipodium formation, and Cdc42 induces the formation of microspikes and filopodia, all of which are dependent on filamentous actin (F-actin) organization (Ridley, 2000 and 2001; Ridley and Hall, 1992; Nobes and Hall,

1995; Johnson and Pringle, 1990). The morphological transformations require only subcellular redistribution of cytoskeletal proteins instead of synthesis or degradation of the respective proteins (Safavi-Abbasi et al., 2001).

Experimental evidence suggests that the cortical cytoskeleton regulates exocytosis (Trifaro and Vitale, 1993). Disruption of F-actin enhances stimulated secretion in pancreatic β-cells (Orci et al., 1972), cultured chromaffin cells (Lelkes et al., 1986), permeabilized mast cells (Koffer et al., 1990), and lactotrope cells (Carbajal and Vitale, 1997). F-actin disassembly takes place in discrete zones of the cell cortex during hormone and neurotransmitter secretion and the areas of exocytosis correspond to cortical areas devoid of F-actin (Vitale et al., 1991; Nakata and Hirokawa, 1992).

The participation of actin-regulatory proteins during the actin reorganization that take place during secretion is suggested by the finding that actin-binding protein such as fodrin (Perrin and Aunis, 1985), scinderin (Vitale et al., 1991) talin and α-actinin (Nguyen et al., 1999) redistribute with cortical F-actin during stimulated secretion. Actin cytoskeleton is crucial for transport of endocytosed molecules. The initial internalization step requires actin cytoskeleton (Lamaze et al., 1997) and the later trafficking of endocytosed molecules is affected by disruption of the actin cytoskeleton (Durrbach et al., 1996). Endocytosed molecules are in general exocytosed at sites of plasma membrane protrusions (Bretscher and Aguado-Velasco, 1998).

1.2.1.4 Actin dynamics in FS cells

As the dynamics of the actin cytoskeleton in non-muscle cells, the actin dynamics in FS cells is also affected by a variety of factors including extra- and intra-cellular

factors, such as serum, hormones and related actin-binding proteins. In the mink anterior pituitary, Cardin et al (2000) found the levels of some hormones influence the morphology of the FS cells via the cytoskeleton reorganization. However, the precise mechanisms underlying the actin dynamic regulation in FS cells are not yet fully understood.

1.2.2 Actin-binding proteins

Due to the involvement of actin-binding proteins in the control of actin cytoskeleton dynamics, we decided to study the participation of these regulatory proteins in the modulation of the actin cytoskeleton organization in FS cells under different experimental conditions. Our previous results have shown that in these cells the reorganizaion of the cell's shape implicated the formation of cellular processes. Therefore, among the several actin-binding proteins, we focused our studies on the role of two actin-binding proteins that mediate the interaction between actin filaments and the plasma membreane: cortactin and α-actinin.

1.2.2.1 Cortactin

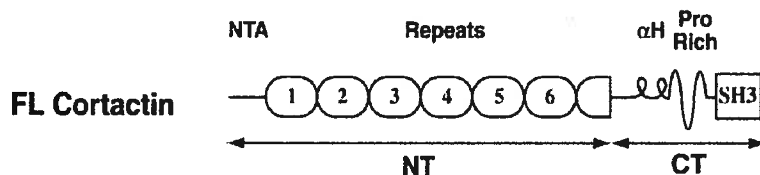
Vertebrate cells exhibit a cortical cytoskeletal network that resides beneath, and is associated with, the inner surface of the plasma membrane. This cortical cytoskeleton is comprised of a dense network of actin filaments and associated actin-binding proteins (Small et al., 1981; Stössel et al., 1981). Cortatin is an actin-binding protein that is enriched in cortical structures such as membrane ruffles and lamellipodia and plays an important role in the dynamic regulation of cortical actin cytoskeleton. The name

cortactin just reflects the cortical subcellular localization and its actin-binding activity (Wu and Parsons, 1993).

1.2.2.1.1 Structural organization

Cortactin, an important actin-binding protein, was initially identified as a tyrosine phosphorylated protein in v-Src infected chicken embryo fibroblasts (Kanner et al., 1990). Cortactin has a unique structure (Wu et al., 1991), comprising several domains (Weed and Parsons, 2001) (Schema 1). The first 84-94 amino-terminal residues are largely unstructured, containing a large number of acidic residues between amino acids 15 and 35 and is referred to as the amino terminal acidic domain (NTA; Weed et al., 2000). The NTA region is followed by a series of six complete 37 amino acid tandemly repeating segments and one incomplete segment of 20 residues in length. The complete repeats form a helix-turn-helix structure (Wu et al., 1991) and are termed cortactin repeats (Sparks et al., 1996a) since they show no sequence similarity to other known repeats except in HS1, a cortactin-like protein. The repeats' region is followed by an α-helical domain, a proline-rich domain abundant in tyrosine, serine and threonine residues, and a Src homology (SH) 3 domain at the distal carboxyl terminus. The cortactin SH3 domain shares significant homology to SH3 domains from Src-family kinases, various adapter and cytoskeletal proteins (Wu et al., 1991; Sparks et al., 1996a). Cortactin genes have now been cloned from a number of diverse organisms. All cortactins are structurally similar to one another, with the SH3 domain showing the greatest amount of evolutionary conservation while the proline-rich domain the least (Schema 2). Only one cortactin gene has been identified to date, and in most cell types, it encodes a single cortactin protein product that often migrates as two separate bands in

SDS-PAGE with relative molecular weights of 80 and 85 kDa (Wu et al., 1991). Northern blot analysis indicates that cortactin mRNA is expressed in nearly all mammalian tissues (Miglarese et al., 1994; Du et al., 1998).



Schema 2. Full-length cortactin is comprised of an N-terminal acidic (NTA) region followed by 6.5 37-amino acid tandem repeats. A predicted α-helical and proline-rich region are followed by an SH3 domain (Kinley et al., 2003).

1.2.2.1.2 The role of cortactin as a dynamic regulator of the actin cytoskeleton

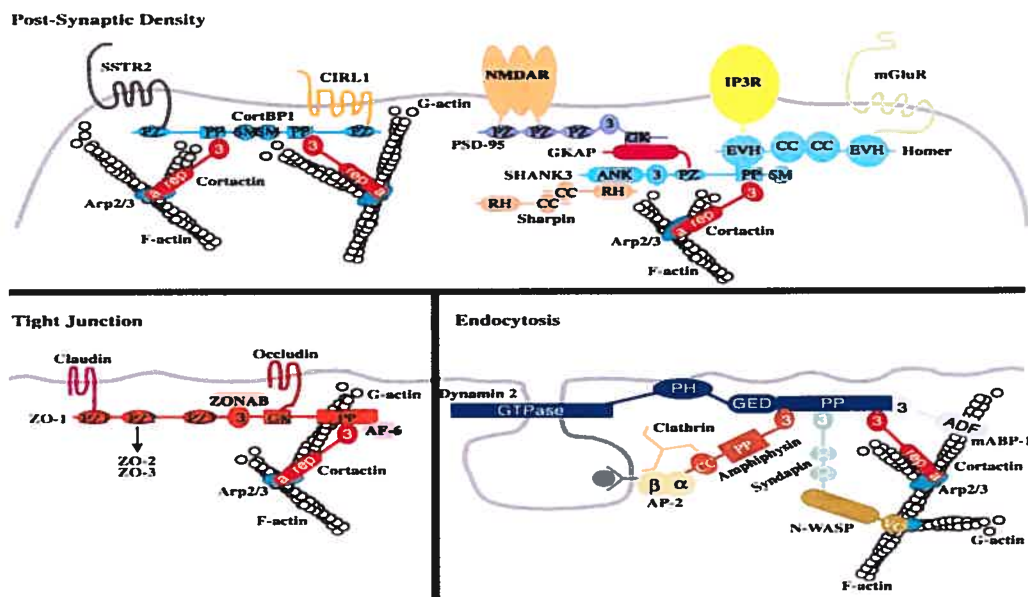
Localization and biochemical studies show that cortactin plays an important role in regulating cortical actin assembly and organization (Weed and Parsons, 2001). In most cell types, cortactin localizes in cytoplasmic punctate structures of unknown composition concentrated at the perinuclear region, and also with F-actin at sites of dynamic peripheral membrane activity (Weed and Parsons, 2001). Cortactin translocates from the cytoplasm to the periphery in response to many of the stimuli that induce its tyrosine phsphorylation, including growth factor treatment, integrin activation and bacterial entry (Ozawa et al., 1995; Weed et al., 1998; Cantarelli et al., 2000). These events also lead to activation of Rac1 (Hartwig et al., 1995; Clark et al., 1998; Mounier et al., 1999), which together with Cdc42 are responsible for controlling the formation of cortical actin networks (Ridley et al., 1992; Kozma et al., 1995). Rac1 activation is required for cortactin translocation to the periphery of the cell (Weed et al., 1998), while cortactin dissociates from cortical actin networks following treatment with

the F-actin disrupting drug cytochalasin D (Wu and Parsons, 1993). Taken together, cortical localization of cortactin is closely associated with Rac1-mediated actin assembly (Weed and Parsons, 2001).

Cortactin interacts directly with F-actin through sequences in the repeats' domain (Wu and Parsons, 1993). Deletion mapping analysis has determined that the fourth cortactin repeat is responsible for F-actin binding and cortical localization (Weed et al., 2000). Cortactin binds to the sides of actin filaments as determined by negative stained electron microscopy of recombinant cortactin/F-actin mixtures and by competition binding experiments with myosin subfragment 1 (Ohoka and Takai, 1998). Recombinant cortactin is found to crosslink F-actin (Huang et al., 1997). Because of its cortical localization and the fact that it is tyrosine phosphorylated in response to ligand stimulation as well as in cellular transformation, cortactin is considered an interesting candidate for a signal-transducing molecule or an effector molecule in the integration of extracellular stimulation with intracellular changes (Grinstein et al., 1989; Erpel and Courtneidge, 1995: Wu and Parsons, 1993).

1.2.2.1.3 Interaction of cortactin with other proteins to regulate the cortical actin cytoskeleton organization

Cortactin serves to link diverse scaffolding protein complexes to Arp2/3 (Weed and Parsons, 2001). These multiprotein scaffolds are associated to the actin cytoskeleton through sequences within the cortactin NTA and repeat domains (Weed and Parsons, 2001) (Schema 3).



Schema 3 Cortactin serves to link diverse scaffolding protein complexes to Arp2/3-driven actin polymerization. Schematic representation of the proposed molecular interactions within the neuronal post-synaptic density (top), epithelial tight junction (bottom left) and in receptor mediated endocytosis (bottom right) (Weed and Parsons, 2001).

A great deal of factors involved in the *de novo* assembly of actin-rich structures have been identified. Among these factors, the Arp2/3 complex has emerged as a key regulator of actin-filament nucleation (Weaver et al., 2001) that is conserved from yeast to humans (Pollard et al., 2000). The Arp 2/3 complex is composed of seven subunits, including two actin-related proteins, Arp2 and Arp3, and five other subunits. It is concentrated in dynamic actin structures such as the leading edges of motile cells (Welch et al, 1997). Cortactin binds to the Arp2/3 complex through its N-terminal domain and stimulates its actin-nucleation activity. Association of cortactin with the Arp2/3 complex is essential and sufficient for cortactin to localize within actin-rich patches. Furthermore, the F-actin-binding activity of cortactin is essential for cortactin to stimulate the Arp2/3 complex (Uruno et al., 2001), suggesting the important relationship between them. Cortactin also inhibits debranching of aged Arp2/3 networks

between Arp2/3 complex and mother/daughter filament networks (Weed and Parsons, 2001). The association of cortactin with mobile cortical actin structures shows a functional role for cortactin in regulating Arp2/3 activity (Dai et al., 2000; Kaksonen et al., 2000). Localization of cortactin to the cell periphery coupled with its ability to stimulate and stabilize Arp2/3-dependent actin polymerization in vitro suggests a role for cortactin in the regulation of actin cytoskeletal dynamics (Weed and Parsons, 2001).

Arp2/3 complex in cells is inactive until cellular signals result in its activation (Goode et al., 2001). In higher eukaryotes, the only known activators of Arp2/3 complex are the Wiskott-Aldrich syndrome family of proteins (WASps) (Goode et al., 2001), which contain a C-terminal acidic domain that binds the Arp2/3 complex. The C-terminal region of WASp also contains a verprolin homology and connecting (VC) domain, which binds monomeric actin (G-actin) (Goode et al., 2001). Cortactin can function cooperatively with WASp proteins in addition to independently activating Arp2/3 complex (Weed and Parsons, 2001). The ability of cortactin to bind and avtivate Arp2/3 complex indicates a role for cortactin in actin-based motility events (Weed and Parsons, 2001). Enhanced cell migration in NIH 3T3 and endothelial cells overexpressing cortactin supports this notion (Patel et al., 1998; Huang et al., 1998).

Cortactin has another role in recruiting other components to the periphery, including perhaps the small GTPases themselves. Weed et al showed that short-term expression of active Rac1 induces cortactin accumulation under the membrane without an obvious accumulation of Rac1 itself (Weed et al., 1998). This indicates that Rac1 can "send" cortactin to the periphery of the cell without being translocated itself. During longer-term expression, however, Di Ciano et al. found strong colocalization of active Rac1 and cortactin in membrane ruffles (Di Ciano et al., 2002). Taken together, F-actin-

binding activity of cortactin, its cortical localization, and its tyrosine phosphorylation indicate that cortactin plays a role in mediating microfilament-membrane interactions in response to signals propagated by receptor and/or nonreceptor protein tyrosine kinases (Wu and Parsons, 1993).

1.2.2.1.4 Phosphorylated status of cortactin

Cortactin is a target for both tyrosine and serine/threonine protein kinases (Weed and Parsons, 2001) and is normally phosphorylated on serine and threonine, but it becomes tyrosine phosphorylated in response to growth factor stimulation and by activated Src (Wu et al., 1991; Maa et al., 1992; Zhan et al., 1993).

In nontransformed cells, cortactin transiently associates with c-Src in response to thrombin activation in platelets (Wong et al., 1992) and in response to FGF-1 in fibroblasts (Zhan et al., 1994). A wealth of data indicates that cortactin tyrosine phosphorylation is closely correlated with Src activity in a number of signaling pathways and the strong correlation between Src activation and cortactin tyrosine phosphorylation in these studies suggest that cortactin is phosphorylated directly by Src (Weed and Parsons, 2001). Src directly phosphorylates cortactin *in vitro* (Huang et al., 1997) on three tyrosine residues in murine cortactin (tyrosine-421, -466, and -482) located within the proline-rich domain (Huang et al., 1998). Cortactin tyrosine phosphorylation requires Rac1-induced cortactin targeting to cortical actin networks, where it is tyrosine phosphorylated at positions 421 and 466 in a hierarchical manner, that is, with tyrosine 421 phosphorylation being required for phosphorylation of tyrosine 466 (Head et al., 2003). Expression of a cortactin construct where tyrosines 421, 466, and 482 were mutated to phenylalanine dramatically reduced cortactin tyrosine

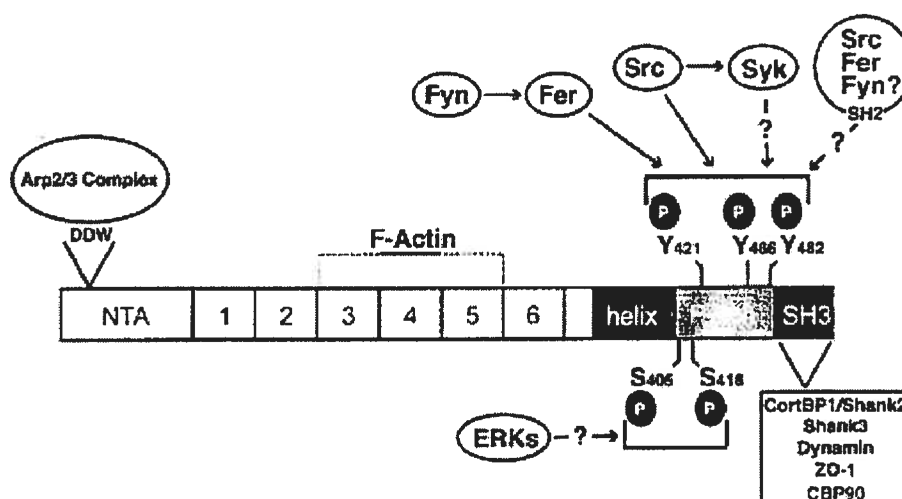
and 482 were mutated to phenylalanine dramatically reduced cortactin tyrosine phosphorylation following expression in v-Src transformed 3T3 fibroblasts, indicating that these residues are targeted by Src *in vivo* (Huang et al., 1998).

Fer is a second nonreceptor tyrosine kinase involved in growth factor-mediated cortactin tyrosine phosphorylation (Weed and Parsons, 2001). NIH 3T3 fibroblasts treated with PDGF lead to Fer activation and cortactin tyrosine phosphorylation (Downing and Reynolds, 1991; Kim and Wong, 1998) that is markedly reduced in fibroblasts derived from Fer $^{-/-}$ mice (Craig et al., 2001). Fer immunoprecipitates with cortactin and the Fer SH2 domain interacts with cortactin in cell extracts, suggesting that Fer directly interacts with and phosphorylates cortactin (Weed and Parsons, 2001).

In addition to tyrosine phosphorylation, serine and threonine phosphorylation may regulate cortactin function (Weed and Parsons, 2001). The EMS1 gene in NIH 3T3 cells encodes the human homologue of cortactin, an 80-85-kDa multidomain actin-binding protein that is a target of both growth factor- and adhesion-regulated signaling pathways (Wu et al., 1991; Schuuring et al., 1993). EMS1 is phosphorylated on serine and threonine residues in untransformed chick embryo fibroblast cells (Wu et al., 1991) and in two human squamous carcinoma cell lines (Van Damme et al., 1997).

As mentioned above, two cortactin species of 80 and 85 kDa are present in many cell types (Wu et al 1991; Schuuring et al., 1993; Katsube et al., 1998; Van Damme et al., 1997). In cells amplified at the 11q13 locus, EGF, serum, or vanadate treatment caused an increase in serine and threonine phosphorylation of EMS1, resulting in an electrophoretic mobility shift from 80 to 85 kDa. EGF- or vanadate-induced increase in serine and threonine phosphorylation of EMS1 also resulted in a change in localization from the cytoplasm to the cell-substratum junctions (Campbell et al., 1999). Several

lines of evidence suggest that the serine/threonine phosphorylation of EMS1 play an independent role in regulation of EMS1 function to tyrosine phosphorylation. Firstly, a wide variety of cell types display both the 80- and 85-kDa forms of the EMS1 protein, and the presence of at least two forms of the protein is conserved in human, mouse, chicken, and Drosophila (Wu et al., 1991; Katsube et al., 1998; Van Damme et al., 1997). In addition, serine and threonine phosphorylation can occur independently of tyrosine phosphorylation, and only serine and threonine phosphorylation correlates with the EMS1 mobility shift (Van Damme et al., 1997) (Schema 4).



Schema 4. Diagrammatic representation of cortactin binding proteins and phosphorylation sites. Binding of Arp2/3 complex occurs through the three amino acid ± DDW -motif within the NTA domain. The actin-binding domain is located within the repeats region, requiring the fourth repeat and possibly adjacent sequences. Tyrosine phosphorylation by Src and Fer occurs within the proline-rich domain at Y421, Y466 and Y482. Putative serine phosphorylation by ERK1/2 occurs at S405 and S418. The SH3 domain interacts with numerous PDZ-containing scaffolding proteins as well with dynamin and CBP90 (Weed and Parsons, 2001).

While phosphorylation of cortactin by Src is thought to be functionally linked to some aspects of actin organization and cell motility, the full significance of Src mediated cortactin tyrosine phosphorylation is not yet clear (Weed and Parsons, 2001). The functional significance of Fer-mediated cortactin tyrosine phosphorylation is also unknown (Weed and Parsons, 2001). In normal cells, cortactin does not contain detectable phosphotyrosine, and the majority of the protein partitions with the detergent-soluble fraction, that is to say with the 'non-cytoskeleton' fraction. In cells expressing wild type v-Src or an SH3 Src deletion, cortactin becomes tyrosine-phosphorylated, and a significant increase in cortactin association to the Triton-insoluble cytoskeletal fraction can be observed (Heidi and Marilyn, 1995).

The tyrosine phosphorylation of cortactin is increased in response to diverse stimuli including receptor tyrosine kinase activation (Maa et al., 1992; Zhan et al., 1994), integrin-mediated cell adhesion (Vuori and Ruoslahti, 1995), oncogenic transformation (Wu et al., 1991; Kanner, et al., 1990), and platelet aggregation (Wong et al., 1992; Fox et al., 1993) and is strongly linked to activation of Src-family kinases. Many of these processes result in extensive cytoskeletal rearrangement, suggesting that tyrosine-phosphorylated cortactin is involved in this reorganization of the actin cytoskeleton (Campbell et al, 1999). In addition to cytoskeletal reorganization induced by stimuli resulting in EMS1 tyrosine phosphorylation, the subcellular localization of EMS1 is also controlled by the small GTPases Ras and Rac1. In untransformed NIH 3T3 cells, EMS1 is found complexed with myosin II and actin, and this complex is disrupted after Ras transformation (He et al., 1998). After growth factor-induced Rac1 activation in Swiss 3T3 cells, cortactin localizes to membrane ruffles, a process that can also be induced by activated Rac1 (Weed et al., 1998). These data indicat that the

subcellular localization of cortactin may be also regulated by mechanisms that are independent of cortactin tyrosine phosphorylation (Campbell et al., 1999).

According to Di Ciano et al (2002), tyrosine phosphorylation of cortactin can be a compensatory process that facilitates the disassembly of the Arp2/3-actin-cortactin complex. Such disassembly, enhanced by Src kinases, can be important for the dynamic recycling of the molecule during cell movement; as the leading edge is propelled forward, cortactin at the base of the lamellipodium may become phosphorylated and detach from actin. After dephosphorylation, cortactin may be rebuilt into the new front (Di Ciano et al., 2002). One role for serine/threonine phosphorylation of cortactin is perhaps in the regulation of EMS1 subcellular localization (Van Damme et al., 1997).

1.2.2.2 α-Actinin

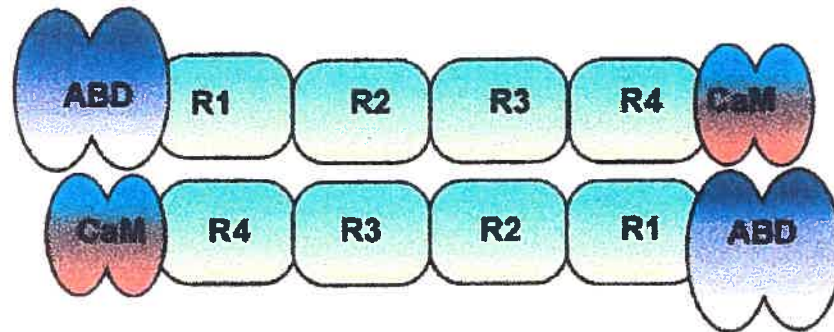
α-Actinin, an ubiquitous actin filament cross-linker belongs to a large family of actin-binding proteins that includes fimbrin, dystrophin, and spectrin (Hammings et al., 1995). It has been identified in most eukaryotic organisms, from human (Beggs et al., 1992; Mills et al., 2001) and mouse (Mills et al., 2001) to fly (*Drosophila melanogaster*) and worm (*Caenorhabditis elegans*) (Fyrberg et al., 1990; Barstead et al., 1991). All family members share a common 27-kDa F-actin binding domain (Puius et al., 1998). At least four human α-actinin genes have been described. One gene (aac1_human) gives rise to two alternative spliced isoforms, the smooth muscle and the cytoskeletal/non-muscle isoforms (Waites et al., 1992). The two isoforms differ in a region that spans the tail of the first EF-hand calcium-binding motif; 27 amino acid residues in the non-muscle isoform were replaced by a distinct stretch of 22 amino acids in the smooth muscle isoform (Waites et al., 1992). As a result of this substitution, the binding of the

non-muscle isoform to actin is inhibited by calcium (Tang et al, 2001), whereas actin interaction with the smooth muscle isoform is calcium-insensitive (Beggs et al, 1992; Waites et al., 1992). Two genes (aac2_and aac3_) encode several α-actinin skeletal muscle isoforms (Beggs et al., 1992). An additional human α-actinin isoform (aac4_) that exhibits 80% sequence identity to the smooth muscle and non-muscle isoforms was recently cloned from a tumor cell line (Honda et al., 1998). The muscle isoforms, α-actinin 2 and α-actinin 3 are localized to the Z-disc of the sarcomeres (Blanchard et al., 1989; Mills et al., 2001). α-Actinin 1 and α-actinin 4 can be found at the leading edge of motile cells, at cell adhesion sites and focal contacts, and along actin stress fibers in migrating cells (Barstead et al., 1991).

1.2.2.2.1 Structural and functional characteristics of α-actinin

The whole length α-actinin has a molecular mass of 93 to 103 kDa (Blanchard et al., 1989). α-Actinin exists as an anti-parallel homodimer, which orients the actin-binding domains at opposite ends, allowing each dimmer to bind two actin filaments, leading to the formation of bundles of actin filaments (Tang, et al., 2001). The N-terminal actin-binding domain of α-actinin consists of two calponin homology (CH) domains, both of which interact directly with the actin filament (Keep et al., 1999; Norwood et al., 2000). At the C-terminus, there are two EF-hands, where the second EF-hand is crucial for calcium binding (Witke et al., 1993; Janssen et al., 1996). The rod domain, which is important for dimerization of α-actinin, is formed by triple-helical repeats (spectrin repeats) and connects the N-and C-terminus. (Djinovic-Carugo et al., 1999; Ylanne et al., 2001). There are usually four spectrin repeats in the rod domain

1999; Ylanne et al., 2001). There are usually four spectrin repeats in the rod domain (Schema 5), but α-actinin from some organisms has only one or two repeats (Virel and Backman, 2004).



