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Research Article

Effects of cytochalasin D on the actin cytoskeleton: association of neoformed actin aggregates with proteins involved in signaling and endocytosis

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Abstract. Cytochalasin D (CD) has been extensively used for assessing the role of the actin cytoskeleton in different biological processes. However, effects of CD have not always been consistent and CD-treated cells have been found to contain irregular spots of F-actin. By transfecting MCF-7 cells with an actin-enhanced yellow fluorescent protein fusion protein we show that, in vivo, CD induces actin aggregation de novo, while simultaneously depolymerizing preexisting actin cytoskeletal components. We also show that CD-induced actin aggregates bind the F-actin-selective drug phalloidin and associate with proteins involved in cell signaling as well as with receptors and endosomal markers (active MAP kinases, paxillin, erbB2, transferrin, Rab-5), but not with clathrin, protein kinase A, protein tyrosine phosphatase 1B, or tubulin. Thus, CD induces new sites of actin aggregation that selectively associate with several important regulatory proteins. Failure of CD to interupt a biological process may therefore not prove that the process is independent of actin aggregation.

Key words. Actin; cytochalasin D; MAP kinase; endosome; transferrin; erbB2; EYFP.

Drugs that interfere with actin polymerization are frequently used for probing the role of the actin cytoskeleton in various biological events [1, 2]. A recent survey revealed that the most widely used of these drugs, cytochalasin D (CD), has been employed in at least 2571 published studies and that its use is steeply increasing. Results obtained with F-actin-disrupting drugs have, however, often been contradictory, in particular with respect to the role of F-actin in endocytosis [3, 4 and references therein]. Moreover, CD treatment has been noted to result in the appearance of actin aggregates in several cell types [4–6].

We have confirmed that CD treatment results in the formation of multiple, irregular aggregates of actin that react with phalloidin, which represents a probe specific for F-actin [7]. We were interested to know whether such aggregates formed at preexisting sites of F-actin or formed de novo during CD treatment. This was studied in live cells transfected with a construct encoding an actin-enhanced yellow fluorescent protein (actin-EYFP) fusion protein. Such fusion proteins have previously been shown to become incorporated in the normal actin cytoskeleton of the cells [7]. Addition of CD to actin-EYFP-transfected cells resulted in gradual breakdown of the preexisting cytoskeleton with concomitant neo-formation of aggregates of actin-EYFP. Moreover, CD-induced actin aggregates were found to associate with molecules involved in signal transduction, receptors, and endosomal markers, but not with other cell proteins investigated. The occurrence of such associations makes difficult the conclusion that biological events which continue to occur in

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CD-treated cells are necessarily independent of actin aggregation.

Materials and methods

Cell culture

MCF-7 cells were cultured as described elsewhere [8] and human umbilical vein endothelial cells (HUVECs; ATCC) were cultured in F-12 K medium (Kaighn's modification; Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (FCS) and 0.4% endothelial cell growth supplement/heparin (ECGS/H-2; PromoCell, Heidelberg, Germany). Cells were treated with 1–10 µg/ml CD (Sigma, St. Louis, Mo.) for 25 min. Controls were treated with equivalent concentrations of vehicle (DMSO). Semiconfluent MCF-7 cells were starved in medium containing no FCS for 24–48 h. Subsequently, the cells were exposed to CD or DMSO for 25 min prior to addition of 15% FCS for an additional 10 min. Cells for Western blotting experiments were grown on plastic Petri dishes, while cells for immunocytochemistry were grown on sterile glass slides and fixed either in a 1:1 mixture of methanol and acetone at –20 °C or in 3.7% paraformaldehyde in phosphate-buffered saline (PBS). Formaldehyde-fixed cells were permeabilized in 1% triton X-100.

