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C3-Induced 3LL Cell Proliferation Is Mediated by C Kinase

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Abstract It has been demonstrated that the third component of complement (C3)[1] and its peptides increase normal and tumour cell proliferation. However, the signal cascade responsible for this phenomenon is still unknown. In this study, we elucidate some of the mechanisms involved in the signalling of C3 stimulation of cell proliferation. We have first investigated the in and out traffic of C3 peptides, then we have identified the subcellular localisation of internalised C3 and, finally, we have explored the role of protein phosphorylation in C3 traffic and in the proliferation of the Lewis lung carcinoma (3LL) cells. Our results indicate that traffic of C3 is not dependent on cytoskeletal integrity and requires protein kinase C-dependent phosphorylation. In addition, proliferation of 3LL cells stimulated by C3 depends on both C3 internalisation and protein-kinase C phosphorylation. J. Cell. Biochem. 94: 635–644, 2005. © 2004 Wiley-Liss, Inc.

Key words: C3; cell proliferation; protein kinase C; internalisation

It has been shown that C3, different fragments of C3, and synthetic peptides of the C3d region of C3 may increase the proliferation rate of normal and neoplastic B cells through a mechanism in which complement receptor CR2 seems to be involved [Hatzfeld et al., 1988]. Previous studies from our and other groups have shown that C3 binds to acceptor sites in Lewis lung carcinoma (3LL) cells without lysing the cells but promoting their proliferation [Kuraya et al., 1990; Luxemburg and Cooper, 1994; Di Renzo et al., 1999]. In a previous study [Di Renzo et al., 1999], we have shown that the internalisation and proliferation effects observed in 3LL cells incubated in normal murine serum (NMS) depend on the presence of the third component of complement (C3). In fact, when anti-C3 antibodies were added to the NMS medium, the proliferation of 3LL cells was abrogated. We have also suggested that 3LL cells do not synthesise C3 and appear to possess a mechanism for clearing potentially harmful C3 molecules from their surface by translocating them into the cell and using them as a growth signal [Morgante et al., 1997; Di Renzo et al., 1999].

However, a number of major questions remain to be addressed. First, what is the mechanism by which C3 is translocated into the cytoplasm from the cell surface? Second what, if any, is the role of the cytoskeleton in this internalisation? Finally, what is the pathway that mediates the C3-dependent proliferation of 3LL cells?

In this article, we provide evidence that: (a) internalisation of C3 does not dependent on actin-based and tubulin-based contractile systems; (b) protein kinase C-dependent phosphorylation is required in the translocation of C3

Abbreviations used: C3[1], third component of complement; NMS, normal murine serum; 3LL, Lewis lung carcinoma; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PBS, phosphate buffered saline; PKA, protein kinase A; PKC, protein kinase C.

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into the cytoplasm and in the increase in cell proliferation; (c) protein kinase A activity is not involved in the C3 traffic and appears irrelevant for cell proliferation; (d) internalised C3 becomes compartmentalised mainly into the cytosol with a minor amount entering the nucleoplasm, and does not appear to be sequestered in small or large vesicles.

We suggest that C3 activates a proliferation signal through a protein kinase C dependent pathway.

MATERIALS AND METHODS

Media and Reagents

Pooled normal mouse serum (NMS), obtained from healthy C57Bl/6 mice, aliquoted in small volumes and stored at −70°C, was used as a source of complement.

12-*O*-tetradecanoylphorbol 13-acetate (TPA, 20 nM), cytochalasin D (5 μM), NH_4Cl (10 mM), chloroquine (10 mM), monensin (10 μM), and trypsin were obtained from Sigma-Aldrich S.r.l., Milano, Italy, staurosporine (50 nM), genistein (70 μM), bisindolylmaleimide (5 μM), forskolin (50 μM), okadaic acid (1 μM), and A3 (20 nM) were obtained from ICN Biomedical, S.r.l, Milano, Italy.

NMS was treated, when indicated, with: (a) methylamine hydrochloride (100 mM) (Sigma-Aldrich) at 37°C for 30 min, followed by dialysis against phosphate buffered saline (PBS) at 4°C (MA-NMS) to avoid covalent binding of C3; or (b) with zymosan (Sigma) (2 mg/ml) for 45 min at 37°C (Z-NMS) in order to allow receptor binding of C3-fragments.

In order to quantify internalised C3, surface bound C3 was removed by incubation with 0.25% trypsin for 10 min at 37°C. This procedure removes all surface bound ligand [Owensby et al., 1989] as determined by cytofluorimetric analysis.