Schema 5. Schematic drawing of an α-actinin dimer. Each molecule consists of an N-terminal actin-binding domain (ABD), a central region with four α-helical spectrin-like repeats (R1–R4) and a C-terminal calmodulin-like (CaM) domain. The molecules in a dimer are aligned in antiparallel fashion (Otey and Carpen, 2004)

1.2.2.2.2 The roles of α-actinin

α-Actinin functions as an actin cross-linking protein abundant at focal adhesions (Maruyama and Ebashi, 1965; Lazarides and Burridge, 1975; Podlubnaya et al., 1975), and has been suggested to play important roles in nascent focal adhesion assembly and stress fibre extensions from the integrin-based cell–substrate adhesion complex. Analyses of green fluorescent proteins (GFP)-α-actinin dynamics showed that once the interaction between the tips of protrusions and extracellular matrix stabilizes, α-actinin begins to localize in small foci at the leading edge, which then grow in size and extend small fibre-like structures toward the cell body (Edlund et al., 2001; Laukaitis et al.,

2001). On the other hand, α-actinin was demonstrated to be critical for the correct positioning of zyxin at focal adhesions, which is suggested to induce actin polymerization at focal adhesions independent of the Arp2/3 complex by forming a complex with a mammalian member of the Ena/VASP family, Mena (Drees et al., 1999; Reinhard et al., 1999; Fradelizi et al., 2001).

The localization of α-actinin in focal adhesion plaques suggestes that it might serve to anchor the network of actin filaments to the plasma membrane (Izaguirre et al., 2001). This is substantiated by the finding that α-actinin associates with the cytoplasmic tail of members of several adhesions receptor families including integrins (Otey et al., 1990; Sampath et al., 1998), cadherins (Knudsen et al., 1995; Nieset et al., 1997), and intercellular adhesion molecules (Carpen et al., 1992; Heiska et al., 1996).

α-Actinin plays an important role in the regulation of cell adhesion by linking actin filaments directly to integrin receptors (Pavalko et al., 1991). α-Actinin constitutes a direct link between the actin cytoskeleton and the cytoplasmic domains of several cell surface receptors. This cytoskeletal connection is implicated in receptor function and anchorage, cytoskeletal reorganization, and concomitant signaling events (Yamada and Geiger, 1997; Pavalko and LaRoche, 1993; Wyszynski et al., 1997). Integrins bind to a number of cytoskeletal proteins through their cytoplasmic domains, including talin, α-actinin, and filamin, all of which bind F-actin (Liu et al., 2000). These integrin-binding proteins bind to another cytoskeletal or scaffold protein, and these hierarchical multiple protein complexes are considered to mediate the attachment of integrins to actin filaments (Miyamoto et al., 1995) (Schema 6).

Schema 6. Possible interactions between structural and signaling proteins within the cell–substratum focal adhesion–actin complex in ECs. Plasma membrane (PM), extracellular matrix (ECM) (Lee and Gotlieb, 2003).

α-Actinin was found to interact with synthetic peptides that correspond to the cytoplasmic tail of β_1, β_3 (Otey et al., 1990 adn 1993) and β_2 (Sampath et al., 1998). Integrin receptor aggregates molecules with which α-actinin interacts indicates that in addition to its role as an actin cross-linking protein, α-actinin also functions as a scaffold to promote protein-protein interactions (Izaguirre et al., 2001). The reported interactions of α-actinin with actin, integrins, vinculin (Wachsstock et al., 1987), zyxin (Crawford, 1992), the p85 subunit of PI3-kinase (Shibasaki et al., 1994), PKN (a fatty acid) and Rho-activated serin/threonine protein kinase (Mukai et al., 1997) are consistent with this possibility.

1.2.2.2.3 Phosphorylation status of α-actinin

α-Actinin is tyrosine-phosphorylated in activated platelets and activated T-cells (Izaguirre et al., 1999; Egerton et al., 1996). Using the recombinant protein, α-actinin is found to be phosphorylated in non-hematopoietic cells and the tyrosine residue at

position 12 in α-actinin is the site of phosphorylation (Izaguirre et al., 2001). In platelets, tyrosine phosphorylation of α-actinin and the focal adhesion kinase (FAK) are closely regulated events suggesting that α-actinin is an FAK substrate and tyrosine phosphorylation of α-actinin by FAK affects the cytoskeleton organization (Izaguirre et al., 2001).

Izaguirre et al (1999) suggest that α-actinin is not a preferred substrate of pp60 src by the finding that trace amounts of phosphotyrosine were incorporated in α-actinin isolated from Rous sarcoma-transformed chicken embryo fibroblasts (Sefton et al., 1981). α-Actinin is tyrosine-phosphorylated in murine T cells activated by T cell antigen receptor ligation (Egerton et al., 1996). These data together suggested that α-actinin tyrosine phosphorylation can also occur in cells other than patelets (Izaguirre et al., 1999). $pp125^{FAK}$ and pp105/α-actinin tyrosine phosphorylation in platelets are closely regulated events (Haimovich et al., 1993; Shattil et al., 1994). According to Izaguirre et al, α-actinin and $pp125^{FAK}$ tyrosine phosphorylation in platelets are dependent on, rather than regulated by the massive cytoskeleton reorganization that takes place in activated platelets (Izaguirre et al., 1999).

1.3 Aim and hypothesis

It is established that tyrosine-phosphorylated cortactin is involved in regulation of the actin cytoskeleton. This is supported by studies using v-src-transformed fibroblasts and thrombin-activated platelets, in which tyrosine-phosphorylated cortactin partitions into the detergent-insoluble cytoskeletal fraction (Ozawa et al., 1995; Okamura and Resh, 1995; Fox, 1993). Furthermore, upon v-Src transformation, cortactin localizes

into abnormal focal adhesions termed rosettes or podosomes (Wu et al., 1991). However, the precise molecular mechanisms underlying the actin cytoskeleton regulation through tyrosine phosphorylation of cortactin are still unclear. Therefore, the present study is aimed at investigating how tyrosine phosphorylated cortactin is involved in the regulation of the actin cytoskeleton dynamics in FS cells.

It is well known that in platelet, tyrosine phosphorylation of α-actinin and the focal adhesion kinase (FAK) are closely regulated events and α-actinin is an FAK substrate. In addition, tyrosine phosphorylation of α-actinin by FAK affects the cytoskeleton organization (Izaguirre et al., 2001). However, whether or not PKA is involved in tyrosine phosphorylation of α-actinin and how α-actinin regulates the actin cytoskeleton dynamics via PKA pathway still remains to be elicited.

Thus in the present study we want to explore how α-actinin is involved in regulating the actin cytoskeleton dynamics in FS cells via PKA pathway and what is the relationship between cortactin tyrosine phosphorylation and PKA pathway.

2 Materials and methods

2.1 Materials

2.1.1 Cell model

The folliculo-stellate-like cell line TtT/GF established from a mouse pituitary tumor (Inoue, et al, 1992) was employed as a model for our experiments. The cells were kindly provided by Dr. U Rennel (Max-Planck Institute, Munich, Germany).

2.1.1.1 Characteristics of the TtT/GF cell line

An isologously transplantable mouse thyrotropic pituitary tumor (TtTb) induced by radiothyroidectomy was found to form lobules composed of glandular cells. The surfaces of the lobules were covered by stellate cells that were immunopositive to glial fibrillary acidic protein (GFAP), which is also positive in pituitary folliculo-stellate cells. Upon culture of the thyrotropic tumor, cells with long processes appeared. These cells showed strong staining for GFAP and were dependent on basic fibroblast growth factor for growth. By repeated passage, the GFAP-positive cells become a stably growing immortal cell line, which is named TtT/GF (Figure B) (Inoue, et al., 1992).

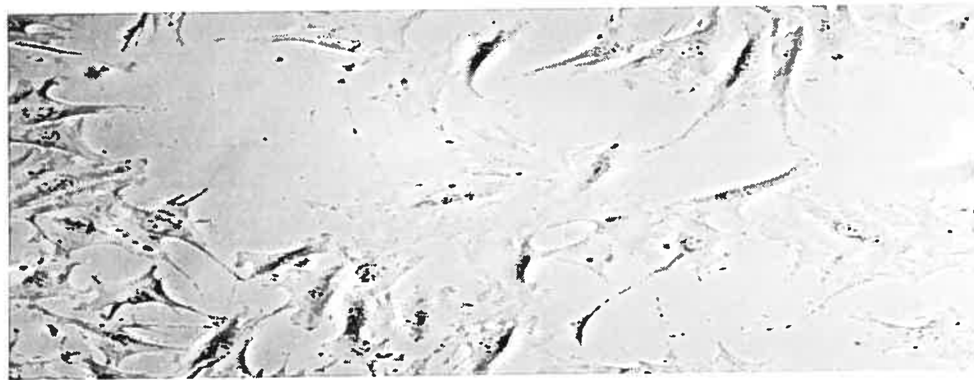


Figure B Light microscopic micrograph of TtT/GF cells. The cells cultured in medium containing FBS are flat and some have crescent appearances. Long cell processes are also evident, giving the cells a star-like shape (kindly provided by Sara Solinet, Department of Pathology and Cell Biology, Université de Montréal).

2.1.1.2 Relationship between the FS cells and TtT/GF cell line

FS cells are characterized by a stellate appearance (Farquhar et al., 1975), formation of follicular lumina (Farquhar, 1975; Kagayama, 1965), the presence of lysosomes and phagocytotic activity (Stokreef et al., 1986; Shiotani, 1980), numerous intermediate filaments in the cytoplasm (Girod and Lheritier, 1986), and the expression of GFAP (Cocchia and Miani, 1980; Ogawa et al., 1990) and of the S-100 protein (Cocchia and Miani, 1980; Nakajima et al., 1980). It is known that the S-100 protein is a dimmer formed by two subunits, S-100α and S-100β. This protein can stimulate prolactin release from cultured clonal PRL cells and from normal dissociated anterior pituitary cells (Ishikawa et al., 1983: Lloyd and Mailloux, 1988). TtT/GF cell line possesses all of these characteristics of the FS cells

2.1.1.3 Reasons for choosing TtT/GF cell line as our model

Our aim is to investigate the molecular mechanisms underlying the regulation of actin cytoskeleton dynamics in FS cells. Because of the morphological and functional similarities between the TtT/GF cell line and FS cells of the anterior pituitary, we decided to choose the TtT/GF cell line as the experimental model for FS cells. In addition, FS cells in the normal pituitary gland are not abundant and do not divide in culture, instead, the TtT/GF cells are easy to grow and they divide in culture.

2.1.2 Antibodies

- Anti-cortactin (p80/85), clone 4F11 mouse monoclonal IgG, was purchased from Upstate (Lake Placid, NY, U.S.A.).

- Anti-α-actinin, mouse monoclonal IgG, was purchased from Chemicon International Co. (Temecula, CA, U.S.A.).

- Anti-actin, rabbit polyclonal IgG, anti-vinculin, mouse monoclonal IgG, anti-myosin light chain, mouse monoclonal IgM, FITC-conjugated goat anti-rabbit IgG, and TRITC-conjugated goat anti-rabbit IgG, were purchased from Sigma (St Louis, MO, U.S.A).

- Anti-p-cortactin [pY421], rabbit polyclonal IgG, was purchased from Medicorp (Montreal, QC, Canada).

- Anti-phospho-tyrosine, clone: PY 20, mouse monoclonal IgG, was purchased from Transduction Laboratories Inc. (Mississauga, ON, Canada).

- Horseradish peroxidase-conjugated donkey anti-mouse IgG (H+L), horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L), FITC-conjugated donkey anti-mouse IgG (H+L), TRITC-conjugated donkey anti-mouse IgG (H+L), were purchased from Jackson ImmunoResearch Laboratories Inc (Missisauga, ON, Canada).

2.1.3 Other reagents

- Dulbecco's Modified Eagle Medium (DMEM) and FBS (fetal bovine serum) were purchased from GIBCO™ Invitrogen Corporation (Burlington, ON, Canada).

- Leupeptin, aprotinin, and forskolin were purchased from Boehringer Mannheim (Laval, QC, Canada). bpV (phen) was purchased from Calbiochem (San Diego, California, U.S.A.). PMSF (phenylmethylsulfonyl fluoride) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Rhodamine-phalloidin was purchased from Molecular Probes (Eugene, Oregon, U.S.A.).

- Genistein was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

- KT 5720 was purchased from Calbiochem (La Jolla, CA, U.S.A.).

2.2 Methods

2.2.1 Cell culture

2.2.1.1 General

The TtT/GF cells were cultured in 25 cm tissue culture flasks with Dulbecco's modified Eagle medium (DMEM, pH 7.2), supplemented with 5% fetal bovine serum (FBS), 44 mM $NaHCO_3$, 10 mM HEPES, penicillin G, and streptomycin sulfate, at 37^0 C in a water-saturated atmosphere of 95% air – 5% CO_2 in CO_2 incubator (model MCO-17A1, Sanyo Electric Co. Ltd., Japan). The culture medium was replaced with new one twice a week.

2.2.1.2 Treatments with drugs and FBS

For biochemical analyses, the cells were cultured in 10 cm diameter plastic Petri dishes. For immunofluorescence microscopy studies, the cells were cultured on 22 mm glass coverslips placed in 35 mm tissue culture dishes.

After an initial 48 h culture period in the presence of serum, the medium was replaced with the same volume of new culture medium in the presence or absence of FBS for another 48 h. Then, the cells were incubated with 30 μM genistein for 0 (as control), 1, 2, and 24 h to test the role of tyrosine phosphorylation of cytoskeletal proteins; or with 15 mM forskolin or 100 nM KT5720 for 0, 0.5, 1, 2, 4, and 8 h (2 and 8 h in the absence of forskolin or KT 5720 as control) to investigate the role of PKA-mediated phosphorylation of the same cytoskeletal proteins. Genistein, forskolin, and KT5720 were dissolved in corresponding volume of dimethyl sulfoxide (DMSO), such that the final concentration of DMSO was less than 0.1%. Preliminary studies in our

laboratory have shown that this concentration of DMSO has no effect on actin polymerization or depolymerization in the TtT/GF cell line.

In another set of experiments, after the initial 48 h incubation period in the presence of 5% FBS, the cells were serum-starved for 48 h and next incubated with culture medium containing 5% FBS for 0 (as control), 0.5, 1, 2, 4, and 8 h to evaluate the effect of serum on the localization of actin, cortactin, and α-actinin.

Following these treatments, the cells were processed for immunofluorescence microscopy or immunoblotting.

2.2.2 Preparation of subcellular fractions

Cells were scraped off with a plastic policeman. The cell suspension was recovered and centrifuged at 2250 rpm (GS-6R Centrifuge, Beckman, USA, GH-3.8 Rotor) at 5^0 C for 5 min. The pellet was washed with PBS (137 mM NaCl, 3 mM KCl, 8mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4), centrifuged and rinsed with PBS again. Finally the suspension was spun down at the same speed as mentioned above and the pellet was kept.

2.2.2.1 Preparation of membrane and cytosol fractions

The pellet was re-suspended in 1.5-2 volumes of modified lysis buffer pH 6.8 (80 mM Pipes, 5 mM EGTA, 1mM $MgCl_2$, 10 μM bpV, 5μg/ml Leupeptin, 5μg/ml Aprotinin, and 2mM PMSF) and homogenized on ice with the aid of a sonicator (up and down 10 seconds each time for eight to ten times, Fisher Sonic Dismembrator, Model 300, Fisher Scientific, Farmington, NY, USA). The homogenate was centrifuged at

1700 rpm (GS-6R Centrifuge, Beckman, USA, GH-3.8 Rotor) at 5^0 C for 5 min and the top layer of debris and the pellet were removed. The supernatant was centrifuged at 15,000 rpm (Beckman Microfuge E™, Beckman Instruments Inc., USA) at 4^0 C for 25 min. The resultant supernatant corresponded to cytosol-enriched fraction and the pellet corresponded to membrane-enriched fraction. Because this membrane-enriched fraction also contains cytoskeletal proteins, the membrane-enriched fraction was suspended in 2 volumes of the lysis buffer solution, and Triton X-100 was added at a final concentration of 1% to get rid of the cytoskeletal contamination. The suspension was next incubated for 30 min at room temperature. After this incubation period, the membrane suspension was centrifuged at 15,000 rpm (Beckman Microfuge E™, Beckman Instruments Inc., USA) at 4^0 C for 15 min and the pellet corresponding to the cytoskeletal protein contamination was discarded.

2.2.2.2 Preparation of cytoskeletal and non-cytoskeletal fractions

Following homogenization of the cell pellet in the lysis buffer, centrifugation and removal of the pellet as explained above (2.2), we added Triton X-100 to the supernatant to a final concentration of 1 % and the cell suspension was incubated for 30 min at room temperature, and then centrifuged at 15,000 rpm (Beckman Microfuge E™, Beckman Instruments Inc., USA) at 4^0 for 15 min. The pellet was considered the cytoskeleton-enriched fraction and the supernatant was considered the non-cytoskeleton-enriched fraction. The cytoskeletal fraction was dissolved in 1.5 volumes of Ripa lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8.0).

2.2.3 Electrophoresis and Western blot

2.2.3.1 Protein measurements

Proteins in the subcellular fractions were measured according to the method of Bradford (1976) by using the dye-binding assay (Bio-Rad Laboratories, Mississauga, ON, Canada). A standard curve was created by using different concentrations of BSA (Bovine Serum Albumin) in PBS and the concentrations of total proteins in each of samples were determined by the standard curve. The samples were prepared for electrophoresis by boiling them in the corresponding volume of SDS sample buffer 2X (8 M urea, 70 mM Tris-HCl pH 6.8, 3% SDS, 0.005% BBP, and 5% β-mercaptoethanol) for 3 min.

2.2.3.2 Preparation of the gels

Proteins in the samples were separated by sodium dodecylsulfate-polyacrylaide gel electrophoresis (SDS-PAGE) using a 4% acrylamide stacking gel and a 10% acrylamide running gel.

2.2.3.3 Electrophoresis and transfer

Ten to twenty μg of proteins of the subcellular fraction samples were loaded per well and electrophoresis was carried out at 70 V for approximately 30 min. When the proteins arrived to the running gel, the voltage was increased to 120 V for 1.5 to 2 h depending on the molecular mass of the proteins of interest. After the migration, the proteins in the gel were electrophoretically transferred at 300 mA for 1.5 h or at 27 V overnight onto nitrocellulose membranes at 4^0 C using a transfer buffer (25mM Tris-HCl pH 8.3, 150mM glycine and 20% (v/v) methanol). After the transfer, nitrocellulose

membranes were colored with Ponceau red (0.2% Ponceau red and 3% TCA in distilled water) for a few seconds at room temperature until the bands were visible and then rinsed with distilled water. The molecular mass of the bands corresponding to the proteins of interest was determined by comparison with the migration of molecular mass standards (SDS-PAGE Standards, High and Low Range, Bio-Rad Laboratories, Missisauga, ON, Canada). Next, the membranes were washed with PBS at room temperature for a few minutes with agitation to remove the Ponceau red.

2.2.3.4 Immunoblotting

To block nonspecific binding, the membranes were incubated in PBS-containing 5% skim milk at 37^0 C for 1 hour. Then the membranes were incubated with one of the following primary antibodies: anti-actin (1:500 dilution), anti-α-actinin (1:1000 dilution), anti-cortactin (1:1500 dilution), anti-p-cortactin (1:1000 dilution), and anti-phospho-tyrosine (1:150-1:300 dilution) overnight at 4^0 C with agitation or for 2h at 37^0C. After the incubation, the membranes were washed with 0.05% Tween20- PBS 15 min each for 4 times at 37^0 C and incubated with the corresponding (anti-rabbit, or anti-mouse IgG) secondary horseradish peroxidase-conjugated antibody (1:2000 dilution) for 1 h at room temperature with agitation. Both primary and secondary antibodies were diluted in PBS-containing 5% skim milk. After being washed in 0.05% Tween 20 - PBS 15 min each for 3 times and in TBS solution (137 mM NaCl, 2.7 mMKCl, 24.8 mM Tris-Base, pH 7.4) for 15 min once at room temperature with agitation, immunoreactive bands were revealed by the chemiluminescenct solution (Lumi-light Western Blotting Substrate, Roch Diagnostics Corporation, Laval, QC, Canada) and the membrane were exposed to Kodak X-OMAT XAR-5 (Eastman Kodak, Rochester, NY, U.S.A.).

2.2.3.5 Densitometry

The bands on the films were scanned with an Astra 1200S scanner (model: UTIUTA-2A, UMAX Data Systems Inc., Industrial Park, Hsinchu, Taiwan). Quantification of the intensity of the bands was performed by using a Scion Image program (Scion Corporation, MD, U.S.A.). The intensity of each immunoreactive bands was normalized to the value obtained at 0h (control) within each experiment. The graphs were made by using Sigmaplot software. Data presented were the mean +/- SEM.of at least three independent experiments.

2.2.4 Immunofluorescence

Cells grown on glass coverslip were subjected to different treatments. Next, the cells were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature followed by washing with PBS six times. Cells were permeabilized in 50% acetone-water, next 100% acetone, and then 50% acetone-water, 5 min each step. After washing with PBS, the cells were treated with 3% skim milk in PBS for 1 hour at room temperature or overnight at 4^0 C to block non-specific binding. After washing with PBS once, the cells were incubated with a mixture of primary antibodies for 1 hour at 37^0 C, rinsed in PBS and further incubated with the fluorescein isothiocyanate (FITC) or rhodamine-conjugated corresponding secondary antibodies at 37^0 C for 1 h. For the detection of F-actin, fixed and permeabilized cells were incubated with rhodamine-phalloidin (1:700 dilution). All antibodies and rhodamine-phalloidin were diluted in 1% skim milk in PBS. Double labelings were made by incubating the cells with anti-cortactin (1:80 dilution) and anti-p-cortactin (1:100 dilution) together or with anti-α-actinin (1:50 dilution) and rhodamine-phalloidin or by incubating the cells with anti-

phospho-tyrosine (1:50 dilution) and anti-cortactin (1:80 dilution) or with anti-α-actinin (1:50 dilution) and anti-phospho-tyrosine (1:50 dilution) at 37^0 C for 1 hour. After the incubation with secondary antibodies, the coverslips were washed with PBS six times and then mounted on micro slides using mounting solution (5% DABCO in 50% Glycerol-PBS). The cells were visualized with Carl Zeiss Axioskop 2 (Carl Zeiss, Jena, Germany) fluorescence microscope. Pictures were taken with 400 ASA TMAX Kodak films.

In some cases images were acquired at magnification of 40 X with a Carl Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) and visualized by using Northern Eclipse program. Image processing was performed with an Adobe Photoshop program.

3 Results

3.1 Effect of serum factors on the dynamics of the actin cytoskeleton in TtT/GF cells via the participation of actin filament-anchoring proteins.

3.1.1 Effect of serum starvation on the actin cytoskeleton of TtT/GF cells.

The actin cytoskeleton is a highly dynamic network composed of actin polymers and a large variety of associated proteins. These actin-binding proteins regulate actin cytoskeleton dynamics by controlling filament formation and crosslinking directed by intra-and extra-cellular stimuli (Hall, 1998; Hatano, 1994). Our lab has shown that folliculo-stellate cell morphology is affected by the endocrine activity of the anterior pituitary (Cardin et al, 2000). We investigated herein the response of the actin cytoskeleton of the folliculo-stellate cell line, TtT/GF cells, to serum factors.

In the presence of serum, TtT/GF cells displayed an elongated fibroblast-like morphology with cytoplasmic projections and pseudopodia (Figure 1A, a, arrow). We observed thin, and sparse actin fibers in the cytoplasm (Figure 1A, a, arrowhead). Conversely, cells grown in serum free medium adopted a polygonal shape and possessed increased cell-cell contacts but less cell membrane projections (Figure 1A, b). In addition, serum-starved cells showed much thicker, denser, and straighter actin fibers throughout the cytoplasm than serum-cultured cells (Figure 1, A, b, arrowhead).

Western blot analyses carried out with actin antibodies showed an immunoreactive band of 43 kDa in TtT/GF cell total homogenates. Serum starvation did not affect the total levels of actin (Figure 1B, TtT+/-). Actin was found in membrane/cytosol (M/C) and cytoskeleton/non-cytoskeleton (CK/NCK) fractions in TtT/GF cells (Figure 1B). Serum withdrawal did not affect actin distribution in membrane/cytosol fractions (Figure 1B, M/C). Conversely, actin association with the cytoskeleton fraction increased upon

serum removal (Figure 1B, CK/NCK). This finding is in agreement with the immunofluorescence observations, showing much thicker and denser actin fibers in the cells cultured in the absence than in the cells cultured in the presence of serum (Figure 1A).

Figure 1 Modulation of the actin cytoskeleton of TtT/GF cells by serum factors.

A). Immunofluorescence studies.

TtT/GF cells were cultured in serum and serum free conditions and then fixed and labeled with rhodamine-phalloidin. In the presence of serum, the cells possessed pseudopodia (arrow) and thin actin filaments (a, arrowhead). The serum-starved cells possessed few cell membrane projections, and thick and straight actin fibers throughout the cytoplasm (b, arrowhead).

B). Immunoblot analyses on the effect of serum on the expression and the association of actin with distinct subcellular fractions.

TtT/GF cells were treated with serum or without serum for 48h. Subsequently, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were obtained and subjected to immunoblotting with actin antibodies. Total actin was not affected by the treatments. Actin associated to membrane/cytosol and cytoskeleton/non-cytoskeleton fractions in TtT/GF cells. No marked effect of serum on actin association with membrane/cytosol fraction was found. However, the association of actin with the cytoskeleton fraction increased significantly in the absence of serum. TtT: TtT/GF whole cell homogenates; M: membrane fraction; C: cytosol fraction; CK: cytoskeleton fraction; NCK: non-cytoskeleton fraction; +: with serum; -: without serum.