Transfections and live-cell observations

For transfections with clonfectin (Clontech, Palo Alto, Calif.), MCF-7 cells were grown on glass-bottomed culture dishes (WillCo Dish Wells, Amsterdam, The Netherlands). When reaching about 70% confluency, cells were transfected with 1.5 µg/ml of an actin-EYFP vector (actin-EYFP; Clontech) under the control of the human cytomegalovirus (CMV) immediate-early promoter using 1.5 µg/ml clonfectin for 8 h [9]. Twenty-four hours after transfection, cells were transferred to CO_2-independent medium without phenol red (GibcoBRL, Life Technologies) at 37 °C and observed in a Molecular Dynamics multiprobe confocal microscope equipped with an MC60 heating stage (Linkam Scientific Instruments, Tadworth, UK).

Immunocytochemistry

Fixed cells were stained with Alexa 488-, Alexa 594-, or coumarin phenyl isothiocyanate (CPITC-)labeled phalloidin (Molecular Probes, Sigma) alone or in combination with monoclonal antibodies recognizing active, dually phosphorylated (Thr202/Tyr204) ERK1/2 (Sigma), Rab5, paxillin (clone 349), clathrin heavy chain, protein kinase A, regulatory subunit I, protein tyrosine phosphatase 1B (PTP1B) (Becton Dickinson, San Diego, Calif.), transferrin receptor (Zymed, San Francisco, Calif.), erbB2 (Pharmingen and Neomarkers, Fremont, Calif.), and tyrosine-tubulin (Sigma). Additionally, affinity-purified polyclonal antibodies to active, dually phosphorylated ERK1/2 (Promega, Madison, Wis.) were used. Antibodies were used at 1–5 µg/ml for 1–2 h at room temperature followed by FITC-, Texas red-, or biotin-labeled antibodies specific for mouse IgG1, IgG2a, IgG2b, or IgG3 subclasses (Southern Biotechnology Associates, Birmingham, Ala.). Biotin-labeled secondary antibodies were detected by aminomethyl coumarin (AMCA-)labeled streptavidin (Vector Laboratories, Burlingame, Calif.). In addition, FITC-, Cy3-, Alexa 488-, or Alexa 594-labeled anti-mouse or anti-rabbit immunoglobulin antibodies were used (Jackson, Molecular Probes). Additionally, nuclei were visualized with bisbenzimide or oxidized paraphenylenediamine staining [10]. Specimens were examined in a Molecular Dynamics multiprobe confocal scanning microscope or in a Leica (Wetzlar, Germany) DMRXA microscope. Controls included use of Western blotting, conventional staining controls [11] and of type-matched monoclonal antibodies.

SDS-PAGE and immunoblotting

Cells were lysed in buffer B [50 mM Hepes pH 7.6, containing 100 mM NaCl, 10 mM EDTA, 1% triton X-100, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride and Complete tablet of protease inhibitors (Roche, Basel, Switzerland)]. After removal of aliquots for protein determination, samples were diluted with 4 × NuPage sample buffer, fortified with 10% NuPage sample reducing agent and heated to 85 °C for 2 min. Lysates and immunoprecipitates were separated on NuPage 10% BisTris gels (Novex, San Diego, Calif.) using NuPage MOPS SDS running buffer in an EI9001 X-cell II Minicell apparatus as recommended by the manufacturer (Novex).

Gels were calibrated with SeeBlue prestained standards (14–191 kDa in the MOPS buffer system; Novex). Electroblotting was performed in a semidry blotting (SDB) apparatus as recommended by the manufacturer (KemEnTec, Copenhagen, Denmark) using PVDF immunoblot membranes (0.2 µm; BioRad, Hercules, Calif.). Membranes were stained with monoclonal antibodies to active dually phosphorylated ERK1/2 (Cell Signaling Tech., Beverly, Mass.) at 1 µg/ml for 1 h and the Western Breeze chemiluminescence kit (Novex) was used in combination with Hyperfilm ECL (RPN2103K; Amersham, Piscataway, N. J.) for detection.