Cell Cultures

3LL cells were obtained from Lewis lung carcinoma as previously described [Lipari et al., 1988]. Cells were cultured at 37°C, 5% CO_2, in RPMI-1640 medium, heat-inactivated foetal calf serum (FCS, 10%, v/v) and L-glutamine 2 mM (Gibco, Grand Island, NY). Subconfluent cells were washed and cultured for 24 h in serum-free AIM-V medium (Gibco) before experiments. When indicated, reagents listed in Table I were added to the cultures. Each agent was used at the optimal concentration as established by preliminary dose–response experiments. At the end of each treatment, cell viability was checked by trypan blue exclusion test; acceptable viability was considered above 90%.

Cytofluorimetric Analysis

Cells (5×10^5) were washed, resuspended in PBS buffer, and incubated for 30 min on ice in the presence of FITC-labelled goat anti mouse C3 antibody (Cappel, MP Biomedicals, Orangeburg, NY) diluted 1:20 in PBS.

Samples resuspended in 200 μl of cold FACS fixing solution were analysed in a cytofluorimeter (Epics XL MCL Coulter Electronics, Hialeah, FL).

ELISA for C3 Detection

ELISA was used to determine the concentration of C3, in both cell-free supernatants and cell lysates (1×10^6 cells/ml). Nunc immunomicrowells Maxi-Sorp (Nunc, Cophenaghen, Denmark) were coated with polyclonal goat anti mouse C3 (Cappel). After blocking with BSA, samples (100 μl) or different dilutions of purified

TABLE I. Reagents Used to Activate or Inhibit PKC and PKA

Reagents (final concentration)	Function
TPA phorbol 12-myristate-13-acetate (20 nM)	Activates protein kinase C in vivo and in vitro, activates Ca^{2+}-ATPase
Staurosporine (50 nM)	A potent inhibitor of protein kinases, inhibits CaM kinase protein kinase A, protein kinase C
Genistein (70 μM)	Inhibits protein kinases by acting as competitive inhibitor of ATP
Bisindolylmameimide (5 μM)	A highly selective cell-permeable protein kinase C inhibitor, acts as competitive inhibitor for the ATP binding site of PKC
Forskolin (50 μM)	Activator of protein kinase A
Okadaic acid (1 μM)	Potent inhibitor of protein phosphatase 1 and 2A
A3 [*N*-(2 aminoethyl)-5-chloronaphthalene-1-sulfonamide hydrochloride] (20 nM)	Inhibitor of protein kinase A

C3 (concentrations ranging from 1 mg/ml to 3.9 ng/ml) were added and incubated at room temperature for 1 h. After washing with PBS containing 0.05% (v/v) Tween 20, the plates were subsequently kept for 45 min at room temperature with 100 μl horseradish peroxidase-conjugated rabbit anti-mouse C3 (Cappel) diluted 1:1,000 in PBS and with 100 μl of *o*-phenylendiamine (Sigma-Aldrich) (0.4 mg/ml in citrate buffer pH 5.0, containing 4 μg/ml of 3% [v/v or w/v] hydrogen peroxide).

After 30 min, the reaction was terminated by the addition of 5 μl of 4N H_2S04 and absorbance at 492 nm measured with a Titertek Multiskan 4SLT (Labinstrument, Town, Austria).

SDS and Immunoblotting Analysis

Supernatants and cell lysates (1×10^6 cells) were obtained from 3LL cultures at different times. Protein content was determined by Bradford assay using bovine serum albumin (BSA) as a standard (Bio-Rad Lab. Richmond, CA). Equal amounts of protein were separated in 6% (cell lysate) and 7.5% (cell supernatant) SDS−PAGE in non reducing conditions by a Mini-Protean II Dual Slab Cell (Bio-Rad Lab.) and blotted onto nitrocellulose sheets. The blots were then saturated with PBS containing skimmed milk (5%, w/v) and Triton X-100 (0.1%) and incubated with peroxidase-conjugated polyclonal goat anti-mouse C3 antibody (Cappel) (diluted 1:1,000). Bound antibodies were visualized using an enhanced chemiluminescent (ECL) detection system (Amersham Int. PLC, Amersham, Bucks., UK).