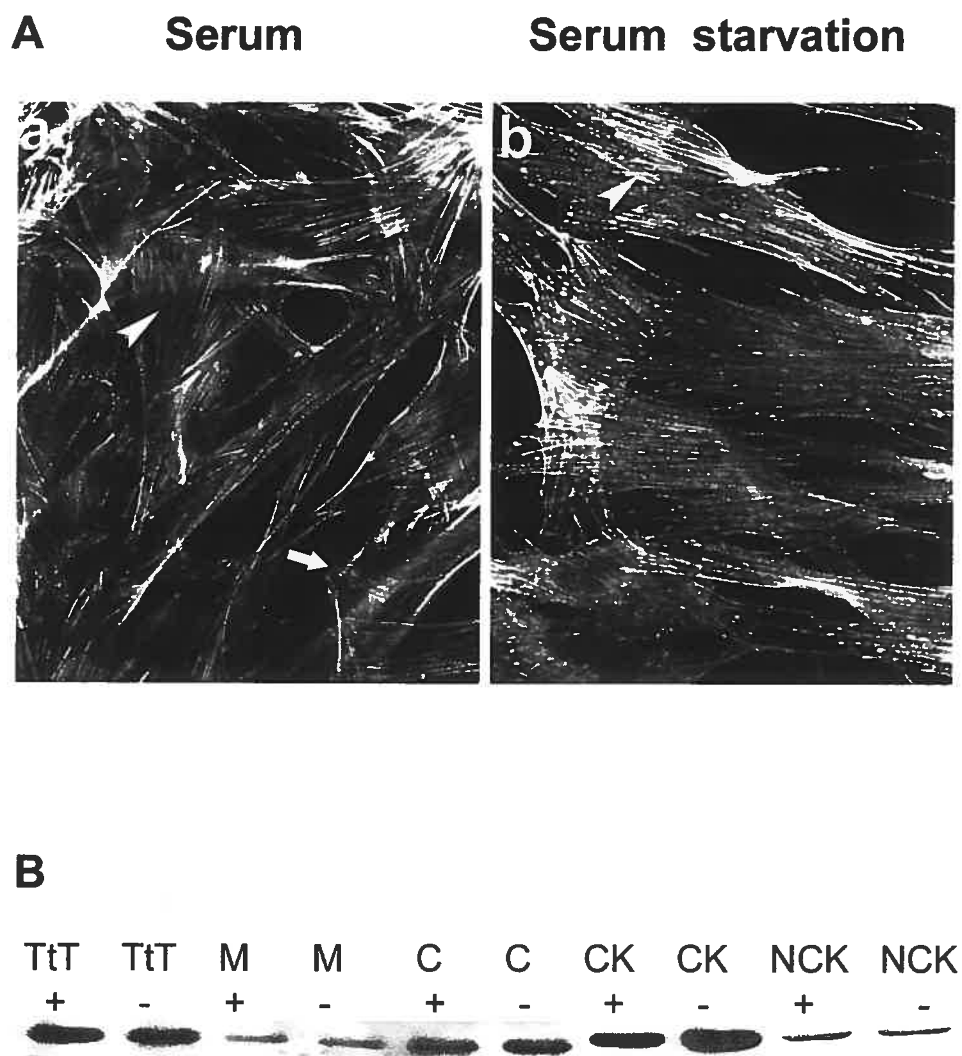


Figure 1

3.1.2 Localization of actin-binding proteins cortactin, α-actinin, vinculin, and myosin in TtT/GF cells cultured either in the presence or in the absence of serum

The assembly and reorganization of the actin cytoskeleton are controlled by a host of actin-binding proteins modulated, in turn, by intra- and extra-cellular stimuli (Aspenstrom, 1999; Cooper, 1991; Hall, 1998; Hatano, 1994; Janmey, 1998; Tapon and Hall, 1997). Therefore, we studied whether the reorganization of the actin cytoskeleton by serum starvation involved actin-binding proteins in TtT/GF cells. Because serum withdrawal changed the spatial organization of the actin cytoskeleton, we decided to investigate the participation of actin anchoring and actin cross-linking proteins. We first carried out immunofluorescence studies in TtT/GF cells cultured either in the presence or in the absence of serum to analyze the potential re-distribution of the following actin-binding proteins: cortactin, α-actinin, vinculin, and myosin.

Cortactin localized to cytoplasmic punctate structures of unknown composition concentrated at the perinuclear region in the TtT/GF cells cultured in the presence of serum (Figure 2, cortactin, a, arrow). In addition, cortactin was associated to dynamic plasma membrane protrusions (Figure 2, a, cortactin, arrowheads). Upon serum withdrawal, cortactin cytoplasmic staining increased (Figure 2, cortactin, a′, arrow) and the whole plasma membrane showed a discontinuous staining (Figure 2, a′, cortactin, arrowhead). Staining of TtT/GF cells cultured in the presence of serum with α-actinin antibodies demonstrated a strong labeling in focal adhesions (Figure 2, α-actinin, b, arrowhead) and along F-actin fibers (Figure 2, α-actinin, b, arrow). Serum-starved TtT/GF cells showed a reduced association of α-actinin with focal contacts and an increased labeling along actin fibers in a distinctive, periodic array (Figure 2, α-actinin,

b′, arrow). Vinculin localized mainly to focal adhesions (Figure 2, vinculin, c, arrowhead) and to stress fibers (Figure 2, vinculin, c, arrow) when TtT/GF cells were cultured in serum-containing medium. Following serum withdrawal, vinculin predominantly localized to stress fibers (Figure 2, vinculin, c, arrow). To investigate the involvement of myosin in the remodeling of actin filaments, we used an antibody against the myosin light chain (MLC). MLC staining was not different in TtT/GF cells cultured with or without serum. In both cases, MLC localized along actin fibers (Figure 2, MLC, d and d′, arrows).

Figure 2 Effect of serum on the localization of the actin-binding proteins cortactin, α-actinin, vinculin, and myosin in TtT/GF cells.

TtT/GF cells were incubated either in the presence or in the absence of serum and next prepared for immunofluorecence using cortactin, α-actinin, vinculin, and myosin light chain (MLC) antibodies. Cortactin localized to pseudopodia in cells cultured with serum (a, arrowheads). In these cells, cortactin cytosolic labeling was punctate in the perinuclear region (a, arrow). Upon serum withdrawal, cortactin labeling was found over the whole plasma membrane (a′, arrowhead) and the cytoplasmic labeling increase (a′, arrow). Alpha-actinin labeling was heavy in focal adhesions of cells cultured in the presence of serum (b, arrowhead). In these cells, α-actinin was also associated with actin stress fibers (b, arrow). Alpha-actinin associated with stress fibers of serum-starved cells (b′, arrow). Vinclulin localized mainly to focal adhesions (c, arrowhead) and also along stress fibers in the presence of serum (c, arrow) and, upon serum removal, vinculin redistributed from focal adhesion to the stress fibers (c′, arrow). Myosin light chain (MLC) localized along F-actin in both serum and serum free conditions (d and d′, arrows).

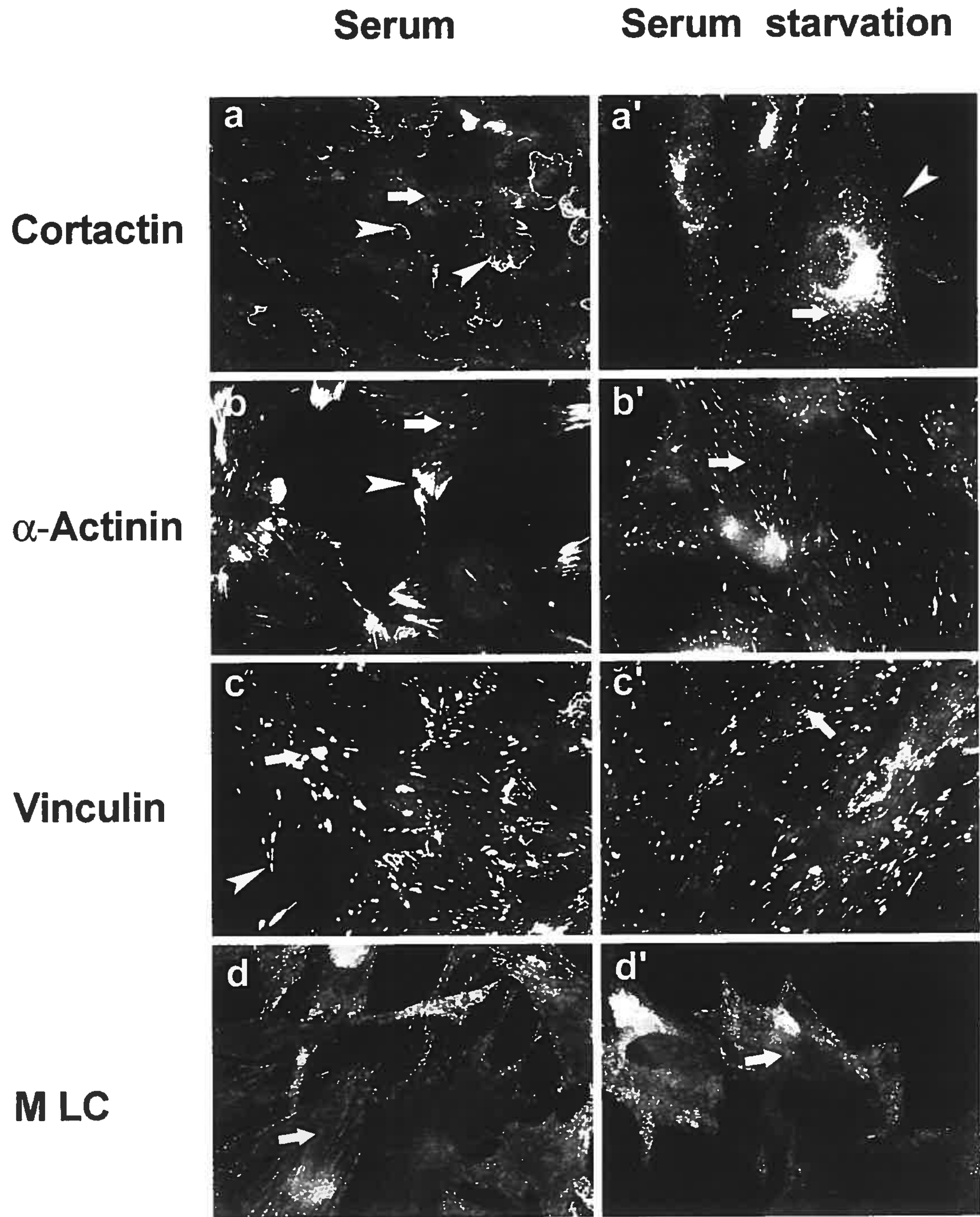


Figure 2

3.1.3 Expression and subcellular distribution of cortactin, α-actinin, vinculin, and myosin light chain in TtT/GF cells cultured either in the presence or in the absence of serum.

To further characterize the changes in cortactin, α-actinin, vinculin, and MLC distribution revealed by immunocytochemistry, and their potential association with the changes in TtT/GF actin cytoskeleton upon serum removal, we analyzed the association of these proteins to different subcellular fractions either in the presence or in the absence of serum by Western blot analyses.

Cortactin is an 85-90 kDa actin-binding protein associated to membrane/cytosol and cytoskeleton/non-cytoskeleton fractions in TtT/GF cells (Figure 3, cortactin). Total cortactin levels were not affected by serum withdrawal (Figure 3, cortactin, TtT/GF +/-). Cortactin translocated from the membrane to the cytosol and from the cytoskeleton to the non-cytoskeleton fraction upon serum withdrawal. Alpha-actinin has a molecular mass of 104 kDa and was recovered only in membrane and cytoskeleton fractions in TtT/GF cells (Figure 3, α-actinin). Total α-actinin increased following serum starvation (Figure 3, α-actinin, TtT/GF +/-). There was a decrease in the association of α-actinin with membrane and cytoskeleton fractions following serum removal (Figure 3, α-actinin). Vinculin has a molecular mass of 116 kDa and its expression was not affected by serum withdrwal in TtT/GF cells (Figure 3, vinculin, TtT/GF +/-). Vinculin distributed in membrane/cytosol and cytoskeleton/non-cytoskeleton fractions in TtT/GF cells. Following serum withdrawal, there were no significant changes in the association of vinculin with these fractions (Figure 3, vinculin). MLC has a molecular mass of 20 kDa. The presence or the absence of serum did not affect the expression of MLC in

TtT/GF cells (Figure 3, MLC, TtT/GF +/-). MLC was only recovered in the particulate fraction (Figure 3, MLC). No influence of serum on MLC intracellular distribution was observed (Figure 3, MLC).

Because our results indicate that cortactin and α-actinin showed the most important changes in the distribution and expression following serum starvation, we focused on these two actin-binding proteins in the next series studies on the dynamics of the actin cytoskeleton in TtT/GF cells.

Figure 3 Effect of serum on the expression and on the subcellular localization of the actin-binding proteins cortactin, α-actinin, vinculin and MLC in TtT/GF cells.

Total cell homogenates, membrane/cytosol, and cytoskeleton/non-cytoskeleton fractions of TtT/GF cells cultured either in the presence or in the absence of serum were subjected to immunoblotting using cortactin, α-actinin, vinculin, and myosin light chain antibodies. Cortactin and vinculin associated with membrane/cytosol and cytoskeleton/non-cytoskeleton fractions. α-Actinin and myosin light chain were recovered only in membrane and cytoskeleton fractions. Cortactin translocated from membrane to cytosol and from cytoskeleton to non-cytoskeleton following serum starvation. Serum withdrawal reduced the association of α-actinin to membrane and cytoskeleton fractions. No detectable effect of serum on the expression and the subcellular localization of myosin light chain were found. TtT: TtT/GF whole cell homogenates; M: membrane; C: cytosol; CK: cytoskeleton; NCK: non-cytoskeleton.

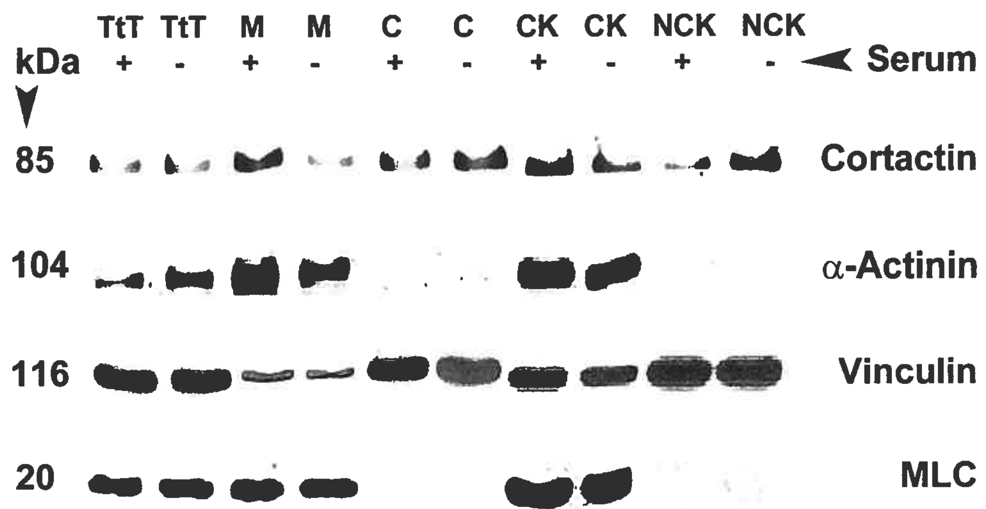


Figure 3

3.2 Participation of cortactin in the remodeling of the actin cytoskeleton by serum factors in TtT/GF cells

3.2.1 Time course studies on the changes in the distribution of cortactin in TtT/GF cells cultured in the presence and in the absence of serum.

To assess the effects of serum factors on the localization of cortactin, TtT/GF cells cultured in the presence of serum were subjected to serum starvation for 48 hours. Next, the cells were challenged with serum-containing medium for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following the treatments, the cells were double-stained with rhodamine-phalloidin and anti-cortactin and viewed with a fluorescence microscope. First, we observed that the presence of serum caused dramatic changes in TtT/GF cell behavior. Cells in serum free media were not motile but shortly after the addition of serum, cell membrane ruffles started to form (Figure 4, actin, arrowhead). This was followed by the appearance of pseudopodia, which indicates the recovery of the fibroblast-like morphology typical of serum-cultured cells (Figure 4).

Serum-starved TtT/GF cells possessed thick actin fibers and the addition of serum to the medium caused the actin fibres to become thinner and shorter (Figure 4, actin). Cortactin displayed a punctate cytoplasmic staining in TtT/GF cells cultured in the absence of serum (Figure 4, cortactin). Cortactin association to the leading edges of membrane ruffles started after a 0.5h incubation period in the serum-containing medium (Figure 4, cortactin, arrowheads). Following 8h in the presence of serum, cortactin distribution was similar to cells cultured for more than 48h in a serum-containing medium (Figure 2, and Figure 4, cortactin).

Figure 4 Effect of serum on the localization of F-actin and cortactin in TtT/GF cells.

TtT/GF cells cultured in serum free conditions were challenged with serum for 0, 0.5, 1, 2, 4, and 8h. The cells were double labeled with rhodamine-phalloidin and anti-cortactin. Upon addition of serum to the culture medium, the cells acquired a star-like shape and membrane protrusions started to appear (arrowheads). Cortactin localized to the leading edge of pseudopodia and other membrane processes (arrowheads).

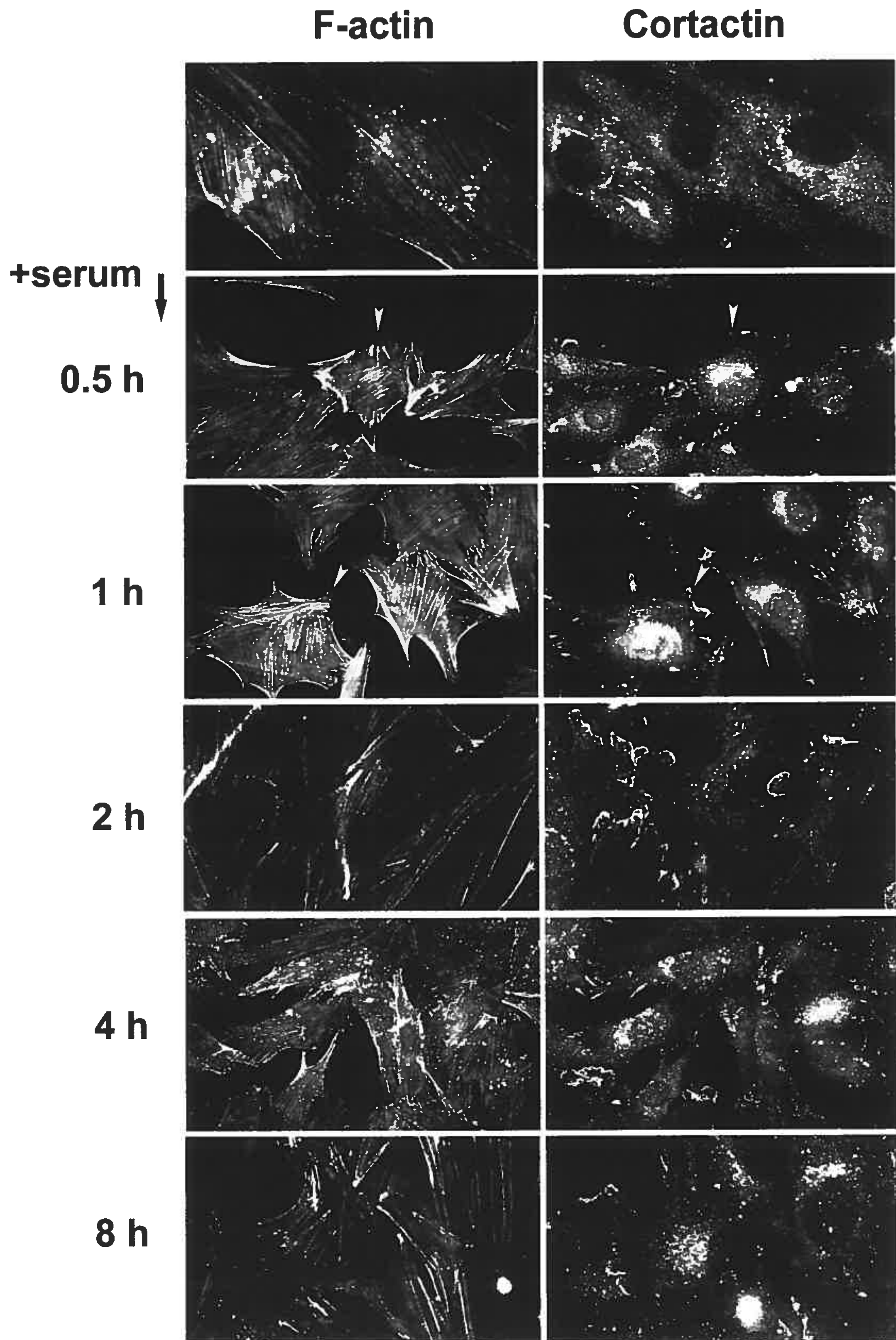


Figure 4

3.2.2 Studies on the phospho-tyrosine status of cortactin under serum and serum-free conditions.

3.2.2.1 Co-localization of cortactin and phospho-tyrosine in TtT/GF cells in the presence and in the absence of serum. Treatment with the tyrosine kinase inhibitor genistein.

Cortactin is a phospho-tyrosine protein. It has been shown that the phosphorylation status of cortactin modifies its subcellular distribution (Di Ciano et al., 2002). To evaluate whether tyrosine phosphorylation of cortactin is involved in the remodeling of the actin cytoskeleton during serum starvation, double labeling immunofluorescence studies with antibodies against cortactin and phospho-tyrosine were performed in TtT/GF cells treated with the tyrosine kinase inhibitor genistein either in the presence or in the absence of serum. As shown before, cortactin localized to cytoplasmic punctate structures concentrated in the perinuclear region (Figure 5, cortactin, C, arrow) and to pseudopodia and cell membrane protrusions in TtT/GF cells cultured in the presence of serum (Figure 5, cortactin, C, arrowhead). Co-localization of cortactin and phospho-tyrosine was evident in pseudopodia and membrane protrusions (Figure 5, C, cortactin and phospho-tyrosine, arrowhead). Genistein treatment for 1h induced the disappearance of pseudopodia, which was followed by the formation of short projections (Figure 5, 1, 2h, arrowheads). The cells recovered the normal shape after 24h in the presence of genistein (not shown). Cortactin labeling was mostly cytoplasmic in cells treated with genistein for 1h. Two hours after genistein treatment, cortactin labeling was associated to the thin cell membrane protrusions (Figure 5, 2h). Phospho-tyrosine labeling of cell membrane protrusions was low in cells cultured in the presence of genistein for one hour (Figure 5, phospho-tyrosine, 1h), but increased after

two hours in the presence of genistein (Figure 5, phospho-tyrosine, 2h). Cortactin and phospho-tyrosine labeling in cells treated with the tyrosine kinase inhibitor for 24hs were similar to the control cells (not shown).

Cortactin and phospho-tyrosine staining at the plasma membrane was weak in serum-starved TtT/GF cells (Figure 6, C, arrowheads). Serum-starved cells treated with genistein for 1-2h showed thin membrane projections (Figure 6, 1-2h). The cells recovered their morphology after 24h in the presence of genistein (not shown). The thin membrane projections induced by the genistein treatment stained positive for cortactin (Figure 6, cortactin 1h) but the phospho-tyrosine labeling of these structures was weak (Figure 6, phospho-tyrosine, 1h). In addition, after 2hs in the presence of genistein, cortactin and phospho-tyrosine labeling was associated with filamentous structures in the cytoplasm (Figure 6, 2h, arrows). Cortactin and phospho-tyrosine distribution in cells incubated for 24h in the presence of genistein were similar to those observed in non-treated cells (not shown).

Figure 5 Localization of cortactin and phospho-tyrosine in TtT/GF cells cultured in the presence of serum. Effect of the tyrosine kinase inhibitor genistein.

TtT/GF cells cultured in the presence of serum were treated with 30μM genistein (final concentration) for 0 (C), 1h, 2h, and 24h (not shown). After treatments, the cells were fixed and double-stained with cortactin and phospho-tyrosine antibodies. Cortactin and phospho-tyrosine localized mainly to the cell periphery (arrowheads) and to the perinuclear area (arrow) in control cells. One hour of genistein treatment reduced cortical cortactin and phospho-tyrosine labeling. Genistein induced the formation of tiny cell membrane protrusions, especially after 2h of treatment (arrowheads). Cortactin and phospho-tyrosine localized to the membrane projections (arrowheads).