Results

CD treatment disrupts the actin cytoskeleton, but leaves aggregates of phalloidin-reactive actin

Staining with fluorescent phalloidin showed that MCF-7 cells possessed a well-developed actin cytoskeleton with numerous subcortical actin filaments and stress fibers

(fig. 1). Upon exposure to CD, all actin filaments were eliminated while multiple, irregular aggregates of phalloidin-reactive material appeared (fig. 1). Increasing doses of CD (up to 10 µg/ml) did not eliminate the actin aggregates (data not shown). CD treatment also induced aggregates of polymerized, phalloidin-reactive actin in HUVECs, while subcortical actin filaments and stress fibers disappeared (fig. 1).

Effects of CD on the actin cytoskeleton of actin-EYFP-transfected cells

We were interested in studying the mechanism by which the aggregates of actin appeared in CD-treated cells. Specifically, we wanted to know whether they appeared at specific preexisting sites of actin polymerization. We therefore transfected MCF-7 cells with a construct encoding actin-EYFP. Transfected cells were observed live in the confocal microscope before and after addition of 1 µg/ml CD or vehicle (DMSO). Control cells displayed a fluorescent actin cytoskeleton that dynamically remodeled. Upon addition of CD, the cells looked relatively unaffected for 10–15 min. Thereafter, the actin cytoskeleton disintegrated over the next 20 min (fig. 2). Stress fibers and actin filaments gradually disappeared. Concomitantly, aggregates of actin-EYFP formed at multiple sites in the cells. There was no association between the actin-EYFP aggregates and preexisting actin-EYFP structures (fig. 2).

Receptors, signaling molecules, and the endosomal marker Rab 5 accumulate at CD-induced actin aggregates

Since previous studies have indicated that CD-sensitive mechanisms are involved in activation and/or transport of

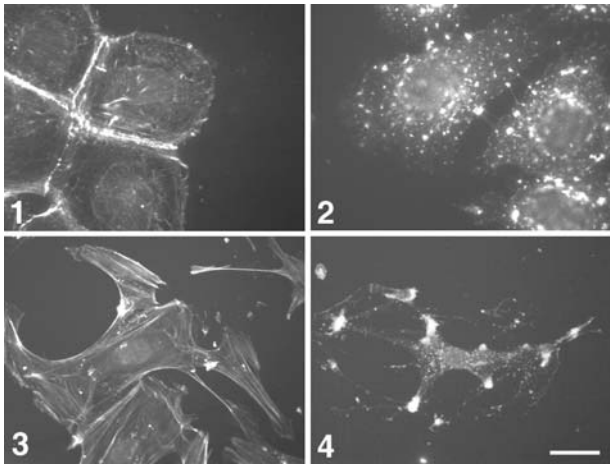

Figure 1. MCF-7 (1, 2) and HUVEC cells (3, 4) pretreated with vehicle (1, 3) or with CD (1 µg/ml) 25 min prior to fixation and staining with fluorescent phalloidin. Note that CD treament results in the appearance of numerous phalloidin-positive aggregates in both cell types. Scale bar, 15 µm.