Proliferation Assay

5×10^4/100 μl 3LL cells were seeded in triplicate into 96-well flat microtitre plates and cultured for 24 h at 37°C, 5% CO_2. Microcultures were pulsed for the last 16 h with 1 μCi/well of [^{3}H]-thymidine (specific activity 126 Ci/mmol) (Amersham). The different treatments did not affect cell viability, as detected by trypan blue exclusion at the end of the [^{3}H]-thymidine pulse period. Cells were harvested onto glass fibre filters and radioactivity was measured in a liquid scintillation counter (Wallac, Milton Keynes, Loughborough, UK).

Laser Scanning Microscopy

Cells (1×10^5), grown on coverslips, were fixed in acetone-methanol (1:1) at −20°C for 10 min. After washing in PBS (3×10 min), cells were incubated for 1 h at room temperature (RT) with an affinity-purified polyclonal goat anti-mouse C3 (200 μg/ml in PBS) (Cappel). After washing in PBS (3×10 min), the bound antibodies were visualised with rabbit anti-goat IgG FITC (400 μg/ml in PBS) (Cappel), 45 min at RT. After washing in PBS (3×10 min), cells were incubated with propidium iodide (10 μg/ml) at RT for 20 min. Following PBS washing (3×10 min) the coverslips were mounted with glycerol (600 μl) + Tris-HCl 0.1M pH 9.2 (400 μl). The samples were analysed by a Zeiss Laser Scan Microscope LSM 510 (Zeiss, Oberkochen, Germany).

Electron Microscopy: Cryoultramicrotomy and Immunolabeling

Cells were embedded as pellets in PBS 0.1M, pH 7.4, containing gelatin (3% w/v) to obtain small blocks (<1 mm^3) that were suspended in 2.3M sucrose and frozen in liquid nitrogen. Ultrathin cryosections were cut on a Reichert cryoultramicrotome, according to Griffits et al. [1984] and Slot et al. [1988]. Immunogoldlabeling was performed by incubating frozen sections for 60 min at room temperature with polyclonal goat IgG fraction to mouse C3 (from Cappel) and subsequently with rabbit antibodies raised against goat immunoglobulin. These antibodies were conjugated with 10 nm gold particles (Biocell Co., Town, UK). Two controls were used: the first antibody was either omitted or replaced by goat IgG to mouse IgG (Sigma). Sections were protected and stained by embedding in 2% methyl cellulose containing 0.2% uranyl acetate and finally studied with a Philips CM-10 electron microscope.

Statistical Evaluation

Mean (M) and standard deviation (SD) were calculated after samples were shown to be homogeneous by calculation of variance coefficients. Significance of difference was determined by Student's *t*-paired test.

RESULTS

Internalisation and Subsequent Recycling of C3

3LL cells were incubated in the presence of NMS for up to 60 min to allow C3 internalisation. After each incubation time any external ligand was eliminated as described in "Materials and Methods." Figure 1A shows that

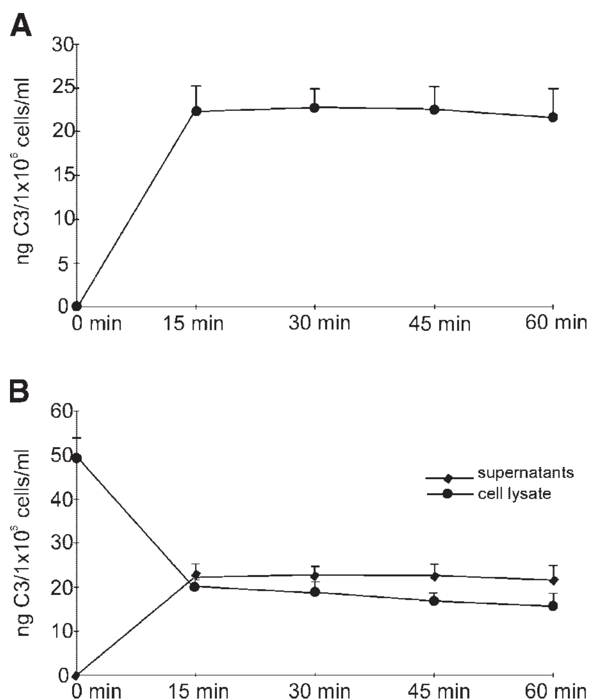

Fig. 1. **A**: Internalisation of C3. Lewis lung carcinoma (3LL) cells were incubated with normal murine serum (NMS) at 37°C for 60 min. Duplicate aliquots taken over time and after washing total bound ligand and internalised ligand were determined by the ELISA technique. A standard curve was obtained using different dilutions of purified C3. This curve was then used to measure the amount of C3 in the samples. Data represent mean ± SD from four experiments. **B**: Release of internalised C3. 3LL cells were allowed to internalise C3 for 15 min at 37°C, then treated with trypsin. Cells were then re-incubated at 37°C in RPMI and sampled at different times to assay for residual C3. The presence of C3 in the supernatants (◇) and in the cell lysates (●) was determined by ELISA as reported in 1A.

the internalisation process was time-dependent and reached a plateau at 15 min.