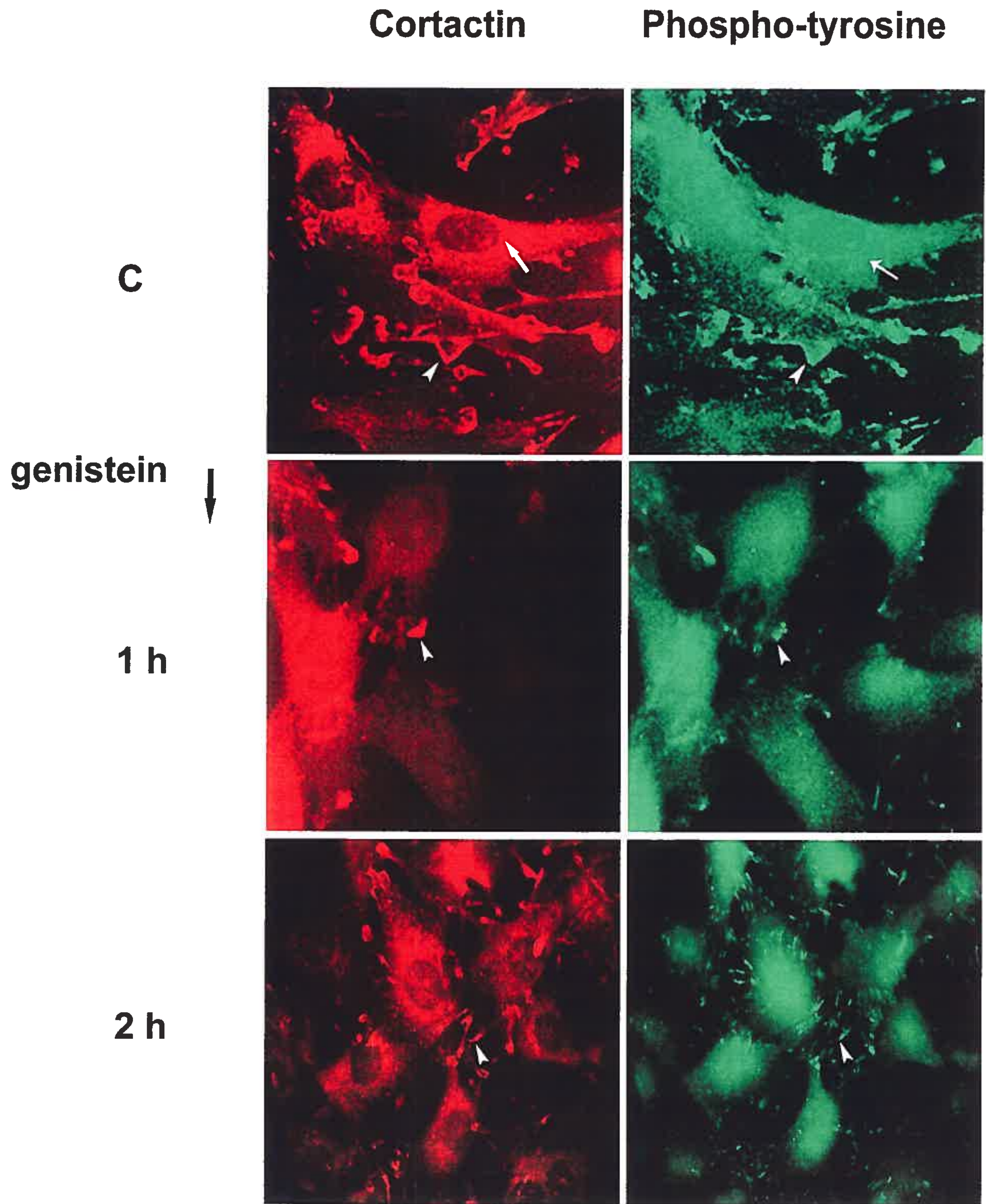


Figure 5

Figure 6 Localization of cortactin and phospho-tyrosine in TtT/GF cells cultured in the absence of serum. Effect of the tyrosine kinase inhibitor genistein.

TtT/GF cells cultured in serum-free conditions were treated with 30μM genistein (final concentration) for increasing period's time: 0 (C), 1, 2, and 24h (not shown). Next, the cells were fixed and double-labelled with cortactin and phospho-tyrosine antibodies. In serum-starved TtT/GF cells, cortactin and phospho-tyrosine staining at the cell membrane was weak (arrowheads) but the cytoplasmic staining was strong (arrow). The cells treated with genistein showed thin and long membrane projections (1h, arrowhead).

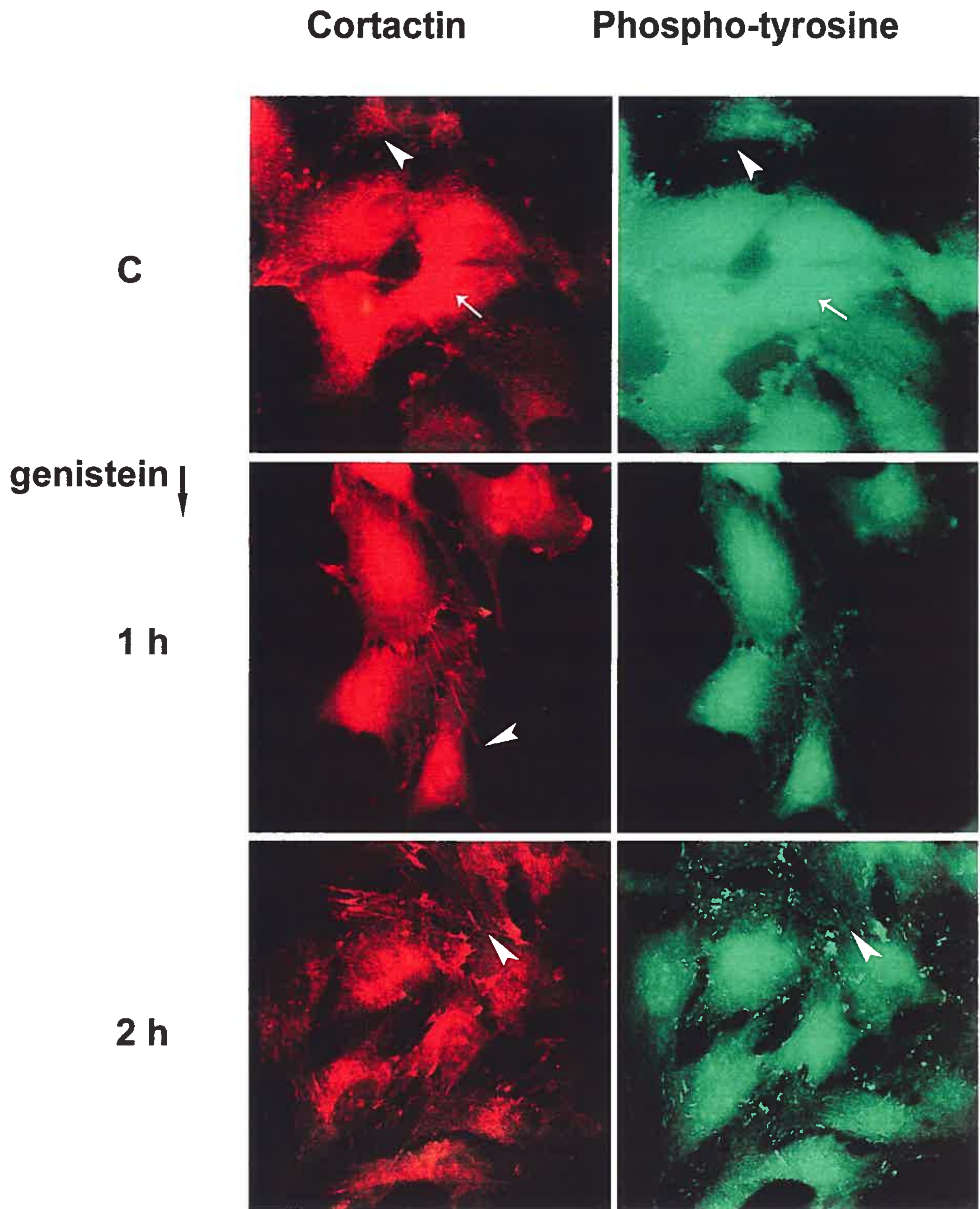


Figure 6

3.2.2.2 Studies on the tyrosine-phosphorylation status of cortactin in cells cultured either in serum or serum-free conditions

To further understand the role of tyrosine phosphorylation of cortactin in serum-induced actin reorganization in TtT/GF cells and because phospho-tyrosine labeling also represents the distribution of other phospho-tyrosine proteins, we carried out immunoblotting analyses with an antibody that only recognizes the tyrosine-phosphorylated form of cortactin, anti-p-cortactin. Anti-p-cortactin showed a 90-kDa immunoreactive band in TtT/GF cells (Figure 7). In the non-cytoskeleton fraction, two bands were observed (Figure 7). P-cortactin was associated with membrane/cytosol and cytoskeleton/non-cytoskeleton fractions in TtT/GF cells. It translocated from membrane to cytosol and from cytoskeleton to non-cytoskeleton fraction following serum removal (Figure 7).

Figure 7 Effect of serum on the expression and the subcellular localization of p-cortactin in TtT/GF cells.

TtT/GF cells were cultured with serum or without serum for 48 hours. Next, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared and subjected to immunoblotting with an antibody that recognizes the tyrosine-phsophorylated form of cortactin (p-cortactin). P-cortactin associated with membrane/cytosol and cytoskeleton/non-cytoskeleton fractions. Serum withdrawal decreased p-cortactin association with membrane and cytoskeleton fractions and increased p-cortactin association with cytosol and non-cytoskeleton fractions. M: membrane; C: cytosol; CK: cytoskeleton; NCK: non-cytoskeleton; +: with serum; -: without serum.

Phospho-cortactin

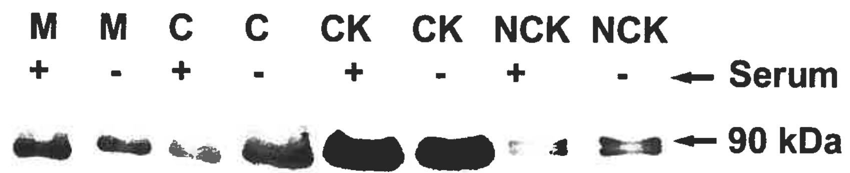


Figure 7

3.2.2.3 Immunofluorescence studies on the co-localization of cortactin and p-cortactin in cells cultured either in the presence or in the absence of serum.

We next studied the localization of cortactin and p-cortactin following serum-induced remodeling of the actin cytoskeleton in TtT/GF cells by immunofluorescence. TtT/GF cells were subjected to serum starvation for 48 hs and next incubated in serum containing medium for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. In serum-starved TtT/GF cells, cortactin and p-cortactin labeling were mostly intracellular, although some labeling was associated with membrane structures (Figure 8A, C). Upon addition of serum to the medium bathing the cells, cortactin and p-cortactin co-localized at the leading edge of membrane ruffles and pseudopodia (Figure 8A, arrowheads).

To study whether tyrosine phosphorylation of cortactin was involved in the serum-induced formation of membrane protrusions that derived of pseudopodia and lamellipodia, TtT/GF cells cultured in the absence of serum were challenged with serum in the presence of genistein (Figure 8B). The tyrosine kinase inhibitor genistein blocked the serum-induced formation of pseudopodia and lamellipodia (Figure 8B). Nevertheless, there was a certain degree of co-localization of cortactin and p-cortactin particularly at the plasma membrane (Figure 8B). In the presence of genistein, cortactin and p-cortactin labeling remains in serum-starved cells (Figure 8A, 8B).

Figure 8.

A) Effect of serum on the localization of cortactin and p-cortactin in TtT/GF cells.

TtT/GF cells were cultured in the absence of serum and next challenged with serum for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labelled with cortactin and p-cortactin antibodies. Following addition of serum, cortactin and p-cortactin association with nascent membrane protrusions increased (arrowheads).

B) Effect of genistein on serum-induced formation of cell membrane protrusions and on the localization of cortactin and p-cortactin.

TtT/GF cells cultured in the absence of serum were challenged with serum in the presence of 30μM genistein (final concentration) for increasing periods of time. Next, the cells were double labeled with cortactin and p-cortactin antibodies. The pictures show that in the presence of genistein, the addition of serum did not induce the appearance of pseudopodia and lamellipodia. Genistein also blocked the association of cortactin/p-cortactin to membrane structures.

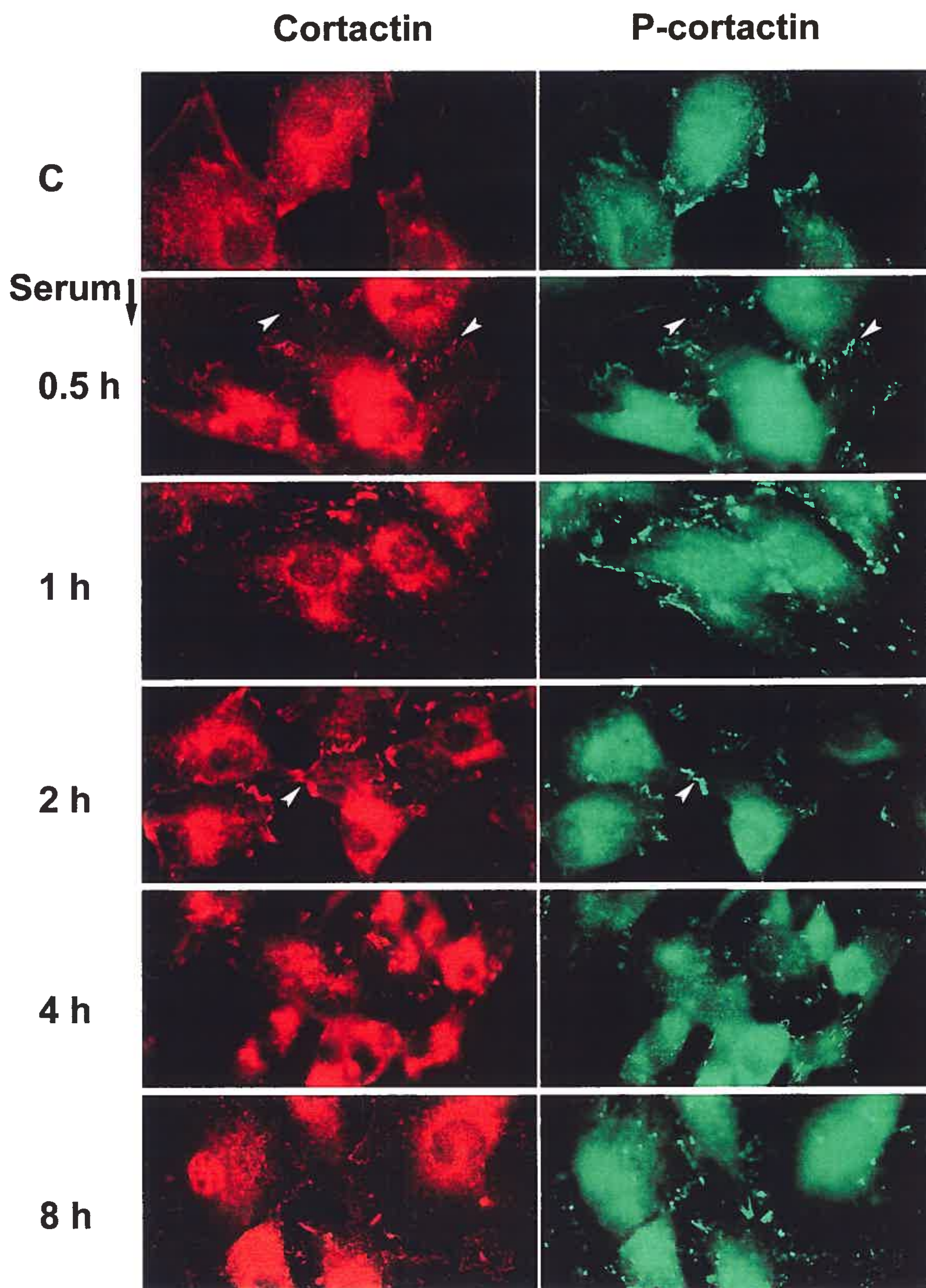


Figure 8 A

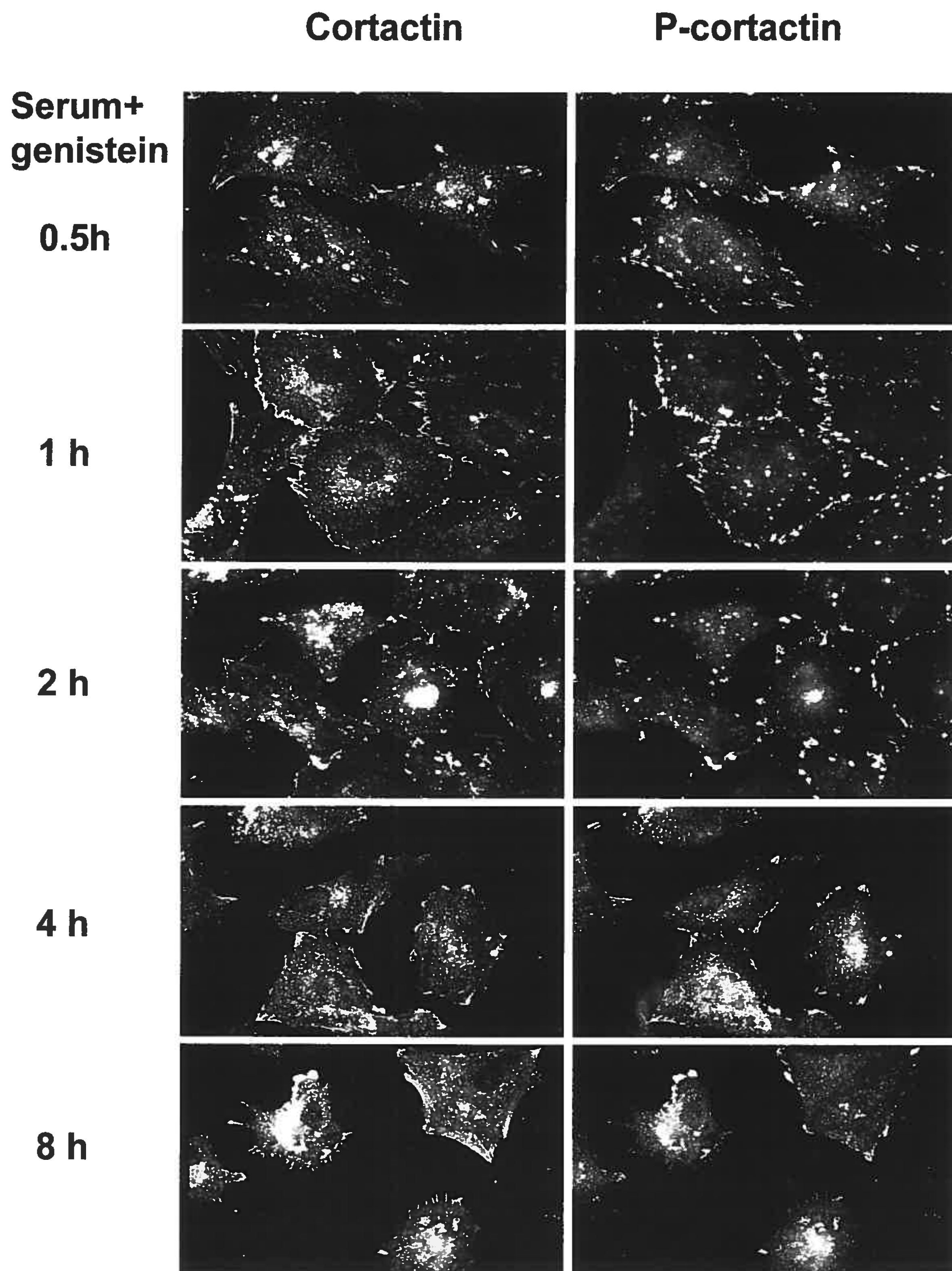


Figure 8 B

3.2.3 The role of c AMP/PKA pathway in the involvement of cortactin in the actin cytoskeleton dynamics in TtT/GF cells.

Although cortactin is a phospho-tyrosine protein, it is also a target of PKA. To investigate whether the cAMP/PKA pathway was involved in the participation of cortactin in the regulation of the actin cytoskeleton in TtT/GF cells, we used two drugs: forskolin, an activator of adenylate cyclase and KT5720, an inhibitor of PKA, to carry out the next series of studies.

3.2.3.1 Effect of forskolin on the expression and on the subcellular localization of cortactin and p-cortactin

Western blot analyses were carried out using cortactin and p-cortactin antibodies in TtT/GF cells treated with forskolin either in the presence or in the absence of serum. As shown in figure 9, cortactin associated with membrane/cytosol and cytoskeleton/non-cytoskeleton fractions in TtT/GF cells. In the presence of serum, forskolin increased cortactin association with the membrane fraction but decreased cortactin association with the cytosol fraction (Figure 9, cortactin). Conversely, forskolin decreased cortactin association with the cytoskeleton fraction but increased cortactin association with the non-cytoskeleton fraction (Figure 9, cortactin). On the other hand, forskolin significantly increased p-cortactin association with the membrane fraction while it increased the association of p-cortactin with the cytosol fraction at 1h and 4h, but decreased it at 2h (Figure 9, p-cortactin). Forskolin decreased p-cortactin association with the cytoskeleton fraction at 0.5h and then increased it at 1h and 4h. Forskolin

significantly increased p-cortactin association to the non-cytoskeleton fraction at 1h (Figure 9, p-cortactin).

In the absence of serum, forskolin decreased cortactin association with the membrane fraction and increased cortactin association with the cytosol fraction (Figure 10, cortactin). Forskolin transiently increased cortactin association with the cytoskeleton fraction, and decreased cortactin association to the non-cytoskeleton fraction (Figure 10, cortactin). Forskolin transiently reduced the amount of p-cortactin associated with membrane and cytosol fractions (Figure 10, p-cortactin). Forskolin also transiently decreased p-cortactin association with cytoskeleton fraction and the association of p-cortactin with non-cytoskeleton fraction (Figure 10, p-cortactin).

Figure 9 Effect of forskolin on the expression and the subcellular localization of cortactin and p-cortactin in TtT/GF cells cultured in the presence of serum.

TtT/GF cells were cultured with serum and next treated with 15mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following the treatments, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared. The samples were subjected to immunoblotting with cortactin and p-cortactin antibodies. The immunoreactive bands were scanned and their intensities were quantified using the Scion Image program. The intensity of each band was normalized using the value at 0 hour for each fraction. Data presented was obtained by quantifying the immunoreactive bands of at least three independent experiments. The values shown are the mean ± SEM. **: $P<0.01$; * $P<0.05$ with respect to the precedent value

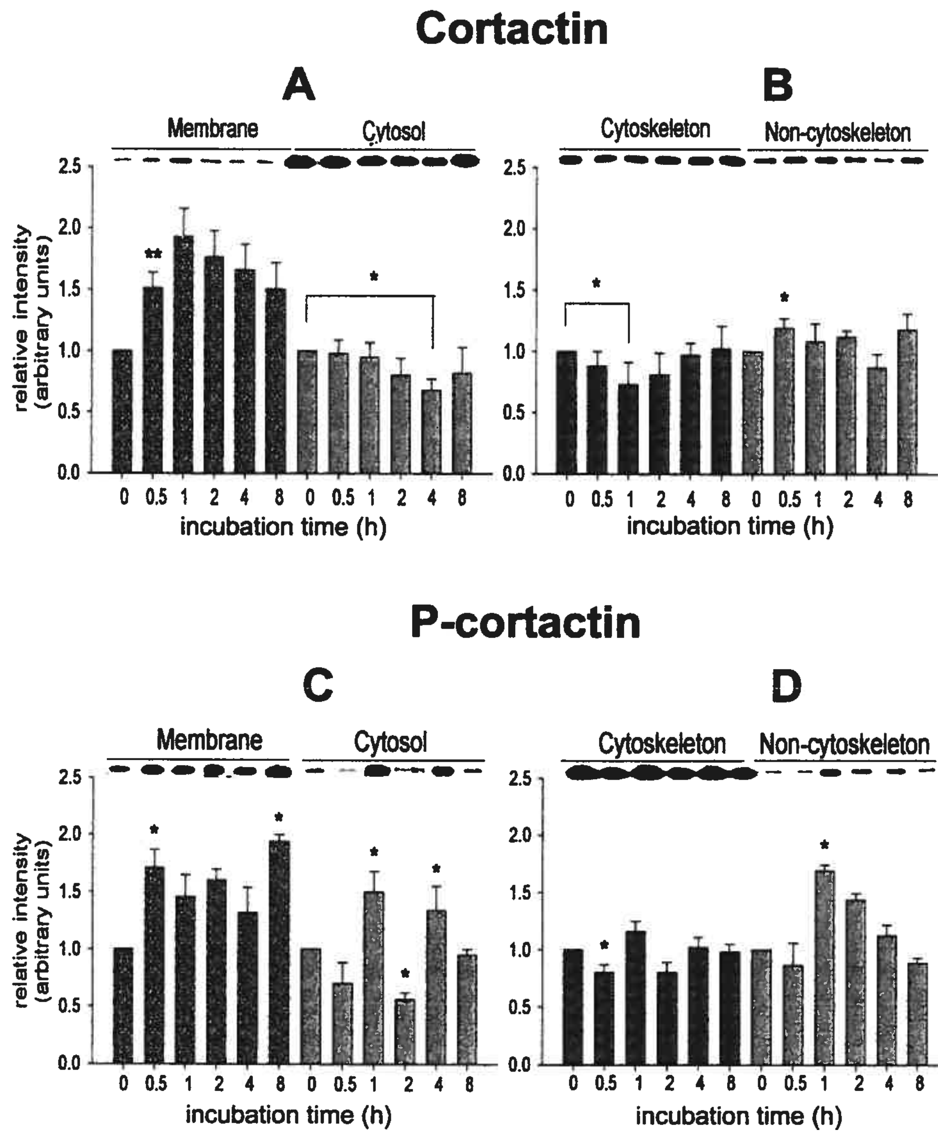
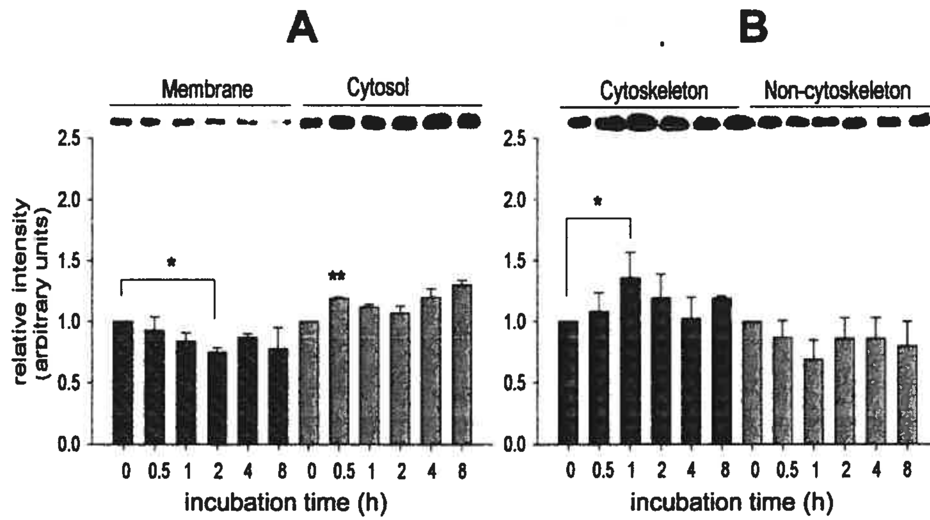


Figure 9

Figure 10 Effect of forskolin on the expression and the subcellular localization of cortactin and p-cortactin in TtT/GF cells cultured in the absence of serum.