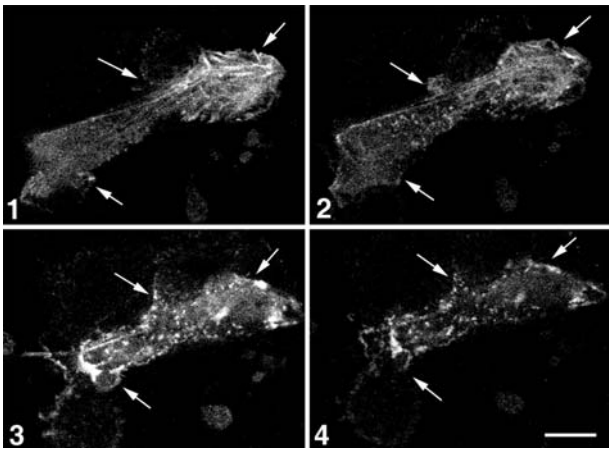

Figure 2. MCF-7 cell transfected with an actin-EYFP-encoding construct. Images were captured at t = 0 (1), t = 15 (2), t = 20 (3), and t = 35 (4) min after the addition of 1 µg/ml CD. Note that irregular aggregates of actin-EYFP start to form haphazardly in the cell after 15 min of exposure to CD, while the preexisting actin filaments disappear. Also note that the aggregates form at sites where preexisting actin filaments are absent or scarce (arrows inserted for orientation). Scale bar, 15 µm.

MAP kinases (MAPKs) [12–15], we probed MCF-7 cells with antibodies recognizing active, dually phosphorylated forms of the MAPKs, ERK (extracellular-signal-regulated kinases) 1 and 2. Serum-starved cells showed only weak staining for active ERKs. However, 10 min of serum stimulation of such cells induced strong staining for active ERKs in the nucleus and perinuclear area (fig. 3). In addition, some serum-stimulated cells showed staining for active ERKs at the cell membrane and over membrane ruffles. In CD-treated cells, active ERKs accumulated around many of the phalloidin-positive aggregates (fig. 3). Immunoblotting studies showed that CD treatment did not impair activation of ERKs in MCF-7 cells (fig. 4).

Since erbB2 as well as focal adhesions have been implicated in MAP kinase activation [13, 14, 16], we next studied if CD affected the distribution of erbB2 or of the focal adhesion protein, paxillin. In control cells, staining for erbB2 occurred at the peripheral cell membrane, while staining for paxillin occurred at focal adhesions together with weaker staining in the perinuclear region. In CD-treated cells, staining for erbB2 became associated with the actin aggregates (fig. 3), while staining for paxillin was more variable. Thus, paxillin no longer marked recognizable focal adhesions but was either associated with actin aggregates or occurred diffusely distributed in the cytoplasm (fig. 5).

These data show that the actin aggregates associate both with MAPK signaling molecules and with receptors involved in MAPK activation. A more variable association with paxillin was noted. Receptor internalization has been shown to be connected to MAPK activation [17, 18]