To study the fate of internalised C3, 3LL cells, previously treated with NMS for 15 min, were incubated again in the same medium, in the absence of any external ligand. At intervals of 15 min C3 was determined in cell lysates and supernatants. Figure 1B shows that the amount of intracellular C3 decreased with time as part of it was released in the supernatant.

Immunoblotting analysis of released C3 (Fig. 2A) shows bands of approximately 190 and 140 kDa as seen with NMS alone. The 190 and 140 kDa bands may represent C3/C3b and C3c, respectively. This result suggests that previously internalised and then released C3, was not degraded in lysosomal compartments. Figure 2B shows immunoblotting analysis, under non reducing conditions, of internalised C3 in 3LL cells. Several bands higher than 190 kDa are present in the extracts. As previously reported form our group [Di Renzo et al., 1999] these bands represent C3 and/or its fragments covalently bound to 3LL cell acceptor sites. Chloroquine, which interrupts recycling of receptors, did not alter C3 release (data not shown).

Effect of Various Agents on C3 Internalisation

The integrity of cytoskeleton is required for vesicle-mediated traffic of proteins; in particular, the actin-based cytoskeleton and microtubular cytoplasmic meshwork appear to be necessary for endocytosis as well as for the vesicle-mediated secretory pathway or exocytosis [Kelly, 1985; Allen, 1995; Qualman et al., 2000]. For this reason, we have examined whether one or both of these two systems were involved in the internalisation of C3 and its subsequent release in the culture medium. We used cytochalasin D to disrupt actin filaments and colchicine to disorganise microtubules.

Table IIA (column 2) shows that treatment of 3LL cells with either colchicine or cytochalasin D did not affect C3 internalisation.

C3 internalisation could be also Na^+ or proton transport-coupled. To test, at least in part, this hypothesis, we have used monensin that exchanges Na^+ for protons at plasma membrane level. The excess of cytosolic Na^+ induces an osmotic enlargement of the vesicular compartment, while the normally acidic pH of some vacuoles rises. In this case also the amount of internalised C3 was not affected (Table IIA). A similar result was obtained in the presence of NH_4Cl, which also alters the pH of various subcellular compartments (Table IIA).

Similarly, C3 release, which could occur either by exocytosis or by a facilitated Na^+ or proton-dependent transport mechanism [Kelly, 1985], was not affected by the presence of both cytochalasin D or colchicine and was also unaffected by treatment with monensin or NH_4Cl (Table IIB).

Role for Protein Kinases in C3 Internalisation Within 3LL Cells

In a previous study we have shown that, incubation of 3LL cells with C3 molecules from NMS, results in internalisation of C3 with a subsequent enhancement of cell growth [Di Renzo et al., 1999].