TtT/GF cells were cultured in the absence of serum and next treated with 15mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following treatments, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared and subjected to immunoblotting with cortactin and p-cortactin antibodies. The immunoreactive bands were scanned and the intensities were quantified using the Scion Image program. The intensity of each band was normalized using the value at 0 hour for each fraction. Data presented was obtained by quantifying the immunoreactive bands of at least three independent experiments. The values shown are the mean ± SEM. **: $P<0.01$; * $P<0.05$ with respect to the precedent value

Cortactin



P-cortactin

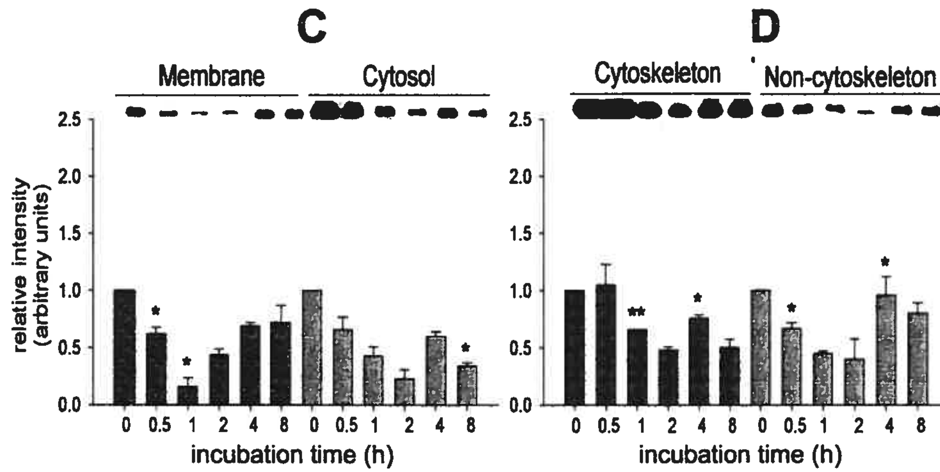


Figure 10

3.2.3.2 Immunofluorescence studies on the localization of cortactin and p-cortactin in TtT/GF cells treated with forskolin.

Double-labeling immunofluorescence studies using cortactin and p-cortactin antibodies were carried out in TtT/GF cells treated with forskolin either in serum or in serum starvation conditions. Forskolin induced the formation of long dendrite-like protrusions in cells cultured in the presence of serum (Figure 11, arrowheads). Cortactin and p-cortactin localized at the tips of these dendrite-like protrusions (Figure 11, arrowheads).

Forskolin treatment of cells cultured in the absence of serum induced the formation of cell membrane protrusions at long incubation times (Figure 12, arrowheads). Cortactin localization at the plasma membrane increased although most cortactin labeling was intracellular (Figure 12, arrowhead) in serum-starved forskolin-treated cell. P-cortactin localized to cell-cell contact sites in forskolin-treated cells (Figure12, arrows).

Figure 11 Effect of forskolin on the localization of cortactin and p-cortactin in TtT/GF cells cultured in the presence of serum.

TtT/GF cells were cultured in serum-containing medium and next treated with 15 mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labelled using cortactin and p-cortactin antibodies. Forskolin induced the formation of dendrite-like structures. Cortactin and p-cortactin localized to the tips of the protrusions (arrowheads).

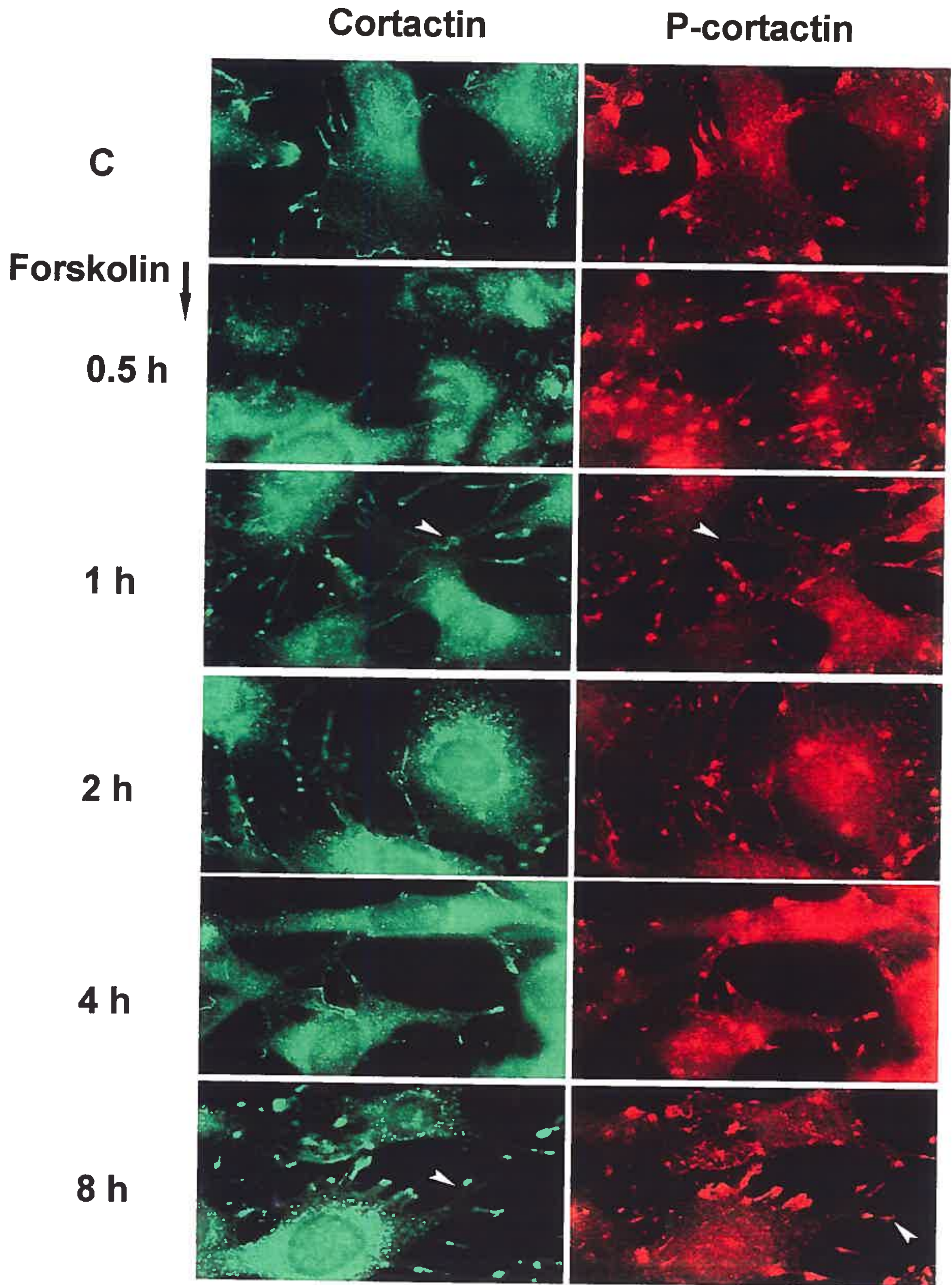


Figure 11

Figure 12 Effect of forskolin on the localization of cortactin and p-cortactin in TtT/GF cells cultured in the absence of serum.

TtT/GF cells were cultured in serum free media. Next, the cells were treated with 15mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labelled using cortactin and p-cortactin antibodies. The cells treated with forskolin in the absence of serum displayed long protrusions (arrowheads). Cortactin localization at plasma membrane decreased and most cortactin labeling was intracellular (asterisk) after forskolin treatment and p-cortactin localized at the contact sites between the cells (arrows).

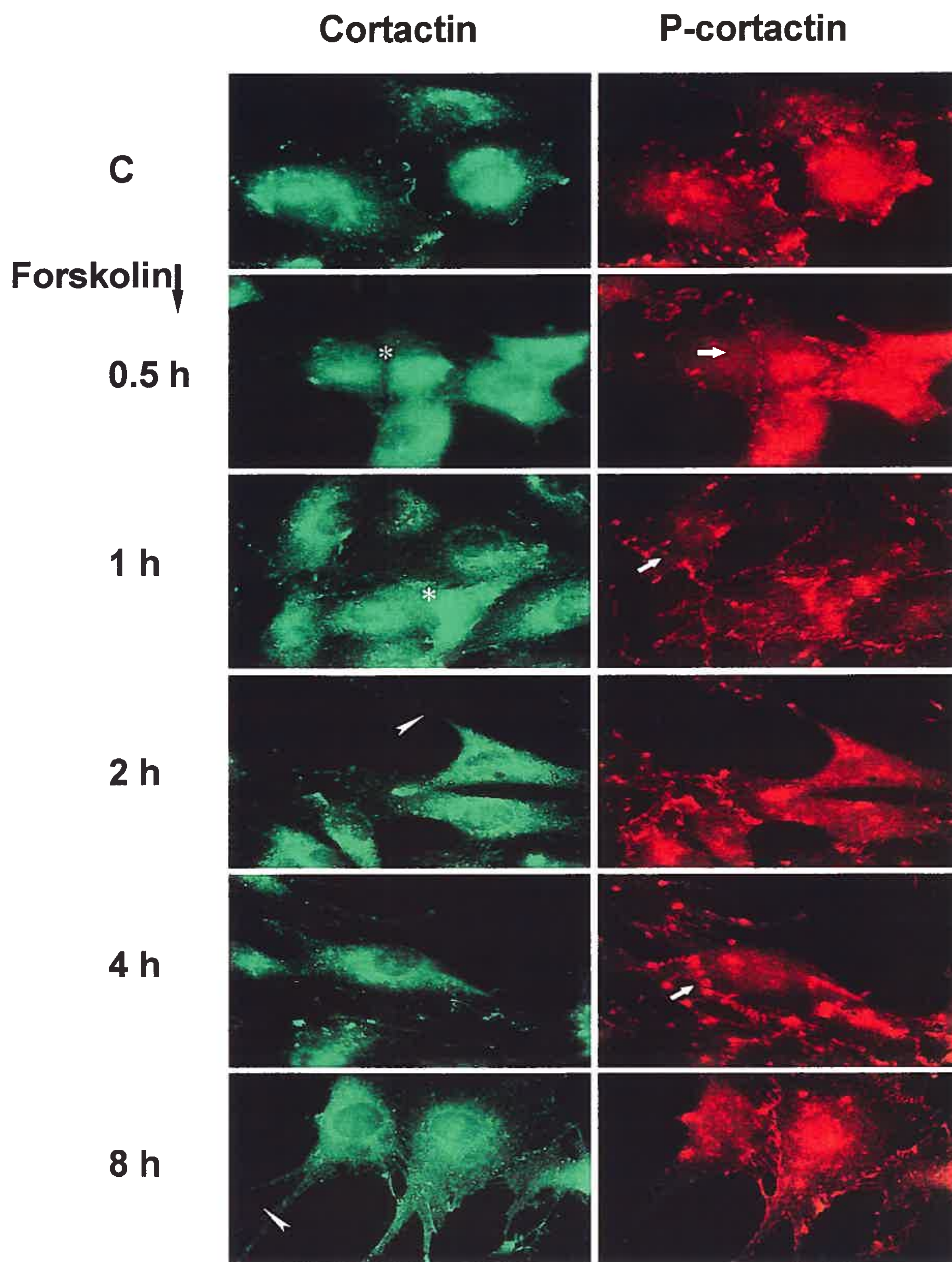


Figure 12

3.2.3.3 Effect of the PKA inhibitor KT5720 on the expression and the subcellular localization of cortactin and p-cortactin

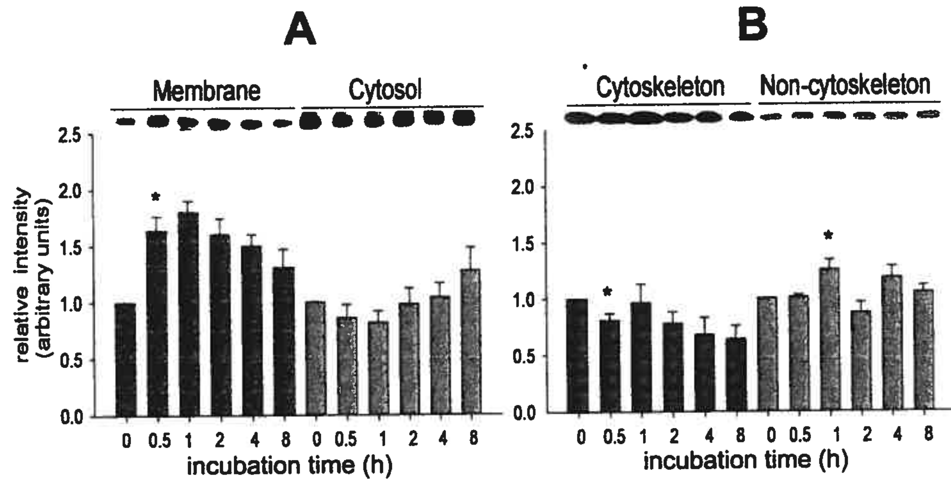
To further explore the role of cAMP/PKA pathway in the cortactin-mediated regulation of the actin cytoskeleton in TtT/GF cells, we inhibited the PKA pathway by employing the compound KT5720 in cells cultured either in the presence or in the absence of serum. Western blot analyses showed a transient increase in cortactin association with the membrane fraction of cells treated with KT5720 and a concomitant decrease in cortactin association with the cytosol fraction (Figure 13, cortactin). KT5720 decreased the amount of cortactin recovered in the cytoskeleton fraction and transiently increased cortactin association with non-cytoskeleton (Figure 13, cortactin). KT5720 increased the amount of p-cortactin recovered in the membrane and cytosol fractions (Figure 13, p-cortactin). In cytoskeleton and non-cytoskeleton fractions, p-cortactin levels showed a decreasing trend in KT5720-treated cells (Figure 13, p-cortactin). P-cortactin levels increased significantly in the non-cytoskeleton fraction at 8h of KT5720 treatment (Figure 13, p-cortactin).

KT5720 had a little effect on the association of cortactin to the membrane/cytosol fraction in cells cultured in the absence of serum. However, KT5720 transiently increased the amount of cortactin recovered in the cytoskeleton and non-cytoskeleton fractions (Figure 14, cortactin). KT5720 induced a biphasic change in the association of p-cortactin with the membrane and cytosol fractions (Figure 14, p-cortactin). KT5720 slightly decreased the amount of p-cortactin association to the cytoskeleton and non-cytoskeleton fractions in serum-starved cells (Figure 14, p-cortactin).

Figure 13 Effect of the PKA inhibitor KT5720 on the expression and the subcellular localization of cortactin and p-cortactin in TtT/GF cells cultured in the presence serum.

TtT/GF cells cultured in serum condition were treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following treatments, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared and subjected to immunoblotting with cortactin and p-cortactin antibodies. The immunoreactive bands were scanned and the intensities were quantified using the Scion Image program. Data were normalized using the value at 0h for each subcellular fraction. Data presented was obtained by measuring the intensities of the immunoreactive bands of at least three independent experiments. The values shown are the mean ± SEM. * $P<0.05$ with respect to the precedent value

Cortactin



P-cortactin

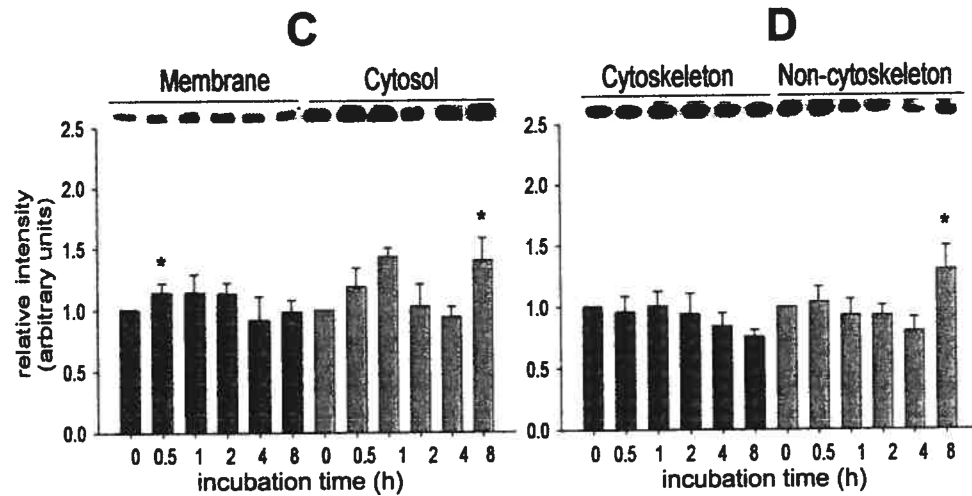
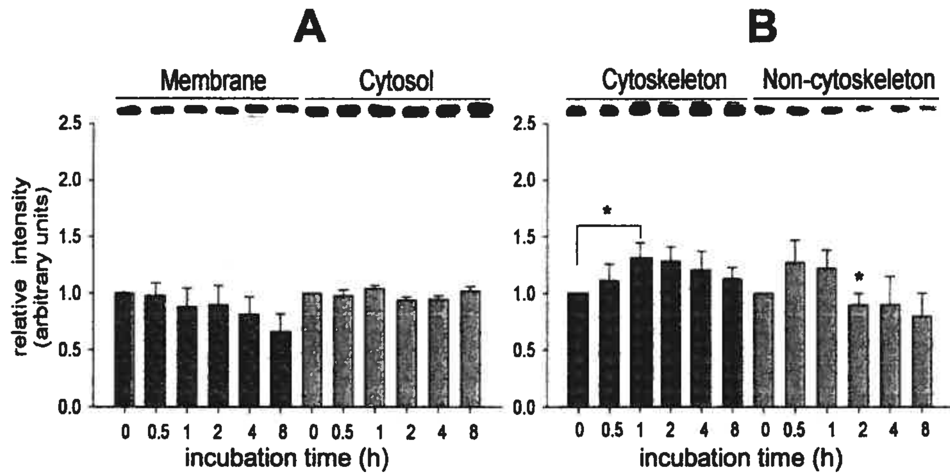


Figure 13

Figure 14 Effect of the PKA inhibitor KT5720 on the expression and the subcellular localization of cortactin and p-cortactin in TtT/GF cells cultured in the absence of serum.

TtT/GF cells were cultured in the absence of serum and next were treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following treatments, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared and subjected to immunoblotting with cortactin and p-cortactin antibodies. The immunoreactive bands were scanned and their intensities were quantified using the Scion Image program. Data were normalized using the value at 0h for each subcellular fraction. Data presented was obtained by measuring the intensities of immunoreactive bands of at least three independent experiments. The values shown are the mean ± SEM. * $P<0.05$ with respect to the precedent value, except where indicated.

Cortactin



P-cortactin

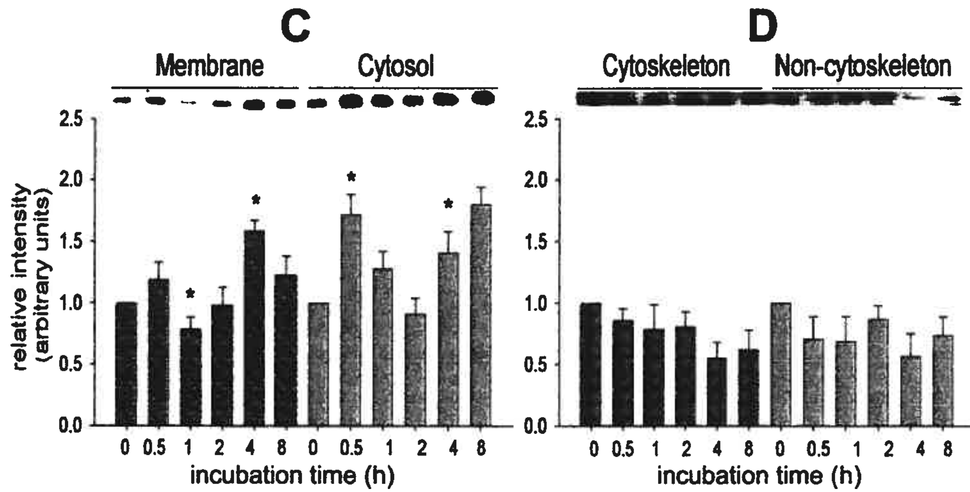


Figure 14

3.2.3.4 Immunofluorescence studies on the localization of cortactin and p-cortactin in TtT/GF cells treated with KT5720 either in the presence or in the absence of serum.

Double labeling immunofluorescence studies were performed after treatment of the cells with KT5720 either in the presence or in the absence of serum. KT5720 caused the formation of short membrane protrusions in cells cultured in the presence of serum (Figure 15, arrowheads). Cortactin and p-cortactin localized to the tips of these membrane structures (Figure 15, arrowheads).

In the absence of serum, KT5720-treated cells lost most of the membrane protrusions (Figure 16). In addition, KT5720 reduced the association of cortactin and p-cortactin to the membrane protrusions (Figure 16).

Figure 15 Effect of KT5720 on the localization of cortactin and p-cortactin in TtT/GF cells cultured in the presence of serum.

TtT/GF cells were cultured in serum-containing medium. Next, the cells were treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labeled using cortactin and p-cortactin antibodies. KT5720 induced the formation of short cell membrane protrusions (arrowheads). Cortactin and p-cortactin localized at the tips of these protrusions (arrows).

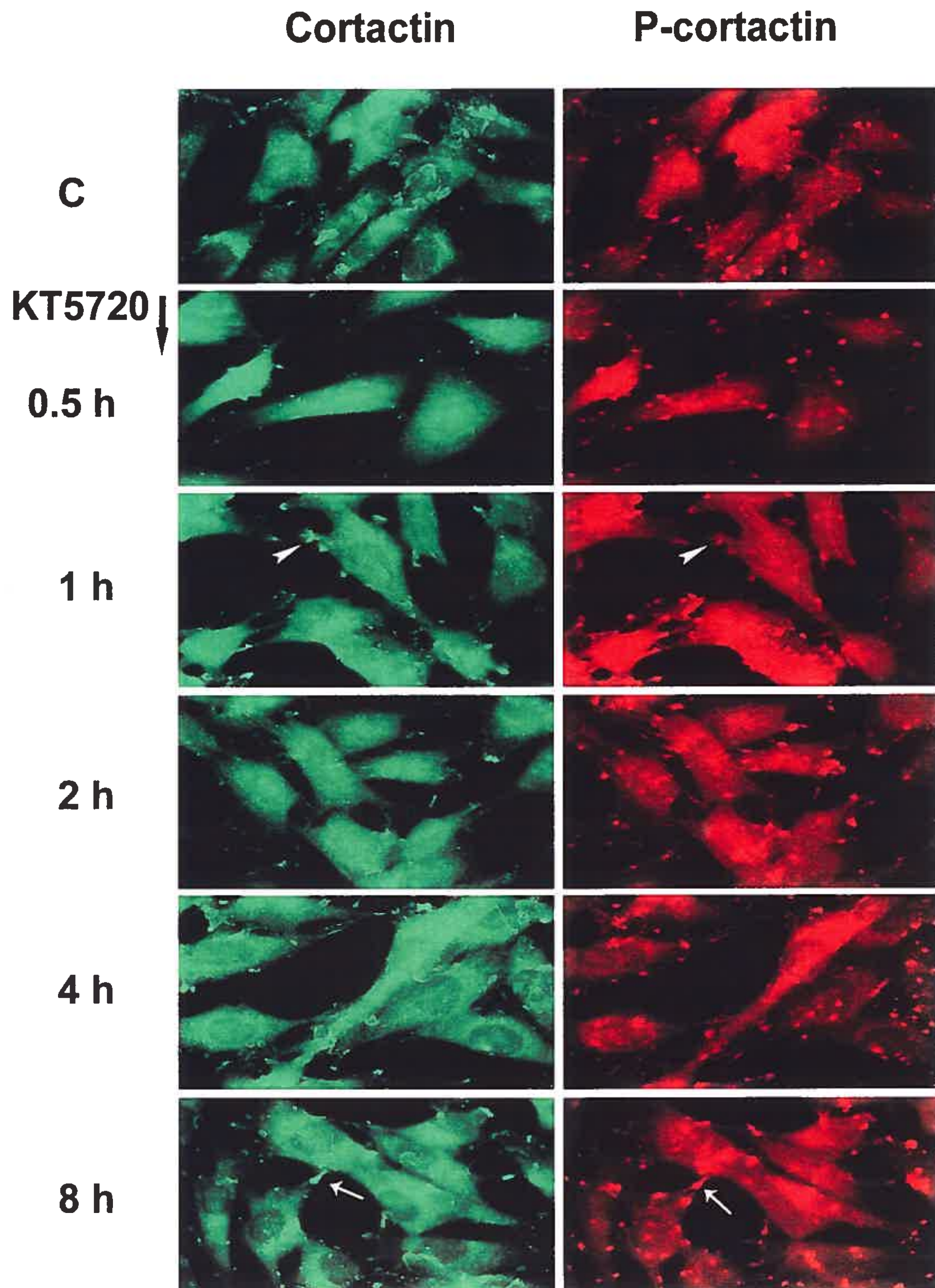


Figure 15

Figure 16 Effect of KT5720 on the localization of cortactin and p-cortactin in TtT/GF cells cultured in the absence of serum.

TtT/GF cells were cultured with serum free medium and treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labelled using cortactin and p-cortactin antibodies. KT5720 reduced the formation of cell membrane protrusions. KT5720-treated cells showed some cortactin labeling at the plasma membrane, but most cortactin labeling was intracellular. P-cortactin labeling association to cortical structures also decreased after KT5720 treatment.