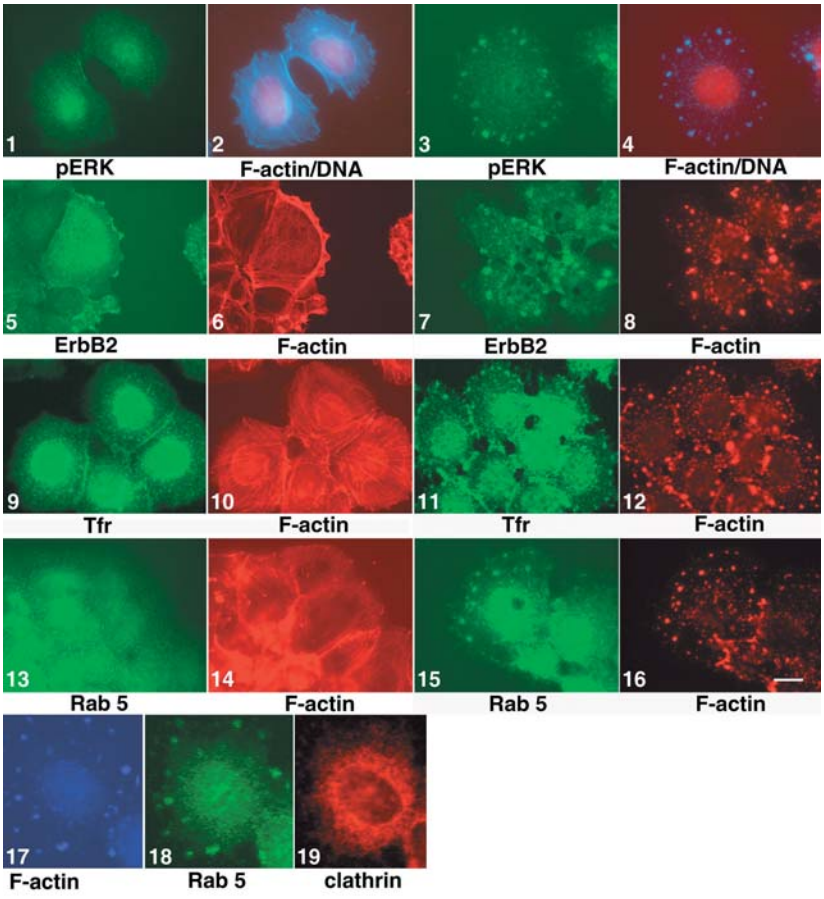

Figure 3. Serum-starved MCF-7 cells pretreated with vehicle (DMSO) (1, 2, 5, 6, 9, 10, 13, 14) or CD (3, 4, 7, 8, 11, 12, 15, 16, 17–19) and then stimulated with serum for 10 min. Cells were stained for active ERK1/2 (pERK), F-actin and DNA (1–4), erbB2 and F-actin (5–8), transferrin receptor (Tfr) and F-actin (9–12) and Rab 5 and F-actin (13–16), and for F-actin, Rab 5, and clathrin (17–19). F-actin was detected with blue-fluorescent CPITC-phalloidin (2, 4, 17) or with red-fluorescent Alexa 594-phalloidin (6, 8, 10, 12, 14, 16), while DNA was revealed with oxidized paraphenylenediamine, producing red fluorescence (2, 4). Note that with the exception of clathrin (19), CD treatment leads to colocalization of the other markers with the F-actin-reactive aggregates. Scale bar, 10 μm.

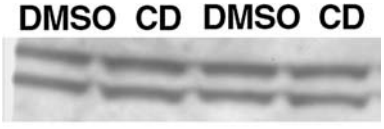

Figure 4. Western blot of serum-starved MCF-7 cells pretreated with vehicle (DMSO) or CD and then stimulated with FCS for 10 min. The blot was immunostained with anti-active MAP kinase antibody. Note that CD treatment was without effect on the intensity of the ERK1 (p44) and ERK2 (p42) bands.

and we therefore examined the association between actin aggregates and molecules connected to endocytosis and endosomal structures. In control cells, staining for clathrin, Rab 5, and the transferrin receptor occurred both at the cell membrane and in the perinuclear region (fig. 3). In CD-treated cells, there was a partial redistribution of Rab 5, and transferrin receptors to the actin aggregates, while no such redistribution was detected for clathrin (fig. 3). Similarly, staining for tubulin, protein kinase A and PTB1B also failed to show any association with actin aggregates in CD-treated cells (figs 3, 5), further underlining that these aggregates show selectivity in their affinity for cellular proteins.

Discussion

Previous studies have documented that CD treatment does not eliminate all polymerized actin, but that irregular phalloidin-reactive aggregates remain [4–6]. However, the importance of this phenomenon for the use of CD as an experimental drug perturbing the actin cytoskeleton has not been addressed. Our studies now show that such actin aggregates associate with a number of proteins involved in signal transduction and endocytosis. Moreover, we showed that this association is not haphazard, since certain proteins like clathrin, tubulin, protein kinase A, and PTB1B do not associate with the aggre-