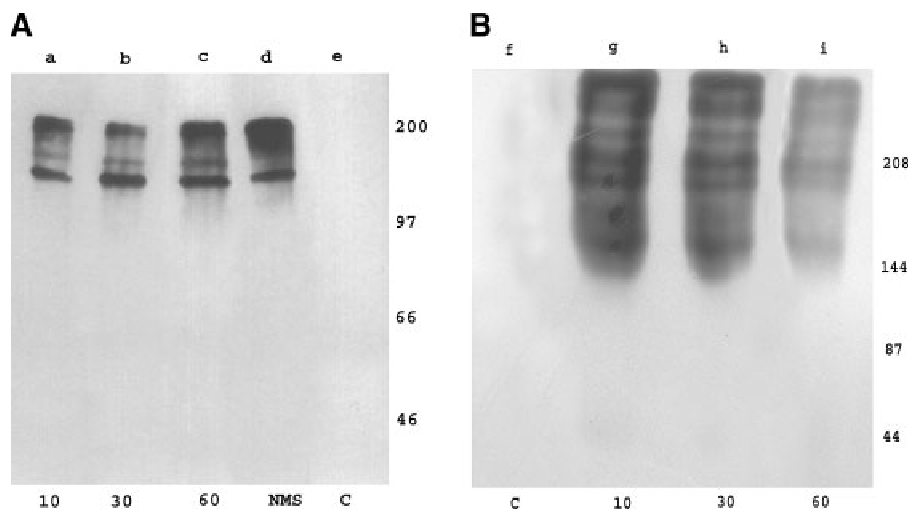

Fig. 2. **A**: Immunoblotting analysis of C3 released in supernatants. 3LL cells were allowed to internalise C3 for 15 min at 37°C, then treated with trypsin. Cells were then re-incubated at 37°C in RPMI and sampled at different times to assay for residual C3. C3 was recovered from 3LL cell supernatants after 10 min (**lane a**), 30′ (**lane b**), and 60′ (**lane c**) and analysed on a 7.5% SDS–PAGE. **Lane d**: NMS without cells. **Lane e**: Supernatant from control cells cultured in the absence of NMS. **B**: Immunoblotting analysis of internalised C3. C3 was recovered from cell lysates of 3LL cells after 10 min (**lane g**), 30′ (**lane h**), and 60′ (**lane i**) and analysed on a 6% SDS–PAGE. **Lane f**: Lysate of 3LL cells cultured in the absence of NMS.

Phosphorylation of a number of proteins is required for peptide translocation across membranes and compartments. Therefore, to study the role of protein phosphorylation on C3 internalisation several reagents were used. Table I summarizes such reagents. They can be divided in four groups: (1) activators of protein kinases C and A (TPA and forskolin, respectively); (2) selective inhibitors of protein kinases C and A (bisindolylmaleimide and A3, respectively); (3) non-selective inhibitors (staurosporine, genistein); and (4) a phosphatases inhibitor (okadaic acid). Confocal microscopy and fluorescent anti-C3 antibodies was used to follow C3 traffic.

Table III and Figure 3C show that activation of PKC with TPA did not alter C3 internalisation. In fact, the C3 fluorescence was similar to that of the cells treated with NMS alone (Table III and Fig. 3B). Importantly, in the presence of bysindolylmaleimide and staurosporine, two well known protein kinase C inhibitors, the internalisation of C3 was completely prevented (Table III and Fig. 3D,E).

On the other hand, both an activator (forskolin) and an inhibitor (A3) of PKA did not change the C3 traffic (data not shown).

In addition, okadaic acid, which increases the level of protein phosphorylation by inhibiting phosphatases, did not interfere with the process (Table III and Fig. 3F).

Finally, binding of C3b to the plasma membrane surface was not significantly altered by any of the above agents, suggesting that protein kinases may act on the internalisation process rather than on a preliminary phase of binding, as also shown by cytofluorimetric analysis (data not shown).

TABLE II. Treatment of 3LL Cells

	A: Effect of different agents on C3 internalisation		B: Release of internalised C3	
Treatment	C3 (bound on 3LL) Cell ng/1 × 10^6	C3 (internalised)	ng C3 residual	ng C3 released
/	230 ± 12	46 ± 7	26 ± 2.4	27 ± 2.2
Cytochalasin D 5 μM	300 ± 15	45.5 ± 8	24 ± 2.7	23 ± 2.5
Colchicine 10 μM	260 ± 16	46.6 ± 4	22 ± 2.9	28 ± 3.5
NH_4Cl 10 mM	260 ± 14	41.8 ± 5	25 ± 2.3	22 ± 2.5
Monensin 10 μM	240 ± 9	40.0 ± 5	20 ± 2.5	22 ± 2.8

TABLE III. C3 Internalisation as Seen by Confocal Microscope

Treatment	Fluorescence
3LL cells − no NMS	−
3LL cells + NMS	+++
3LL cells + NMS + TPA	+++
3LL cells + NMS + bisindolylmaleimide	−
3LL cells + NMS + staurosporine	−
3LL cells + NMS + okadaic acid	++

−, negative; +, low positive fluorescence; ++, moderate positive fluorescence; +++, intense positive fluorescence.