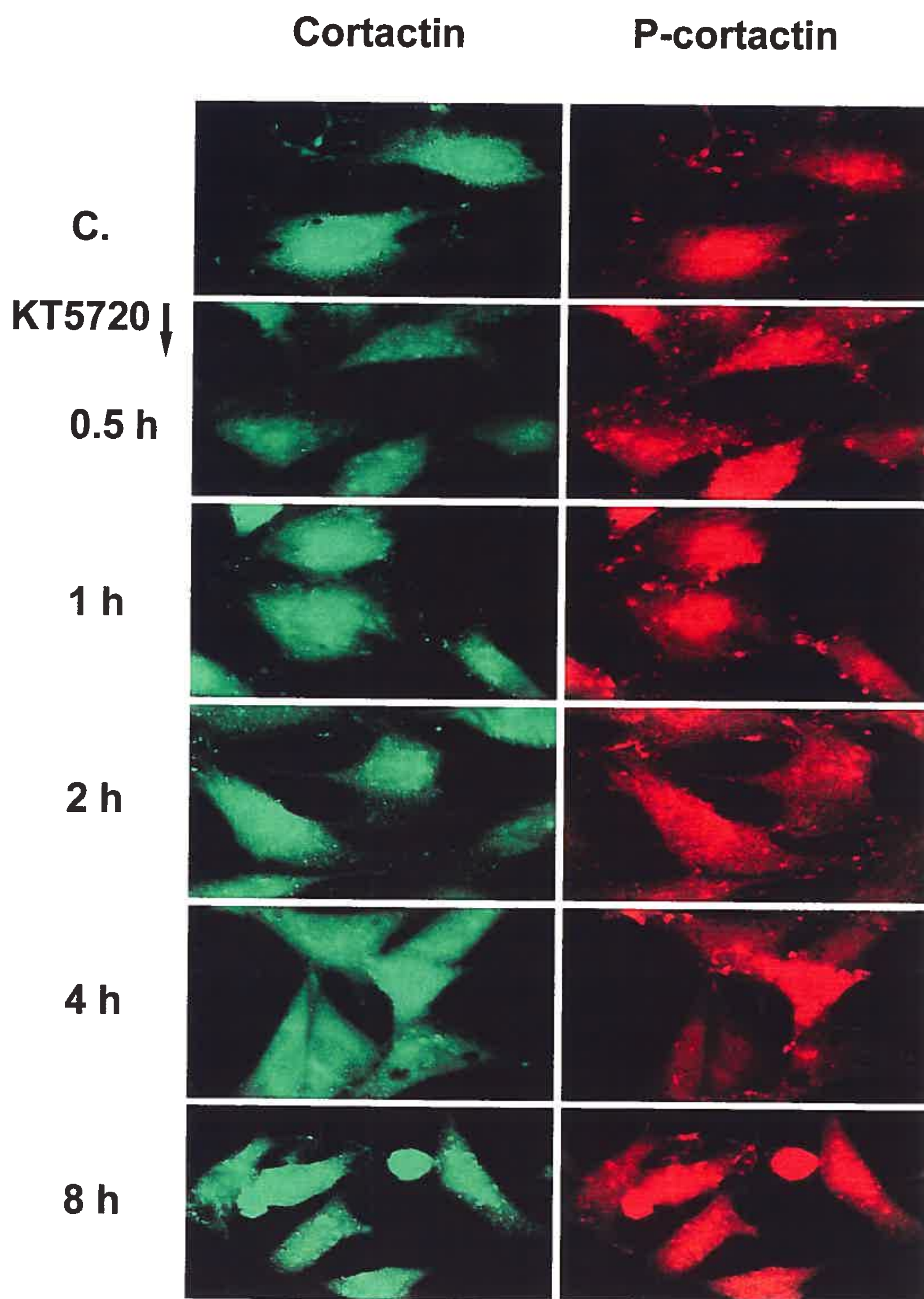


Figure 16

3.3 Participation of α-actinin in the modulation of the actin cytoskeleton dynamics by serum factors in TtT/GF cells

3.3.1 Time course studies on the localization of α-actinin in TtT/GF cells cultured in the presence and in the absence of serum.

As we have shown before (see sections 1.2 and 1.3), α-actinin intracellular localization in TtT/GF cells was affected by serum withdrawal. To assess whether α-actinin is involved in the remodeling of the actin cytoskeleton by serum factors, we performed immunofluorescence studies in TtT/GF cells cultured in serum-free conditions and next, challenged with serum. Alpha-actinin localized mainly to actin filaments in serum free conditions (Figure 17, C, arrowheads). Alpha-actinin association to focal adhesions increased following serum addition to the medium bathing the cells (Figure 17, arrow). These changes in α-actinin distribution were concomitant with the reorganization of actin from thick actin bundles to thin bundles and the reappearance of membrane-based structures.

Figure 17 Effect of serum on the localization of F-actin and α-actinin in TtT/GF cells.

TtT/GF cells were cultured in serum free medium and next, challenged with serum for 0 (C) to 8 hours. The cells were double-stained with rhodamine-phalloidin and α-actinin antibodies. In the absence of serum, actin fibers were thick and abundant and α-actinin localized along F-actin (C, arrowheads). Addition of serum caused the redistribution of F-actin to membrane structures (arrow) and the re-localization of α-actinin to focal adhesions (arrow).

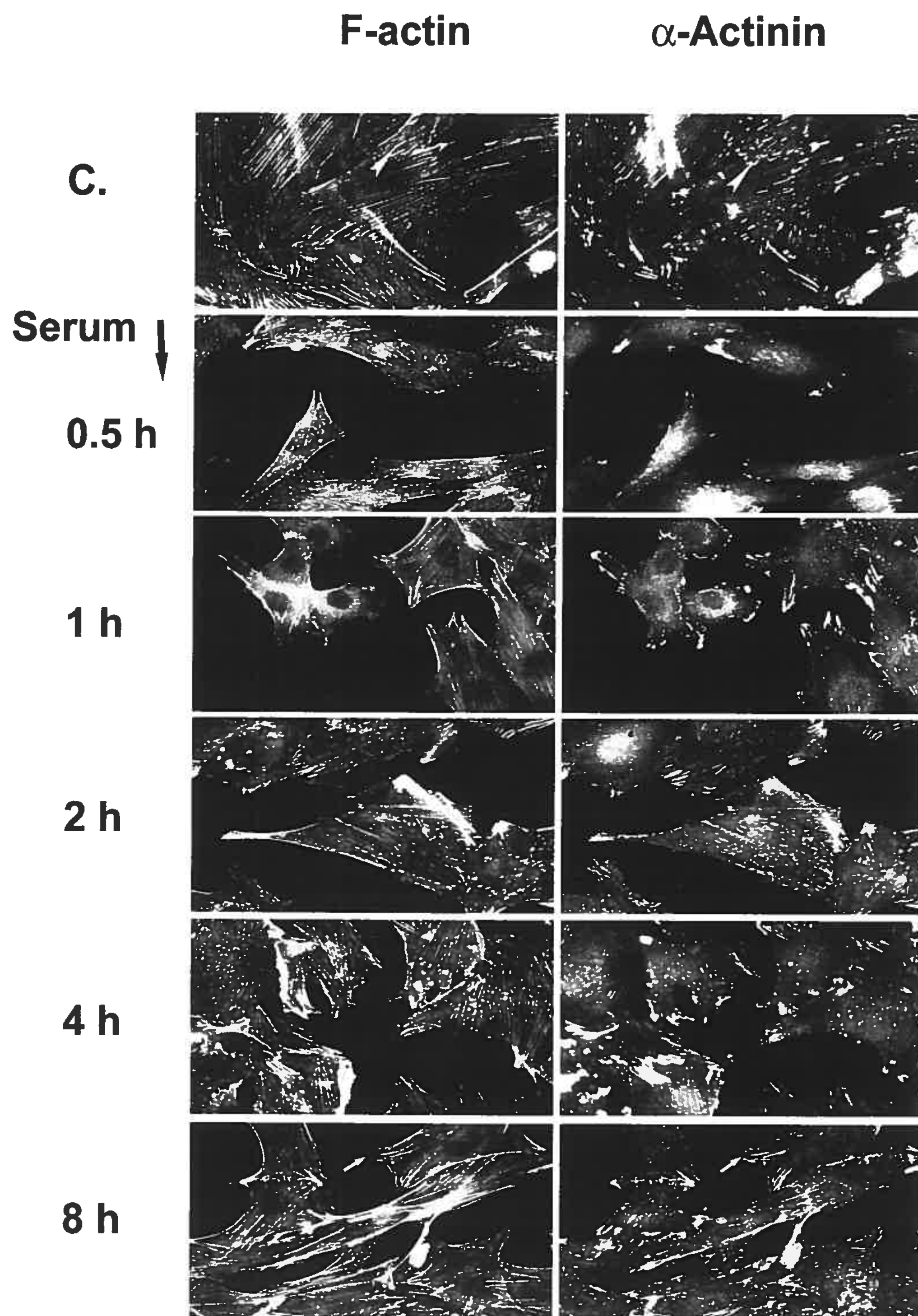


Figure 17

3.3.2 Studies on the phospho-tyrosine status of α-actinin in TtT/GF cells cultured under serum and serum-free conditions.

To investigate if and how tyrosine phosphorylation of α-actinin affects TtT/GF cell morphology, immunofluorescence studies were carried out in TtT/GF cells treated with the tyrosine kinase inhibitor genistein either in the presence or in the absence of serum. Alpha-actinin localized mainly to focal adhesions in the cells incubated in the presence of serum (Figure 18, C, arrowhead). Genistein treatment induced a reduction in the size of cortical contacts where α-actinin and phospho-tyrosine colocalized (Figure 18, arrows).

In the absence of serum, α-actinin and phospho-tyrosine localized to actin stress fibers (Figure 19, C, arrows). Less α-actinin and phospho-tyrosine localized to focal adhesions when serum-starved genistein treated cells were compared to cells cultured in serum containing medium (see Figure 18 C and 19 C). The serum-starved, genistein-treated cells showed elongated protrusions that were positive for α-actinin and phospho-tyrosine staining (Figure 19, arrowheads).

Figure 18 Effect of genistein on the localization of α-actinin and phospho-tyrosine in TtT/GF cells cultured in the presence of serum.

TtT/GF cells cultured in serum-containing medium were treated with 30μM genistein (final concentration) for 0 (C), 1h, 2h, and 24h. The cells were double-labeled with α-actinin and phospho-tyrosine antibodies. Alpha-actinin and phospho-tyrosine localized mainly to focal adhesions in control cells (arrowheads). Genistein treatment caused re-localization of phospho-tyrosine and α-actinin from focal adhesion to short cell membrane protrusions (arrows).

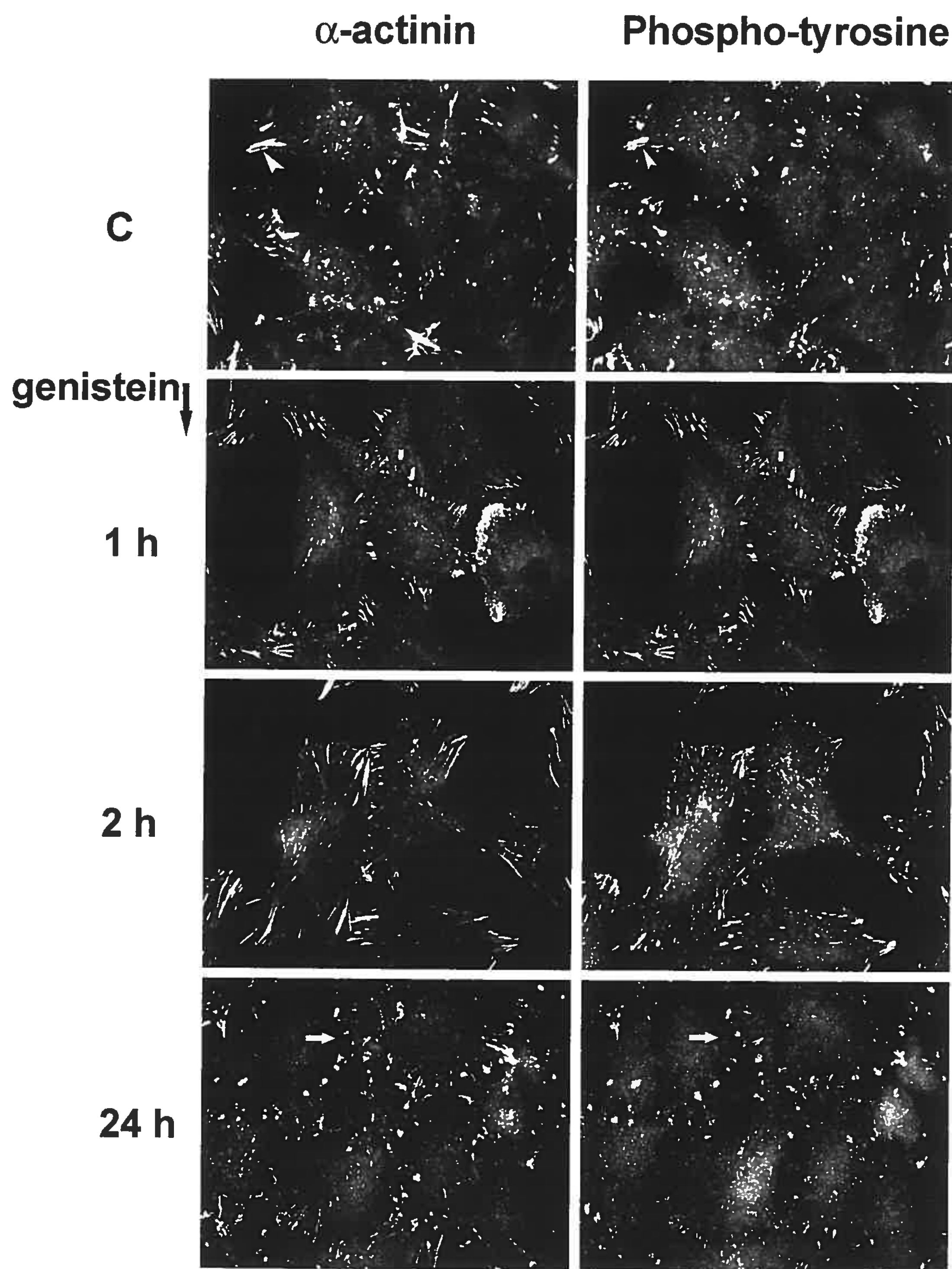


Figure 18

Figure 19 Effect of genistein on the localization of α-actinin and phospho-tyrosine in TtT/GF cells cultured in the absence of serum.

TtT/GF cells cultured in serum free medium were treated with 30μM genistein (final concentration) for 0 (C), 1h, 2h, and 24h. The cells were double-labeled with α-actinin and phospho-tyrosine antibodies. The serum-starved cells showed association of α-actinin and phospho-tyrosine with actin filaments (C, arrows). Following genistein treatment, there was a reduction in α-actinin association with stress fibers. In these cells, α-actinin labeling was found at thin membrane projections (arrowheads), where it co-localized with phospho-tyrosine.

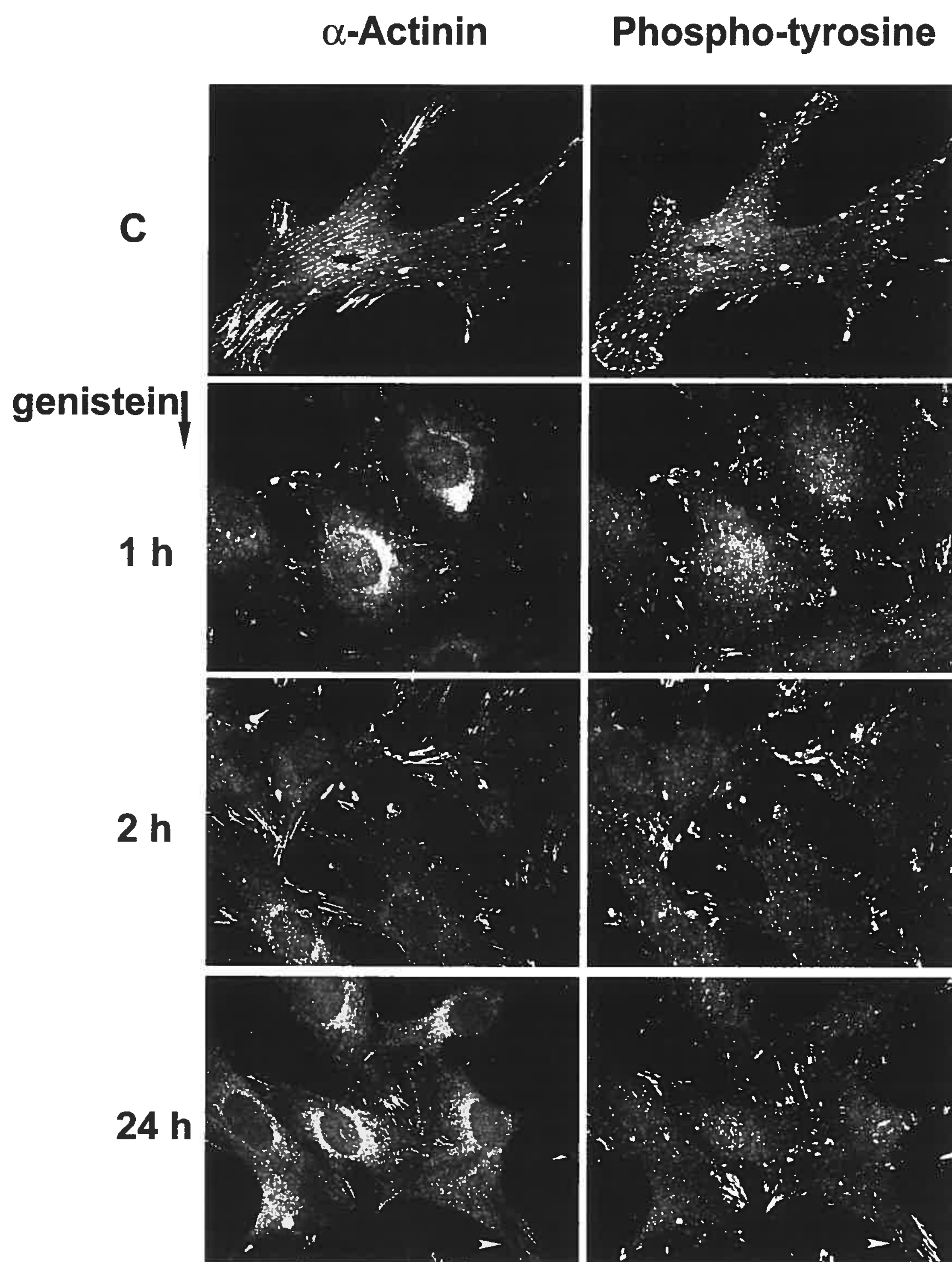


Figure 19

3.3.3 Involvement of cAMP/PKA pathway in the implication of α-actinin in the actin cytoskeleton dynamics in TtT/GF cells.

To evaluate the participation of the PKA pathway in the α-actinin-mediated actin remodeling in TtT/GF cells, two compounds affecting the PKA-dependent pathway, the adenylate cyclase activator forskolin and the PKA inhibitor KT5720 were used

3.3.3.1 Effect of forskolin on the expression and the subcellular localization of α-actinin in TtT/GF cells cultured in either serum containing or serum free medium

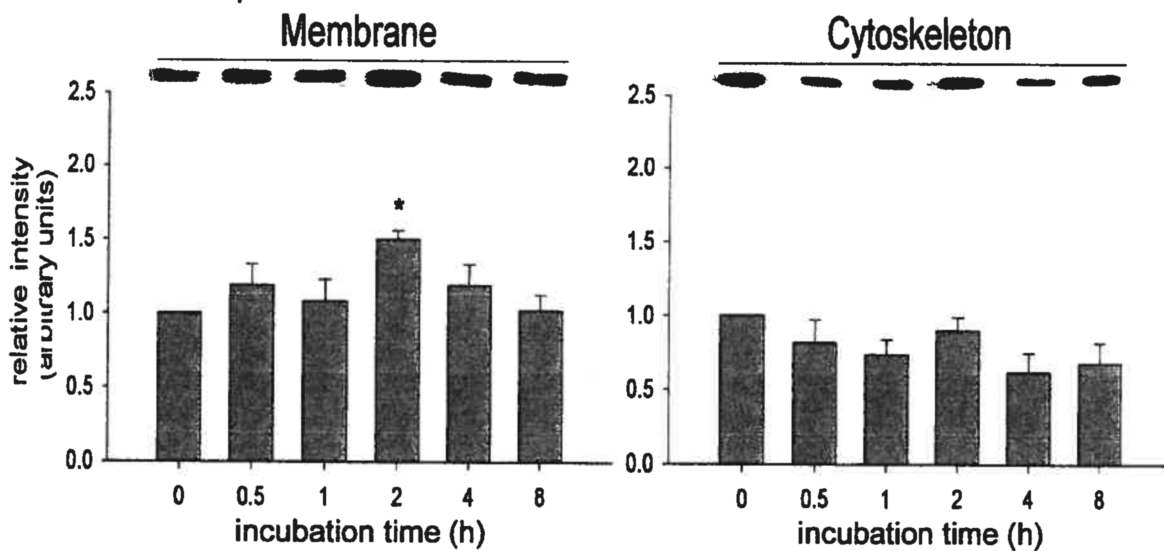
Western blot analyses were performed in TtT/GF cells treated with forskolin either in the presence or in the absence of serum (Figure 20, A and B). Alpha-actinin was only associated with membrane and cytoskeleton fractions. Forskolin increased the amount of α-actinin recovered in the membrane fraction in both serum and serum-free conditions (Figure 20, A and B). In cells cultured in the presence of serum, the increase in α-actinin level was transient, while in serum-starved cells, the increase was sustained (Figure 20, A and B). In the cytoskeleton fraction of cells cultured in the presence of serum, there was a decreasing trend in the association of α-actinin (Figure 20, A). Forskolin increased the level of α-actinin in the cytoskeleton fraction from 2h, in serum-starved cells (Figure 20, B).

Figure 20 Effect of forskolin on the expression and the subcellular localization of α-actinin in TtT/GF cells.

TtT/GF cells were cultured with either serum-containing (A) or serum free media (B) and treated with 15mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following treatments, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared and subjected to immunoblotting with α-actinin antibodies. The immunoreactive bands were scanned and their intensities were quantified. Data were normalized using the value at 0h for each subcellular fraction. Data presented was obtained from at least three independent experiments. The values shown are the mean ± SEM. * $P<0.05$ with respect to the precedent value

α-Actinin

A Serum-cultured cells



B Serum-starved cells

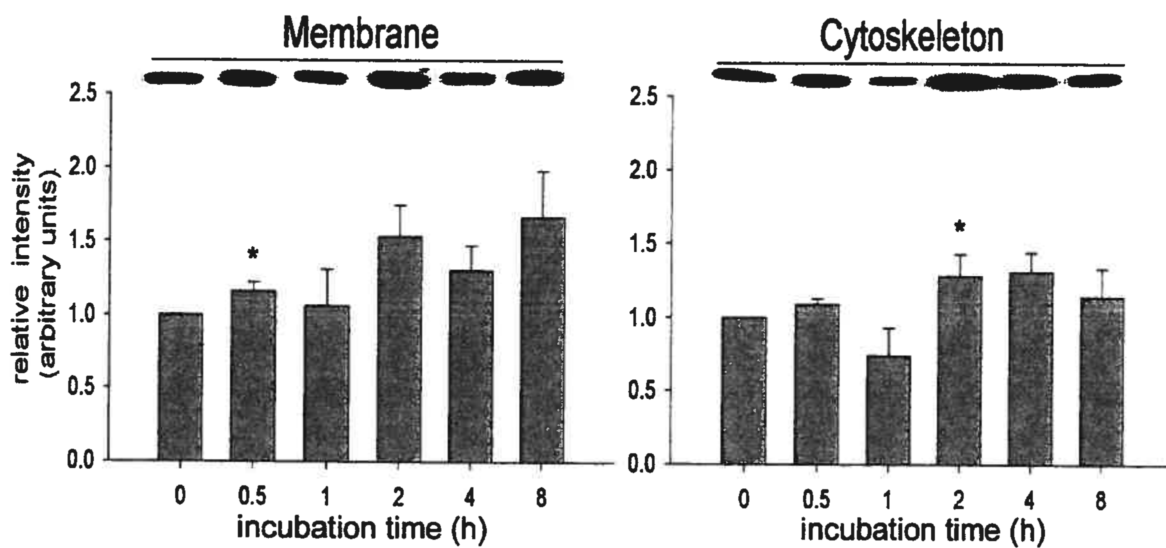


Figure 20

3.3.3.2 Immunofluorescence studies on the localization of α-actinin in TtT/GF cells treated with forskolin.

Next, we carried out immunofluorescence studies in TtT/GF cells treated with forskolin either in the presence or in the absence of serum. Forskolin induced the appearance of dendrite-like protrusions (Figure 21, arrowhead) in cells cultured in the presence of serum. Alpha-actinin localized mainly at the tips of the protrusions (Figure 21, α-actinin, arrowhead) and also along F-actin (Figure 21, arrows).

In serum free conditions, forskolin also induced the formation of cell membrane protrusions (arrowhead). Alpha-actinin localized at the tips of the protrusions (Figure 22, arrowhead) and also in the cytoplasm of serum-starved, forskolin-treated cells (Figure 22, arrow).

Figure 21 Effect of forskolin on the localization of F-actin and α-actinin in TtT/GF cells cultured in the presence of serum.

TtT/GF cells cultured in serum-containing medium were treated with 15 mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labelled using rhodamine-phalloidin and α-actinin antibodies. Forskolin induced the appearance of dendrite-like protrusions (arrowhead). Alpha-actinin localized mainly at the tips of the protrusions (arrowhead) and also along F-actin (arrow).