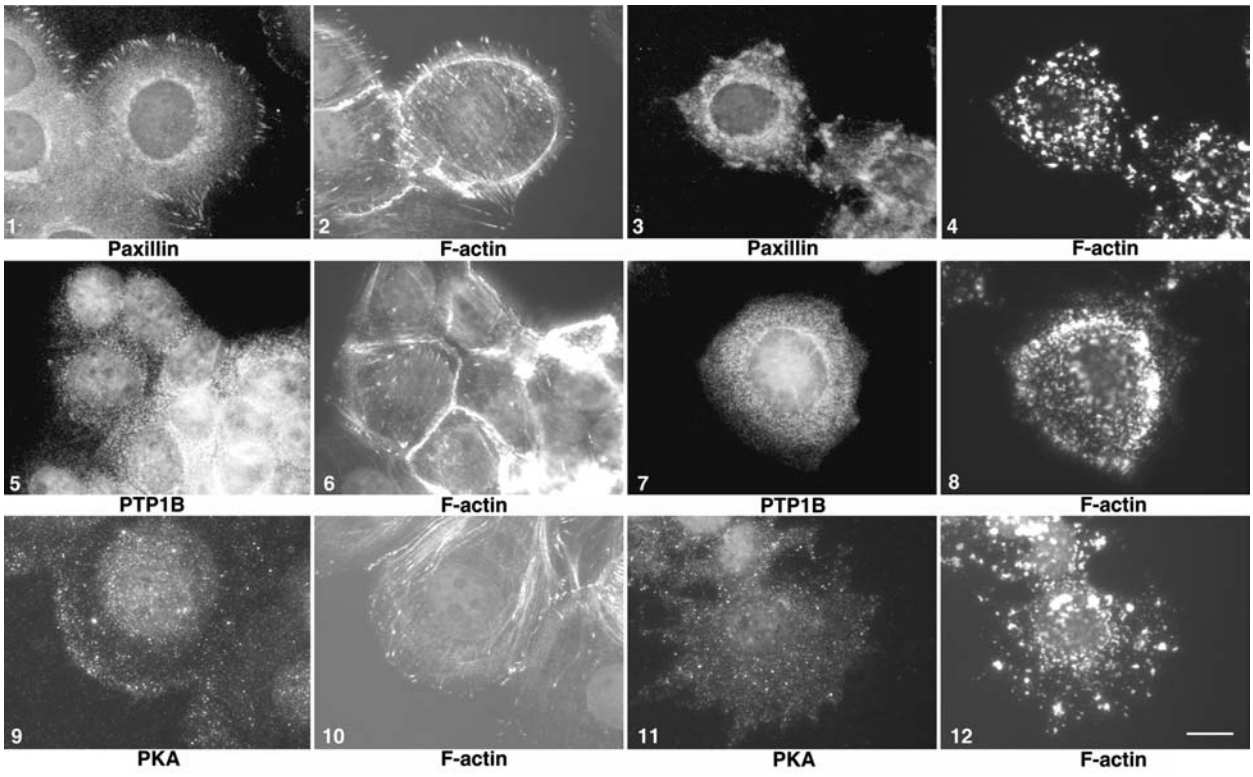

Figure 5. Serum-starved MCF-7 cells pretreated with vehicle (DMSO) (1, 2, 5, 6, 9, 10) or cytochalasin D (3, 4, 7, 8, 11, 12) and then stimulated with serum for 10 min. Cells were double-stained for paxillin and F-actin (1–4), PTP1B and F-actin (5–8), or protein kinase A, regulatory subunit I (PKA) and F-actin (9–12). Note that paxillin is a good marker for focal adhesions in control cells (1) but is eliminated from these sites by CD treatment (3). Also note that CD treatment leads to colocalization of paxillin and actin aggregates in some cells (cf text), but that PTP1B or PKA never colocalize with the phalloidin-positive aggregates. Scale bar, 15 µm.

gates. Finally, using living cells, we documented that the aggregates form de novo. Consequently, certain cellular components must be trapped by the neo-formed actin aggregates. Interestingly, recent data have shown that endocytotic vesicles move using F-actin tails [19, 20] and that endocytosis and endosomal structures are associated with MAP kinase activation [17, 18]. Interestingly, all proteins that associate with CD-induced actin aggregates also localize to endosomal structures and/or are involved in MAPK activation. One might therefore speculate that endocytotic vesicles become entrapped by de-novo-formed actin aggregates in CD-treated cells. Most important, however, our data urge considerable caution in interpreting effects of CD on cells. Specifically, concluding that activities that are not inhibited by CD are independent of the formation of phalloidin-reactive aggregates may not be possible.