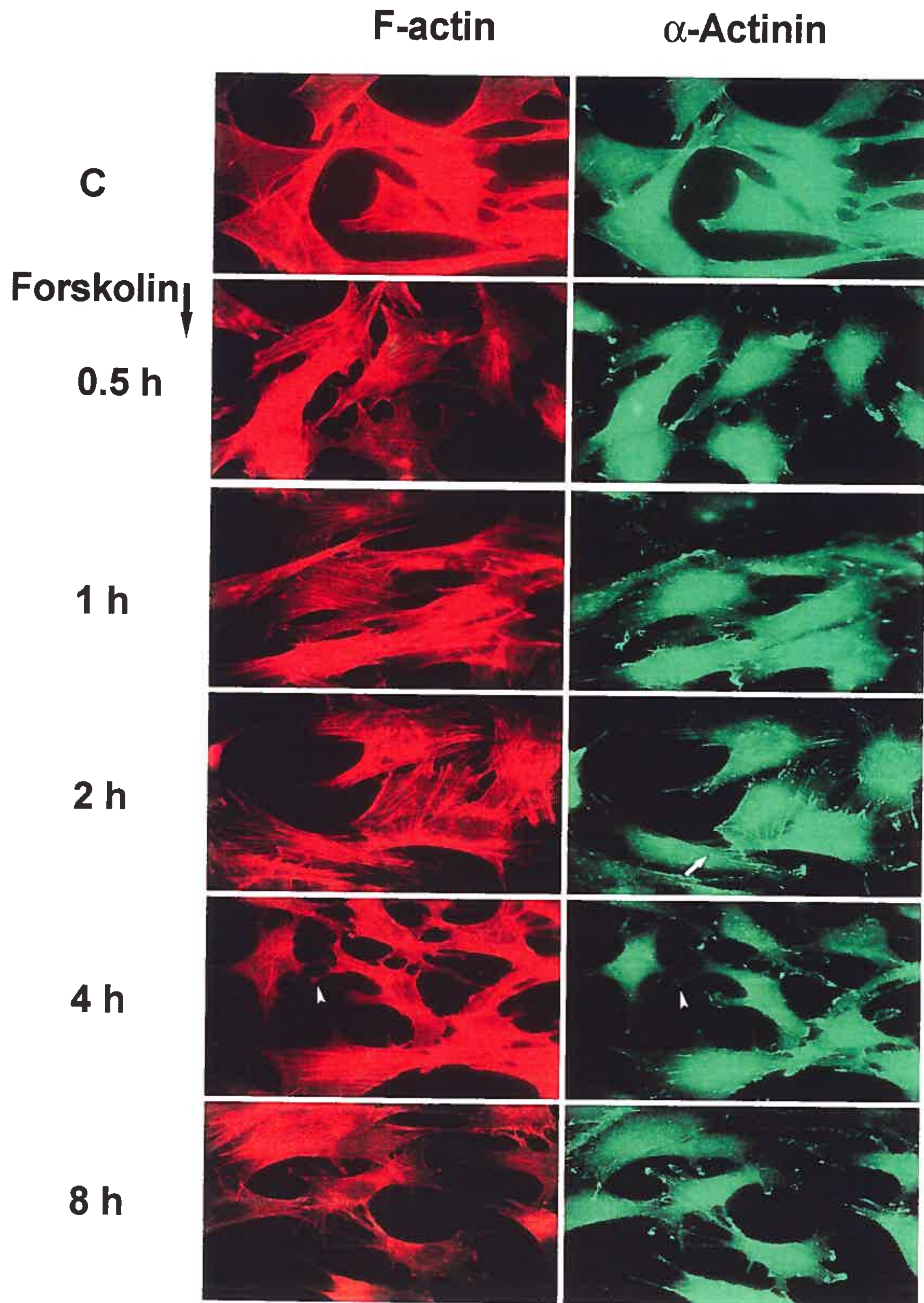


Figure 21

Figure 22 Effect of forskolin on the localization of F-actin and α-actinin in TtT/GF cells cultured in the absence of serum.

TtT/GF cells cultured in serum free medium were treated with 15mM forskolin (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labelled using rhodamine-phalloidin and α-actinin antibodies. Forskolin induced the formation of cell membrane protrusions (arrowhead). Alpha-actinin localized at the tips of the protrusions (arrowhead) and also in the cytoplasm in these cells (arrow).

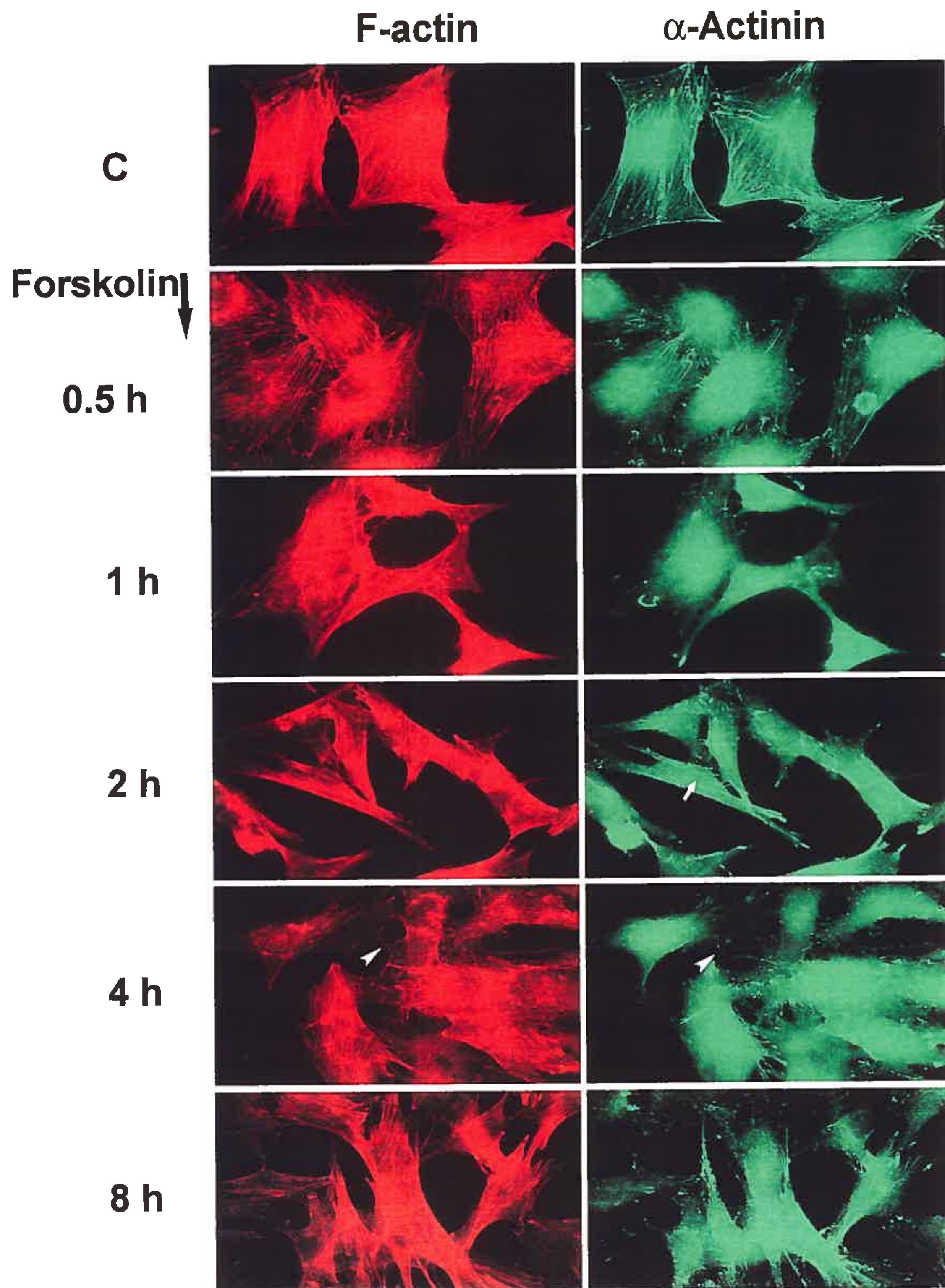


Figure 22

3.3.3.3 Effect of the PKA inhibitor KT5720 on the expression and the subcellular localization of α-actinin in TtT/GF cells cultured in either serum containing or serum free medium

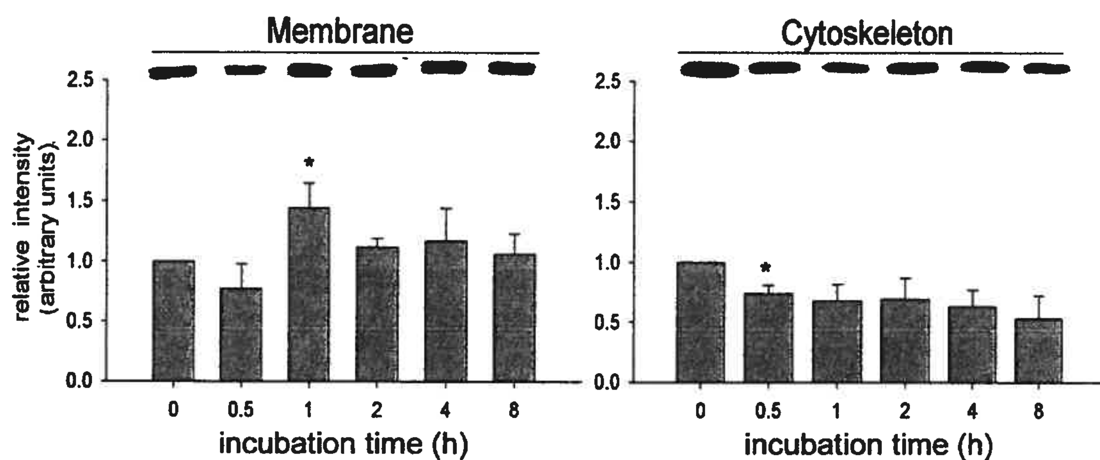
Western blot analyses were carried out in TtT/GF cells treated with KT5720 either in the presence or in the absence of serum. KT5720 transiently increase the association of α-actinin to the membrane in serum-treated cells (Figure 23, A). KT5720 decreased the level of α-actinin association with the cytoskeleton fraction (Figure 23, A).

In the absence of serum, KT5720 caused a transient and small increase in the level of α-actinin association with the membrane fraction and reduced the association of α-actinin to the cytoskeleton fraction after 4h treatment with KT5720 (Figure 23, B).

Figure 23 Effect of the PKA inhibitor KT5720 on the expression and the subcellular localization of α-actinin in TtT/GF cells.

TtT/GF cells cultured either in serum containing (A) or in serum free (B) medium were treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. Following treatments, membrane/cytosol and cytoskeleton/non-cytoskeleton fractions were prepared and subjected to immunoblotting with α-actinin antibodies. The immunoreactive bands were scanned and their intensities were quantified using the Scion Image program. Data were normalized using the value at 0h for each subcellular fraction. Data presented was obtained from at least three independent experiments. The values shown are the mean ± SEM. * $P<0.05$ with respect to the precedent value.

α-Actinin



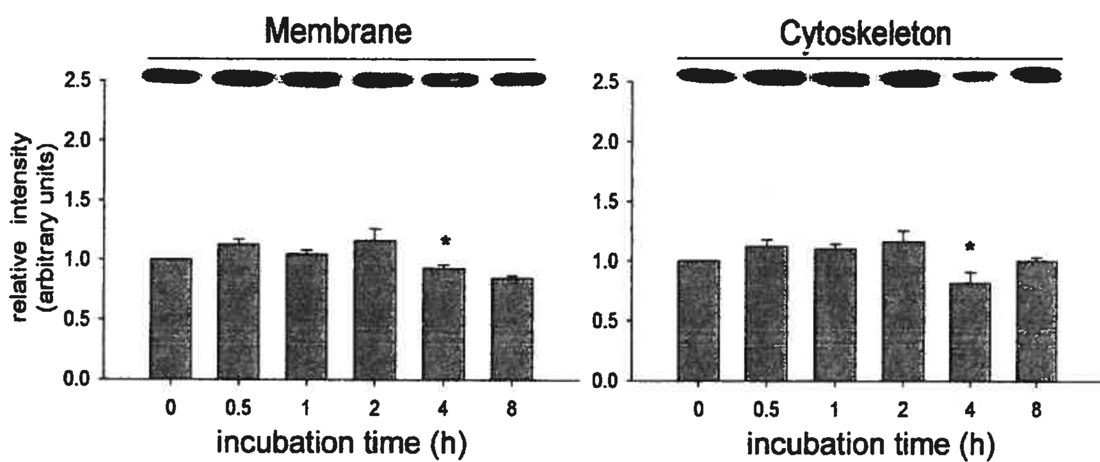


Figure 23

3.3.3.4 Immunofluorescence studies on the localization of α-actinin in TtT/GF cells treated with KT5720 in the presence or in the absence of serum

To know if α-actinin affected TtT/GF cell morphology via cAMP/PKA pathway, immunofluorescence studies were carried out in TtT/GF cells treated with KT5720 in the presence or in the absence of serum. Under serum conditions, short time incubation with K T5720 a ccentuated t he p olygonal s hape of T tT/GF c ells (Figure 24). Al pha-actinin localization to the focal adhesions increased in cells treated with KT5720 (Figure 24, arrowhead). Alpha-actinin localization along F-actin decreased, especially at 2h (Figure 24, arrows).

Inhibition of PKA with KT5720 did not affect the shape of cells cultured in the absence of serum (Figure 25). However, KT5720-treated cells showed depolymerization of actin filaments (Figure 25, arrowhead) and a lower α-actinin staining on actin filaments than control cells (Figure 25, arrows).

Figure 24 Effect of KT5720 on the localization of F-actin and α-actinin in TtT/GF cells cultured in the presence of serum.

TtT/GF cells cultured in serum-containing medium were treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labeled using rhodamine-phalloidin and α-actinin antibodies. KT5720 induced the acquisition of an elongated morphology. KT5720 decreased the intensity of the F-actin staining, especially at 2h and 4h. Alpha-actinin association with focal adhesion increased following KT5720 treatment (arrowhead). Alpha-actinin localization along F-actin decreased at 2h and 4h of KT5720 treatment (arrows).

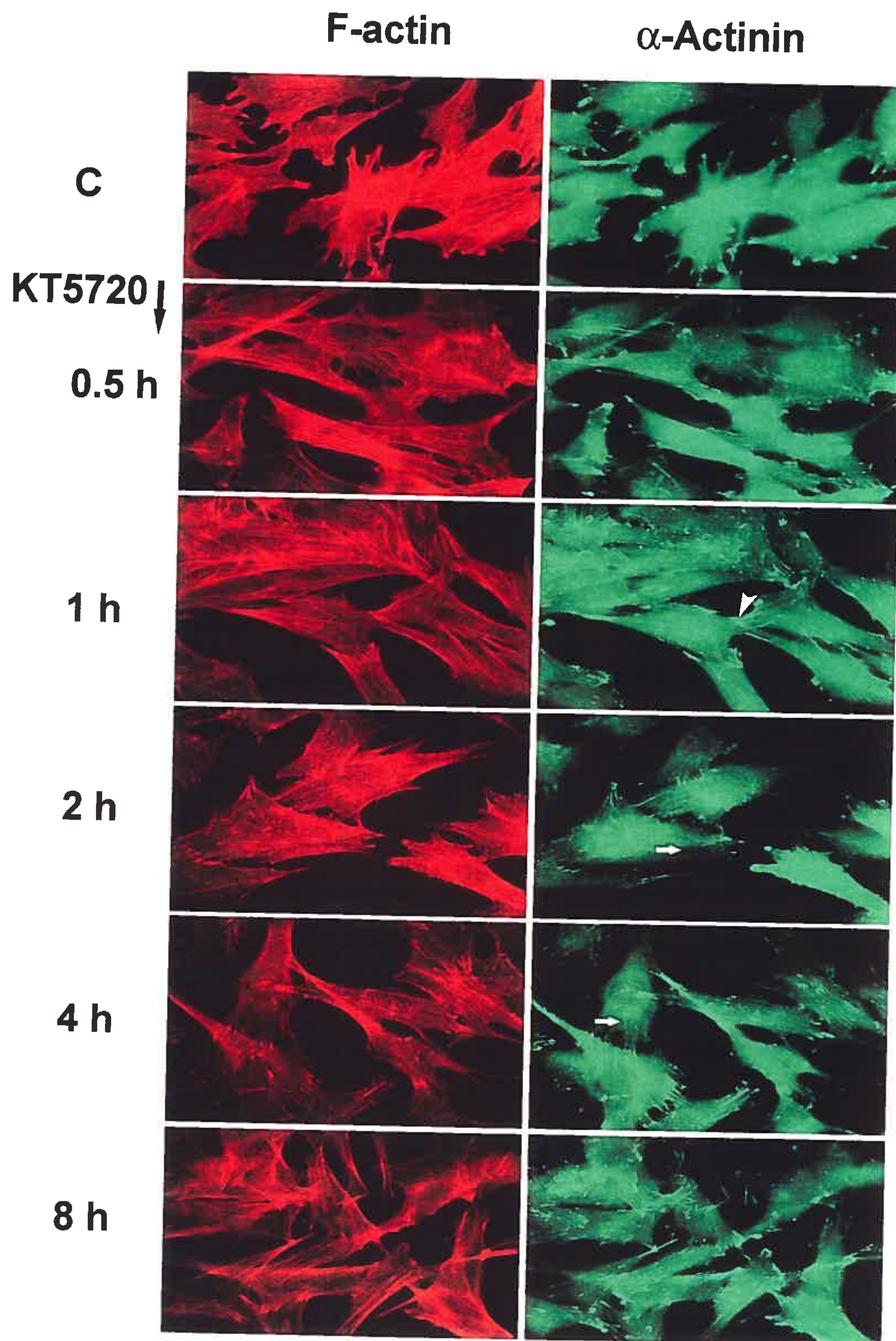


Figure 24

Figure 25 Effect of KT5720 on the localization of F-actin and α-actinin in TtT/GF cells cultured in the absence of serum.

TtT/GF cells cultured in serum free medium were treated with 100nM KT5720 (final concentration) for 0 (C), 0.5h, 1h, 2h, 4h, and 8h. The cells were double-labeled with rhodamine-phalloidin and α-actinin antibodiies. In the presence of KT5720, the cells acquired a polygonal shape. Signals of F-actin depolymerized such as rhodamine-phalloidin positive clumps (arrowhead) were evident from 0.5h of treatment with KT5720 and during that time α-actinin localization along F-actin decreased (arrows).

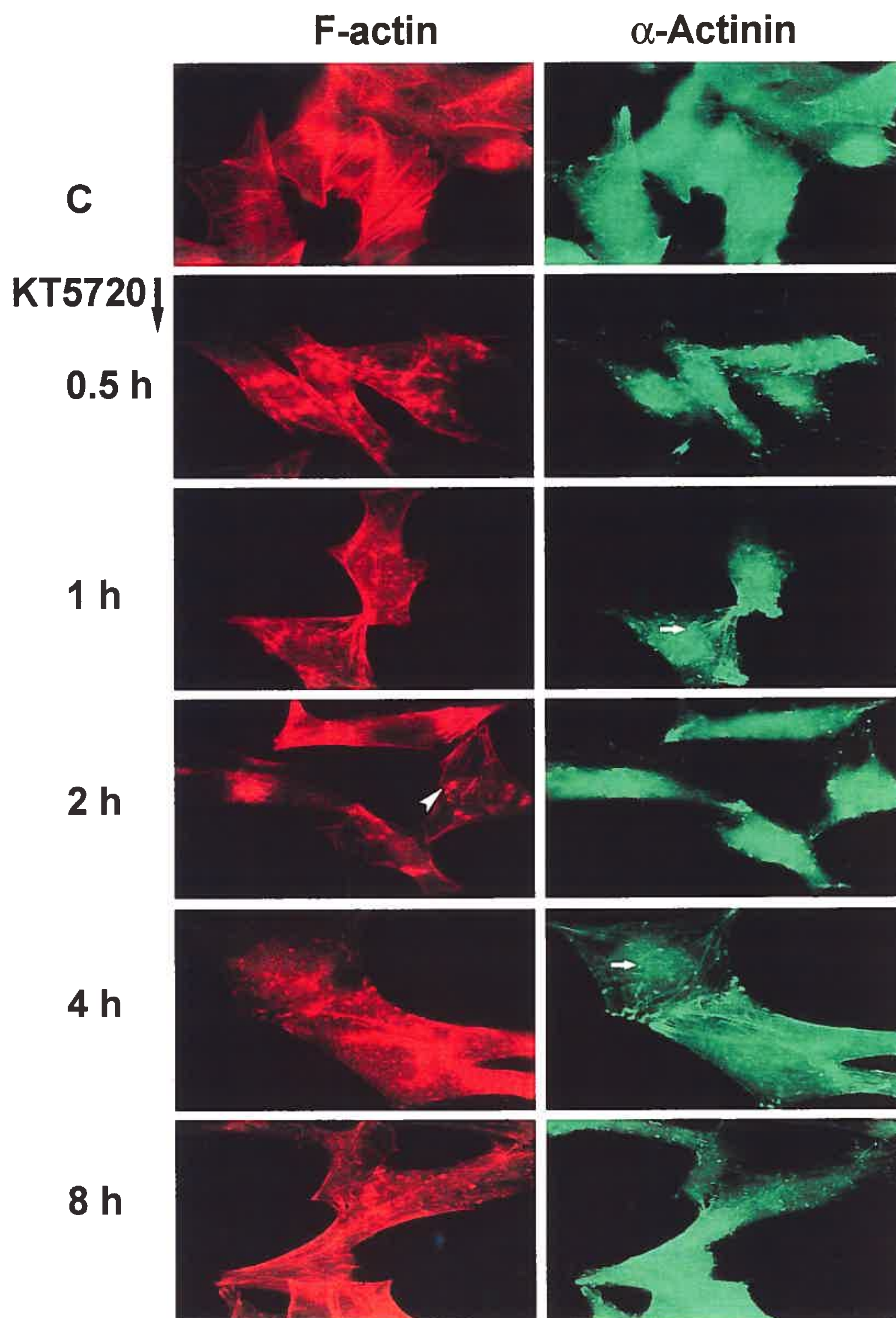


Figure 25

4 Discussion

It has been reported that FS cell's morphology is affected by the secretory activity of the anterior pituitary (Cardin et al, 2000). Because cell morphology is dependent on the actin cytoskeleton, changes in FS cell's morphology must be driven by alteration of the actin cytoskeleton. Yet, the precise mechanisms underlying the actin dynamics in FS cells are not fully understood. In non-muscle cells, most actin filaments are highly dynamic structures that are constantly assembled, disassembled, and reorganized as the cell changes its shape, divides, crawls, and adheres to a substratum or to neighbouring cells and adapts to the environment (Bershadsky and Vasiliev, 1988; Bray, 1992; Mitchison and Cramer, 1996; Small et al., 1996 and 1999; Stössel, 1993 and 1994; Theriot, 1994; Welch et al., 1997). The remodelling of the actin filaments is controlled by a host of actin-binding proteins regulated by external and internal stimuli (Aspenstrom, 1999; Cooper, 1991; Hall, 1998; Hatano, 1994; Janmey, 1998; Tapon and Hall, 1997). Here, we show that, the actin dynamics of FS cells is affected by serum factors.

In the present study, we have performed biochemical and morphological analyses and demonstrated the participation of the actin-binding proteins, cortactin and α-actinin in the regulation of the actin cytoskeleton dynamics in the FS cell line, the TtT/GF cells, by serum factors. We have used the tyrosine kinase inhibitor genistein and the adenylate cyclase activator forskolin and the PKA inhibitor KT5720 to perform Western blot analyses and immunofluoresence microscopy studies to explore the mechanisms underlying the involvement of cortactin and α-actinin in the regulation of actin dynamics by serum factors in TtT/GF cells. We found that tyrosine kinase and PKA-

dependent pathways regulate cortactin and α-actinin-mediated remodelling of the TtT/GF cells' actin cytoskeleton by serum factors.

4.1 Effect of serum factors on the dynamics of the actin cytoskeleton in TtT/GF cells via the participation of distinct actin-binding proteins

We know that the actin cytoskeleton is responsible for the cell shape. It has been reported that folliculo-stellate cell morphology is affected by the hormonal milieu (Cardin et al, 2000). We investigated here whether the TtT/GF cells'actin cytoskeleton was sensitive to serum factors. TtT/GF cells displayed an elongated fibroblast-like shape, membrane protrusions and pseudopodia when cultured in the presence of serum. Cortical actin filaments were important but cytoplasmic actin filaments were thin and sparse. Cells cultured in serum free medium adopted a polygonal shape, lost most of the membrane ruffles and tended to be organized in epithelial-like sheets. Cells grown in serum free medium were not mobile but shortly after the addition of serum, cell membrane protrusions started to grow and were followed by the formation of pseudopodia, indicating the reappearance of the fibroblast-like morphology. Additionally, serum-starved cells showed much thicker, denser, and straighter actin fibers throughout the cytoplasm than serum-cultured cells. These results indicate that TtT/GF cell actin cytoskeleton was affected by serum factor, and as a consequence of this, the morphology of the cells changed.

The dynamics of the actin cytoskeleton is controlled by a set of binding proteins. Therefore, we studied the involvement of such binding proteins in the serum-induced reorganization of the actin cytoskeleton in TtT/GF cells. Because there are at least 100

distinct actin-binding proteins, we restricted our studies to actin-binding proteins that are involved in the regulation of plasma membrane-actin filament association and in determining the thickness of actin fibers. Cortactin, is an actin-binding protein involved in the generation of membrane ruffles, pseudopodia and lamellipodia. Alpha-actinin participates in the attachment of actin fibers to focal adhesions and in the organization of actin stress fibers. Vinculin is involved in the organization of the focal adhesions and myosin in the building of actin stress fibers. Our results showed the participation of some of these proteins in the actin cytoskeleton reorganization induced by serum factors.

Serum withdrawal induced significant changes in the distribution of cortactin and α-actinin: cortactin translocated from membrane ruffles, pseudopodia and lamellipodia to the whole cell membrane and α-actinin relocated from focal adhesions into stress fibers.

The two other actin-binding proteins, vinculin and myosin light chain did not show significant changes in both their expression and subcellular localization upon serum withdrawal. Collectively, our results suggest that serum induced the remodelling of the actin cytoskeleton in TtT/GF cells partly via cortactin and α-actinin relocation. Therefore, we focused our studies on the role of cortactin and α-actinin in mediating the reorganization of the actin cytoskeleton in TtT/GF cells by serum factors.

4.2 Participation of cortactin in the modulation of actin cytoskeleton dynamics in TtT/GF cells by serum factors

The actin filaments underlying the plasma membrane determine the shape and movement of the cell. Actin-based structures can form many different cell surface

projections, including microvilli, filopodia and lamellipodia, which help the cells to move on solid substrates. Cortactin is an actin-binding protein and cortical structures such as membrane ruffles and lamellipodia are enriched in cortactin. Indeed, cortactin plays an important role in regulating cortical actin assembly and organization (Weed and Parsons, 2001). In most cell types, cortactin localizes with F-actin at sites of dynamic peripheral membrane activity and, in cytoplasmic punctate structures of unknown composition concentrated at the perinuclear region (Weed and Parsons, 2001). Cortactin migrates from the cytoplasm to the periphery in response to many of the stimuli that induce its tyrosine phosphoryation, such as growth factor treatment, integrin activation and bacterial entry (Ozawa et al., 1995; Weed et al., 1998; Cantarelli et al., 2000). We found that cortactin labeling was associated to active membrane regions such as membrane ruffles, pseudopodia and lamellipodia in serum-cultured TtT/GF cells. In addition, cortactin displayed a punctuate cytoplasmic staining that was denser in the perinuclear area. These results are in agreement with cortactin localization described in other cell types. When TtT/GF cells were deprived of serum factors, cortactin distribution was affected. Cortactin displayed a discontinuous distribution along the whole plasma membrane. Particularly strong was the labeling at cell-cell contact regions of the membrane. The spotty intracellular cortactin staining in serum-starved cells was sometimes more diffused than in serum-cultured cells. This reorganization of cortactin was supported by the biochemical studies.

Our results also showed that, upon serum addition of serum-starved cells to the culture medium, membrane activity i.e., the development of ruffles, pseudopodia and lamellipodia reappeared and that this coincided with the re-association of cortactin with the active membrane regions. These results indicate that cortactin is actively implicated

in the formation of membrane projections driven by serum factors in TtT/GF cells.

4.2.1 Tyrosine phosphorylation of cortactin regulates the actin cytoskeleton dynamics in TtT/GF cells in the presence of serum factors

To gain further insight into the role of cortactin in the modulation of cortical actin structures in TtT/GF cells, we studied the implication of tyrosine phosphorylation in cortactin relocation by serum factors. Cortactin is a tyrosine phosphorylated protein, and its phosphorylation status affects its association with the plasma membrane and its ability to induce actin filament branching in other cells types (Weed and Parsons, 2001). Cortactin is also a target of both tyrosine and serine/threonine protein kinases (Weed and Parsons, 2001). Cortactin is normally phosphorylated on serine and threonine residues, but it becomes tyrosine phosphorylated when the cells are stimulated by growth factors or when the tyrosine kinase Src is activated (Wu et al., 1991; Maa et al., 1992; Zhan et al., 1993). Phosphorylation of cortactin by Src is thought to be functionally linked to some aspects of actin organization and cell motility, however, the full significance of Src-mediated cortactin tyrosine phosphorylation is not yet clear (Weed and Parsons, 2001). In normal cells, cortactin does not contain detectable phospho-tyrosine, and the majority of the protein partitions with the detergent-soluble fraction, which is known as the 'non-cytoskeleton' fraction. In cells expressing wild-type v-Src or an SH3-deleted Src, cortactin becomes tyrosine-phosphorylated, and a significant increase in cortactin association to the Triton-insoluble cytoskeletal fraction is observed (Heidi and Marilyn, 1995). Di Ciano et al (2002) showed that tyrosine phosphorylation of cortactin may be a compensatory process that facilitates the disassembly of the Arp2/3-actin-cortactin complex. Such a disassembly, enhanced by Src kinases, is important for the dynamic

recycling of cortactin during cell movement. As the leading edge is pushed forward, cortactin at the base of the lamellipodium may become phosphorylated and detach from actin. After dephosphorylation, cortactin may be rebuilt into the new front (Di Ciano et al., 2002). We observed that inhibition of tyrosine kinases not only blocked the reappearance of membrane ruffles induced by serum but also the concomitant redistribution of cortactin as well. The result may indicate that blockage of tyrosine phosphorylation of cortactin blocked its capacity to bind to actin filaments, therefore inhibiting the formation of membrane ruffles where cortactin-associated branching activity is needed.

To further evaluate the role of tyrosine phosphorylation of cortactin in the remodelling of the actin cytoskeleton in serum and serum-free conditions, we performed double labelling immunofluorescence studies with cortactin and phospho-tyrosine antibodies on TtT/GF cells treated with the tyrosine kinase inhibitor genistein in the presence and in the absence of serum. As described above, in the presence of serum, cortactin localized to pseudopodia and other cell membrane protrusions and to cytoplasmic punctuate structures concentrated at the perinuclear region. Co-localization of cortactin and phospho-tyrosine was apparent in the pseudopodia and membrane protrusions. Short-term genistein treatment reduced cortactin and phospho-tyrosine labelling. Soon after, short and small cell membrane protrusions that stained positively for cortactin and phospho-tyrosine replaced the wide membrane ruffles typical of serum-cultured cells. Compared to serum-cultured cells treated with genistein, the genistein-treated, serum-starved cells showed a weaker staining of cortactin and phospho-tyrosine that were associated with thin and long membrane projections. Taken together, the results suggest that tyrosine phosphorylation of cortactin is responsible for the

association of cortactin to the cell pulling in areas where active actin branching takes place.

However, we have to take into consideration that the phospho-tyrosine antibody does not allow us to discriminate between several tyrosine phosphoraylated proteins. Thus, we performed immunofluorescence and Western blot analyses using an antibody that is specific for cortactin phosphorylated in tyrosine 421 (p-cortactin). P-cortactin associated to membrane/cytosol and cytoskeleton/non-cytoskeleton fractions in TtT/GF cells. P-cortactin redistributed from membrane to cytosol and from cytoskeleton to non-cytoskeleton following serum removal. These results suggest that serum induced the tyrosine phosphorylation of cortactin and that their tyrosine phosphorylated proteins becomes associated with the cytoskeleton and cell membranes. Interestingly, we found that serum-starvation did not decrease the levels of p-cortactin in TtT/GF cells. Immunofluorescence studies demonstrated that p-cortactin was associated to the whole plasma membrane and to cytoplasmic regions in serum-starved cell, whereas in serum-cultured cells, p-cortactin was concentrated in membrane ruffles, pseudopodia and lamellipodia. When we studied the distribution of p-cortactin in serum-starved cells challenged with serum, we observed that cortactin and p-cortactin co-localizition at the leading edges of membrane ruffles and pseudopodia increased during serum treatment. The presence of genistein in the culture medium blocked the association of p-cortactin to the membrane ruffles, yet the levels of p-cortactin were not very much affected. The results suggest that a tyrosine kinase activated by serum factors is involved in the translocation of p-cortactin to the sites of active actin branching such as the membrane ruffles and the pseudopodia.

4.2.2 Cortactin is involved in the modulation of the actin cytoskeleton dynamics via the cAMP/PKA pathway

A variety of signalling molecules utilize the PKA pathway to regulate actin cytoskeleton dynamics and cell migration (Howe, 2004). While some of these intracellular events are stimulated by PKA, others are inhibited by the kinase. Agonists of cAMP/PKA signaling cause significant changes in cellular architecture, such as dissolution of stress fibers and induction of a stellate morphology in neurons and other cells (Dong et al., 1998; Ramakers and Moolenaar, 1998; Edwards et al., 1993).

Though cortactin is a phospho-tyrosine protein, it is also phosphorylated at serine and threonine residues (Weed and Parsons, 2001). We used two drugs: forskolin, an activator of adenylate cyclase and KT5720, an inhibitor of PKA to investigate the role of the cAMP/PKA pathway in the participation of cortactin and p-cortactin in the regulation of the actin cytoskeleton in TtT/GF cells.

Western blot analyses on TtT/GF cells treated with forskolin either in the presence or in the absence of serum showed that both cortactin and p-cortactin were associated to membrane/cytosol and cytoskeleton/non-cytoskeleton fractions. In the presence of serum, forskolin increased the association of cortactin to the membrane fraction but decreased cortactin association to the cytosol fraction. Forskolin transiently decreased cortactin association with the cytoskeleton fraction. These findings indicate that forskolin in the presence of serum factors induced a redistribution of cortactin from cytosol to membrane and from cytoskeleton to non-cytoskeleton fraction. The changes in the levels of p-cortactin associated to the subcellular fractions following treatment were not as clear-cut as in the case of cortactin. However, we observed an increasing trend in the levels of p-cortactin in the membrane and the non-cytoskeleton fractions.

The immunofluorescence studies on TtT/GF cells treated with forskolin showed that in the presence of serum, forskolin caused a marked change in TtT/GF cell morphology. TtT/GF cells showed long dendrite-like protrusions. Co-localization of cortactin and p-cortactin was found at the tips of these dendrite-like protrusions. The findings suggest that the increased levels of cortactin and p-cortactin associated with the dendrite-like protrusions corresponded to the increased association of cortactin and p-cortactin to the membrane fraction following treatment of the cells with forskolin.

In the absence of serum, forskolin acts differently. It decreased cortactin association with the membrane fraction and increased cortactin levels in the cytosol fraction. In cytoskeleton/non-cytoskeleton fractions, forskolin increased cortactin in the cytoskeleton fraction and decreased it in the non-cytoskeleton fraction. The results are just the opposite we found in cells incubated in the presence of serum. Upon serum withdrawal, forskolin transiently reduced the level of p-cortactin in membrane/cytosol and cytoskeleton/non-cytoskeleton fractions. In the absence of serum, the cells treated with forskolin displayed longer protrusions than control cells. Less cortactin localized at the leading edge of the membrane ruffles. At long incubation times, p-cortactin was associated with the contact sites between the cells.

It is possible that certain tyrosine kinases responsible for cortactin phosphorylation are up regulated by forskolin in the presence of serum while they are down regulated by forskolin in the absence of serum. In addition, phosphorylation of cortactin at serine/threonine may influence cortactin phosphorylation at tyrosine residues. The relationship between these two types of phosphorylations in cortactin remains to be further explored. Moreover, serine/threonine phosphorylation of other proteins modulating the tyrosine phosphorylation of cortactin might also occur.

To further investigate the role of cAMP/PKA pathway in the regulation of cortactin phosphorylation during serum-induced remodelling of TtT/GF cells, we blocked PKA pathway using the PKA inhibitor KT5720 either in the presence or in the absence of serum. Immunofluorescence studies performed in TtT/GF cells treated with KT5720 either in the presence or in the absence of serum, showed that in serum-cultured cells, KT5720 caused TtT/GF cells to display short protrusions with cortactin and p-cortactin being localized at the tips of the protrusions. In the absence of serum, KT5720-treated cells were deprived of cell membrane protrusions and there was a reduced colocalization of cortactin and p-cortactin at the plasma membrane.

Western blot analyses showed that in the presence of serum, KT5720 increased cortactin levels in the membrane fraction and decreased cortactin levels in the cytosol fraction. KT5720 decreased cortactin levels in the cytoskeleton fraction and increased them in the non-cytoskeleton. KT5720 slightly increased p-cortactin levels both in membrane and cytosol fractions. Association of p-cortactin to the cytoskeleton and non-cytoskeleton fractions showed a decreasing trend following treatment of the cells with KT5720. However, p-cortactin levels increased significantly in the non-cytoskeleton fraction after 8h of KT5720 treatment. Interestingly, if we compare the effects of the activation and of the inhibition of the cAMP/PKA pathway on the association of cortactin to the membrane/cytosol and to the cytoskeleton/non-cytoskeleton fractions, we observe that in both cases there was an increase in cortactin levels in the membrane and in the cytoskeleton fractions. These results are hard to interpret taking into account that opposite effects on PKA induced the same effects on cortactin. One possibility would be that different signalling pathways beyond PKA are affected. In fact this is what is suggested by the distribution of p-cortactin. Forskolin that increased cAMP

levels, and therefore, PKA activity, also increased the association of p-cortactin to the membrane and the cytoskeleton fractions, whereas, KT5720, and therefore, the inhibition of PKA activity, did not have significant effect. These findings may indicate that activation of PKA indirectly facilitates the tyrosine phosphorylation of cortactin and that this mediates cortactin association with the membrane. Instead, inhibition of PKA has no effect on the tyrosine phosphorylation status but still cortactin associated with the membrane. It is tempting to speculate that because cortactin is also phosphorylated in serine/threonine, that PKA phosphorylates cortactin and this enhances tyrosine phosphorylation of the actin-binding protein. Instead, the absence of serine-threonine phosphorylation of cortactin will not affect its tyrosine phosphorylation status.

In the absence of serum, KT5720 had little effect on the association of cortactin to the membrane/cytosol fractions. However, KT5720 transiently increased the levels of p-cortactin in the cytoskeleton fraction. KT5720 had a biphasic effect on the association of p-cortactin to the membrane and the cytosol fractions.

These results indicate that there might be some factors in serum that alter the response of the cells to the effect of KT5720. First, in the presence of serum, KT5720 increased cortactin association to the membrane but did not affect too much its association with the cytoskeleton. The opposite effect was observed in the absence of serum. Moreover, p-cortactin association to membrane was not affected in the presence of serum but it was very much affected in the absence of serum.

4.2.3 Conclusion

A variety of serum factors influence the actin cytoskeleton dynamics. Epidermal growth factor (EGF) exerts its effects in the target cells by binding to the plasma

membrane EGF receptor, resulting in the activation of its tyrosine kinase activity and subsequent receptor autophosphorylation, which is essential for the interaction of the receptor with its substrates (Boonstra et al., 1995). EGF-induced early signal transduction causes rapid remodelling of the actin microfilament system in a variety of cells (Rijken et al., 1991; Peppelenbosch et al., 1993). The EGF receptor itself is an actin-binding protein (den Hartigh et al., 1992), which causes a rapid actin depolymerisation, and the formation of membrane ruffles (Rijken et al., 1991). These membrane ruffles function as the first site of signal transduction after EGF binding, and thus are thought to be signal transduction structures (Boonstra et al., 1995). This is also the case for other growth factors, such as nerve growth factor (Paves et al., 1990) and platelet-derived growth factor (PDGF) (Arvidsson et al., 1992; Kundra et al., 1994). The receptors for various growth factors, including PDGF (Frackelton et al., 1984; Ek and Heldin, 1984), insulin (Kasuga et al., 1982) and insulin-like growth factor I (Sasaki et al., 1985) are all protein tyrosine kinases. In our case, cortactin localized to active areas of the cell cortex and serum withdrawal induced its translocation from the active membrane region to the whole membrane and to the cell interior. Based on our results, we suggest that cortactin could be a signal-transducing molecule or an effector molecule in the integration of extracellular stimulation with cell morphology.

4.3 Participation of α-actinin in the modulation of actin cytoskeleton dynamics by serum factors in TtT/GF cells

α-Actinin plays an important role in the regulation of cell adhesion by linking actin filaments directly to integrins (Pavalko et al., 1991; Liu et al., 2000). In addition, α-

actinin constitutes a direct link between the actin cytoskeleton and the cytoplasmic domains of several cell surface receptors. This cytoskeletal connection is implicated in receptor function and anchorage, cytoskeletal reorganization, and concomitant signaling events (Yamada and Geiger, 1997; Pavalko and LaRoche, 1993; Wyszynski et al., 1997).

Analyses of green fluorescent protein (GFP)-α-actinin dynamics showed that once the interaction between the tips of protrusions and extracellular matrix stabilizes, α-actinin begins to localize in small foci at the leading edge of the protrusions, which then grow in size and extend small fiber-like structures toward the cell body (Edlund et al., 2001; Laukaitis et al., 2001). The localization of α-actinin in focal adhesion plaques suggestes that it might serve to anchor the network of actin filaments to the plasma membrane (Izaguirre et al., 2001). This is substantiated by the finding that α-actinin associates with the cytoplasmic tail of members of several adhesion receptor families including integrins (Otey et al., 1990; Sampath et al., 1998), cadherins (Knudsen et al., 1995; Nieset et al., 1997), and intercellular adhesion molecules (Carpen et al., 1992; Heiska et al., 1996). Alpha-actinin functions as an actin cross-linking protein abundant at focal adhesions (Maruyama and Ebashi, 1965; Lazarides and Burridge, 1975; Podlubnaya et al., 1975), and has been suggested to play important roles in nascent focal adhesion assembly and stress fibre extensions from the integrin-based cell–substrate adhesion complex. In agreement with this finding, our results of double labeling immunofluorescence studies showed that α-actinin was associated with focal adhesions of TtT/GF cells cultured in the presence of serum. In serum free conditions α-actinin localized mainly to actin stress fibers. When serum starved cells were incubated with serum, we observed a translocation of α-actinin from the cytoplasmic actin filaments to

focal adhesions. This relocation of α-actinin was accompanied by a marked change in TtT/GF cell morphology and behavior: the transformation from a polygonal shape to an elongated cell with the concomitant switch from a non-mobile to a mobile cell.

4.3.1 Tyrosine phosphorylation of α-actinin regulates the actin cytoskeleton dynamics in TtT/GF cells in the presence of serum factors

It is well known that α-actinin is tyrosine-phosphorylated in activated platelets and activated T-cells (Izaguirre et al., 1999; Egerton et al., 1996). Using the recombinant protein, α-actinin was found to be phosphorylated at the tyrosine residue in position 12 (Izaguirre et al., 2001). In platelets, tyrosine phosphorylation of α-actinin and the focal adhesion kinase (FAK) are closely regulated events. It was suggested that α-actinin is a FAK substrate and tyrosine phosphorylation of α-actinin by FAK affects the cytoskeleton organization (Izaguirre et al., 2001). Tyrosine phosphorylation reduced the binding of α-actinin to actin (Izaguirre et al, 2001). These data establish that α-actinin is a novel FAK substrate, and as such, is likely to transduced FAK-dependent signals that regulate the organization of the cytoskeleton (Izaguirre et al, 2001). According to Izaguirre et al (1999), α-actinin and $pp125^{FAK}$ tyrosine phosphorylation in platelets are dependent on the massive cytoskeleton reorganization that takes place in activated platelets. Alpha-actinin and $pp125^{FAK}$ tyrosine phosphorylation are dependent on protein kinase C activation (Haimovich et al, 1993; Shattil et al, 1994). PI3-kinase inhibitors also abrogate $pp125^{FAK}$ and α-actinin phosphorylation in platelets adherent to fibrinogen (Ji and Haimovich, 1999).

The smooth and non-muscle α-actinin isoforms are localized along stress fibers, in focal adhesion plaques, and in adherens junctions (Waites, et al, 1992; Knudsen et al, 1995). In addition to its role as an actin cross-linking protein, α-actinin may complement the activity of $pp125^{FAK}$ and serve as a scaffold to promote protein-protein interactions (Clark and Brugge, 1995). The reported interactions of α-actinin with actin, integrins, vinculin, zyxin, the p85 subunit of PI3-kinase, and PKN, a fatty acid and Rho-activated serine/threonine protein kinase, are consistent with this possibility (Clark and Brugge, 1995; Wachsstock et al, 1987; Crawford et al, 1992). As a scaffold protein that is closely associated with both transmembrane adhesion receptors and cytoskeletal proteins, α-actinin may be an attractive regulatory target. Calcium binding to the EF hand modules in α-actinin decreases the interaction between α-actinin and actin (de Arruda et al, 1990; Witke et al, 1993). Fukami et al. (1992 and 1996) have shown that the skeletal muscle isoform of α-actinin binds phosphatidylinositol 4,5-bisphosphate and that the actin gelating activity of the protein was enhanced by the phospholipid. More recently, Greenwood et al. (2000) reported that in rat embryonic fibroblasts, phosphatidylinositol 3,4,5-triphosphate (IP_3), a lipid product of phosphatidylinositol 3-kinase, binds to α-actinin. Immunoprecipitation studies suggested that the binding of IP_3 to α-actinin decreased the binding affinity of α-actinin for β_3 and β_1 integrins (Greenwood et al., 2000). This correlated with a re-localization of α-actinin and actin to the cell cortex and was accompanied by the dissolution of actin stress fibers. These data raise the possibility that the interaction between α-actinin and its ligands may be regulated by more than one mechanism.

To investigate whether and how tyrosine phosphorylation of α-actinin was

involved in the relocation of α-actinin following serum starvation in TtT/GF cells, we performed immunofluorescence studies in cells treated with the tyrosine kinase inhibitor genistein either in the presence or in the absence of serum using α-actinin and phospho-tyrosine antibodies. Alpha-actinin localized mainly in focal adhesions in the cells incubated in the presence of serum, where it co-localized with phospho-tyrosine. The punctuate cytoplasmic staining of α-actinin also co-localized with phospho-tyrosine. Genistein treatment of serum-cultured cells caused the re-localization of α-actinin and phospho-tyrosine from focal adhesions to the cell interior. After 24h of genistein treatment, the cells acquired short protrusions where α-actinin and phospho-tyrosine co-localized. In the absence of serum, most α-actinin and phospho-tyrosine localized to stress fibers. The serum-starved cells showed membrane protrusions at 2h and 24h of genistein treatment. These results together suggest that serum affects the localization of α-actinin via the activation/inactivation of tyrosine kinases. In the presence of serum, and thus, when tyrosine kinases are activated, α-actinin associates to focal adhesion. In contrast, when serum factors are absent, there is a decrease in tyrosine kinase activity, which causes the release of α-actinin from the focal adhesions and its association with the actin stress fibers. This possibility is substantiated by the experiments using genistein. Therefore, the expression patterns and localization of actin and the cross-linking protein α-actinin, two proteins that co-localize to focal adhesions and stress fibers in adherent nonmuscle cells, are readily modulated by extracellular factors. These factors include insulin and extracellular matrix molecules, which modulate the level of expression of α-actinin and actin in nonmuscle cells (Gluck et al, 1992). It is possible that other factors present in serum, such as EGF and FGF modulate the tyrosine

phosphorylation of α-actinin, thus determining its association/dissociation from focal adhesion and its participation in the organization of actin stress fibers.

4.3.2 α-Actinin is involved in the actin cytoskeleton dynamics via cAMP/PKA pathway

We used forskolin and KT5720 to study whether the PKA pathway modulates the participation of α-actinin in the serum-induced remodelling of the actin cytoskeleton in TtT/GF cells. Western blot analyses performed in TtT/GF cells treated with forskolin either in the presence or in the absence of serum show that α-actinin was only associated to membrane and cytoskeleton fractions. Forskolin transiently increased the level of α-actinin in the membrane fraction in cells cultured in the presence of serum; while in serum-starved cells, the increase was sustained. In the presence of serum, there was a decreasing trend in α-actinin association with cytoskeleton fraction, while in serum-starved cells, forskolin increased the levels of α-actinin in cytoskeleton. In summary, forskolin increased the association of α-actinin to the membrane fraction in the presence of serum and to the membrane and cytoskeleton fractions in serum starving conditions. The results indicate that PKA activation increase the association of α-actinin to particulate fractions.

The immunofluorescence studies carried out in TtT/GF cells treated with forskolin either in the presence or in the absence of serum revealed that in cells cultured in the presence of serum, forskolin induced the formation of dendrite-like protrusions. Alpha-actinin localized mainly at the tips of the protrusions and also along F-actin. In serum free condition, more cell protrusions were induced by forskolin. Alpha-actinin localized

at the tips of the protrusions and also in the cytoplasm. Taken together, these results suggest that activation of PKA modulates the association of α-actinin to different cytoskeleton and membrane structures depending on the presence or absence of serum factors.

To further explore the role of cAMP/PKA pathway in the participation of α-actinin in the modulation of actin cytoskeleton dynamics in TtT/GF cells, we performed Western blot analyses in TtT/GF cells treated with the PKA inhibitor KT5720 either in the presence or in the absence of serum. In serum-treated cells, KT5720 decreased the association of α-actinin to the membrane fraction and then increased it. KT5720 decreased the levels of α-actinin associated with the cytoskeleton fraction. In the absence of serum, KT5720 caused a transient increase in the level of α-actinin in the membrane fraction and reduced the association of α-actinin to the cytoskeleton fraction after 4h treatment. If we compare the Western blot analyses from cells treated with forslolin and KT5720, we observe the opposite effects of these two compounds on the subcellular distribution of α-actinin in serum starving condition. However, in serum-cultured cells, these two drugs cause the same effects. Our results suggest that some factors in serum must modulate the response of the TtT/GF cells to forskolin or/and KT5720.

To evaluate whether the association of α-actinin to different subcellular fractions was correlated with the appearance of distinctive structures, we carried out immunofluorescence studies in the TtT/GF cells treated with KT5720 in the presence and in the absence of serum. In the presence of serum, KT5720 induced the appearance of cells with an elongated shape. Alpha-actinin localization to the focal adhesion

decreased after 0.5h incubation in the presence of KT5720 followed by an increase in the association of α-actinin with actin fibers. KT5720-treated serum-starved cells possessed the typical polygonal shape, but clamps of α-actinin and F-actin were formed at longer incubation times, indicating that KT5720 induced the disassembly of F-actin. However, although we observed the KT5720-induced disassembly of F-actin in serum free conditions, we did not find a decrease in the levels of α-actinin in the cytoskeleton fraction.

4.3.3 Conclusion

Collectively, our results show that α-actinin involvement in the modulation of TtT/GF cell morphology by serum factors particularly implicates tyrosine kinases. However, the precise molecular mechanisms by which the PKA-mediated pathway modulates the association of α-actinin with certain actin-based structures either in the presence or in the absence of serum is not clear and needs further studies.

4.4 General conclusion

Our investigations suggest that the dynamics of the actin cytoskeleton, which underlies the changes in the cell shape, the appearance of distinctive cell membrane structures and the capacity of locomotion are mediated at least in part by the actin-binding proteins cortactin and α-actinin in TtT/GF cells. The data also support the notion that the phosphorylation status of these proteins affected either by tyrosine kinases or the cAMP/PKA pathway regulates the interaction of cortactin and α-actinin with the membrane and/or the cytoskeleton resulting in the formation of different actin-dependent

structures and, in this way, causing the appearance of a characteristic phenotype. In addition, we found that the "behavior" of the actin-binding proteins, cortactin and α-actinin was different depending on the presence or the absence of serum factors. In spite of the work already done in the dynamics of the actin cytoskeleton, much remains to be achived to determine how the cell environment is linked to the modulation of the actin cytoskeleton dynamics in TtT/GF cells. In this way, our work should be completed by the investigation of the serum factors, such as EGF, FGF, PDGF that may be implicated in the impact of "serum" on the actin cytoskeleton dynamics in TtT/GF cells.

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