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1 Sampath P. and Pollard T. D. (1991) Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. Biochemistry **30:** 1973–1980
2 Spector I., Braet F., Shochet N. R. and Bubb M. R. (1999) New anti-actin drugs in the study of the organization of the actin cytoskeleton. Microsc. Res. Techn. **47:** 18–37
3 Qualmann B., Kessels M. M. and Kelly R. B. (2000) Molecular links between endocytosis and the actin cytoskeleton. J. Cell Biol. **150:** F111–F116
4 Fujimoto L. M., Roth R., Heuser J. E. and Schmid S. L. (2000) Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. Traffic **1:** 161–171
5 Brett J. G. and Godman G. C. (1986) Cytoskeletal organization affects cellular responses to cytochalasins: comparison of a normal line and its transformant. Tissue Cell **18:** 175–199
6 Schliwa M. (1982) Action of cytochalasin D on cytoskeletal networks. J. Cell Biol. **92:** 79–91
7 Small J.-V., Rottner K., Hahne P. and Anderson K. I. (1999) Visualising the actin cytoskeleton. Microsc. Res. Techn. **47:** 3–17
8 Mortensen K., Skouv J., Hougaard D. M. and Larsson L.-I. (1999) Endogenous endothelial cell nitric oxide synthase modulates apoptosis in cultured breast cancer cells and is transcriptionally regulated by p53. J. Biol. Chem. **274:** 37679–37684
9 Mortensen K. and Larsson L.-I. (2001) Quantitative and qualitative immunofluorescence studies of neoplastic cells transfected with a construct encoding p53-EGFP. J. Histochem. Cytochem. **49:** 1363–1367

10 Oriol R. and Mancilla-Jiminez R. (1983) Fluorescent staining of nuclei and amyloid substance: two useful properties of p-phenylenediamine. J. Immunol. Methods **62:** 185–192
11 Larsson L.-I. (1988) Immunocytochemistry, Theory and Practice, CRC, Boca Raton, Fla
12 Cazaubon S., Chaverot N., Romero I. A., Girault J.-A., Adamson P., Strosberg A. D. et al. (1997) Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and -independent pathways. J. Neurosci. **17:** 6203–6212
13 Della Rocca G. J., Maudsley S., Daaka Y., Lefkowitz R. J. and Luttrell L. M. (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade: role of focal adhesions and receptor tyrosine kinases. J. Biol. Chem. **274:** 13978–13984
14 Aplin A. E., Stewart S. A., Assoian R. K. and Juliano R. L. (2001) Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J. Cell Biol. **153:** 273–281
15 Arnautov A. M. and Nikolskii N. N. (1998) Transport into the cell nucleus of p42 and p44 isoforms of MAP kinase induced by epidermal growth factor requires the presence of intact active cytoskeleton. Tsitologiia **40:** 639–647
16 Alroy I. and Yarden Y. (1997) The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. FEBS Lett. **410:** 83–86
17 DeFea K. A., Zalevsky J., Thoma M. S., Déry O., Mullins R. D. and Bunnett N. W. (2000) β-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. J. Cell Biol. **148:** 1267–1281
18 Pierce K. L., Maudsley S., Daaka Y., Luttrell L. M. and Lefkowitz R. J. (2000) Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and non-sequestering G protein-coupled receptors. Proc. Natl. Acad. Sci. USA **97:** 1489–1494
19 Merrifield C. J., Moss S. E., Ballestrem C., Imhof B. A., Giese G., Wunderlich I. et al. (1999) Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat. Cell Biol. **1:** 72–74
20 Taunton J., Rowning B. A., Coughlin M. L., Wu M., Moon R. T., Mitchison T. J. et al. (2000) Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. **148:** 519–530