Cell Proliferation in Response to Protein Kinase C Inhibitors

The potential involvement of protein kinases in the proliferation response of 3LL cells after incubation with NMS was investigated by treating the cells with activators or inhibitors of protein kinases. When 3LL cells were treated with TPA (20 nM) in the presence of C3 a significant stimulation of cell proliferation (1.5-fold) was observed as compared to cells treated with C3 alone (Fig. 4). Importantly, TPA alone did not effect the proliferation of 3LL cells (data not shown). PKC inhibitors prevented this increase in proliferation. When cells were treated with staurosporine, bisindolylmaleimide or genistein, the proliferation reached values similar to those of the control untreated cells (Fig. 4). None of these inhibitors affected the basal proliferation of the cells (Fig. 4). In contrast, selective activation of PKA with forskolin or inhibition with A3 had no effect on C3-induced proliferation of 3LL cells, suggesting that PKA may be not involved in the proliferation (Fig. 4).

To determine whether phosphatases modulated 3LL cells proliferation okadaic acid was used. Figure 4 shows that, in the presence of

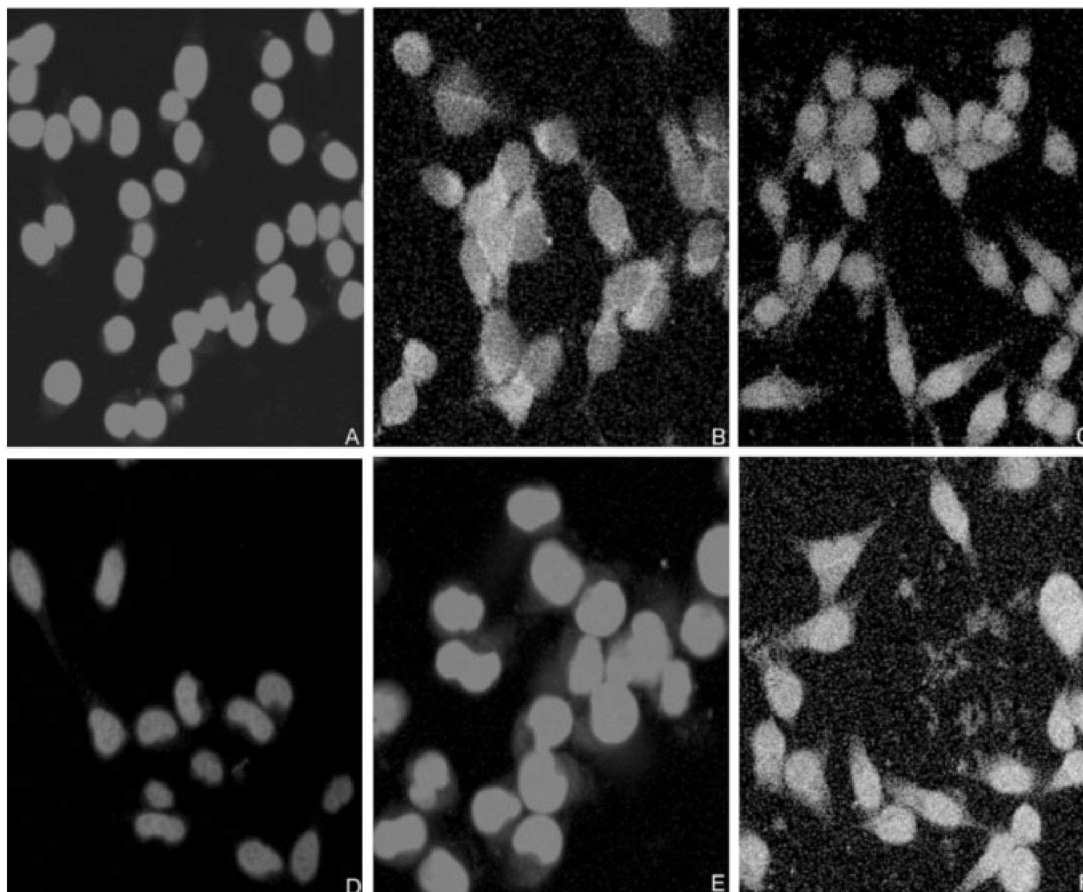

Fig. 3. Laser scanning confocal microscopy images of 3LL cells treated with FITC-labelled antibodies against C3. 3LL cells were incubated as described in Figure 1A in the presence or absence of different agents. The green fluorescence indicating the presence of C3 increases or disappears after treatment with different agents. **A**: 3LL cells − no NMS. **B**: 3LL cells + NMS. **C**: 3LL cells + NMS + TPA. **D**: 3LL cells + NMS + bisindolylmaleimide. **E**: 3LL cells + NMS + staurosporine. **F**: 3LL cells + NMS + okadaic acid. Propidium iodide was used to evidence cell nuclei (red staining).

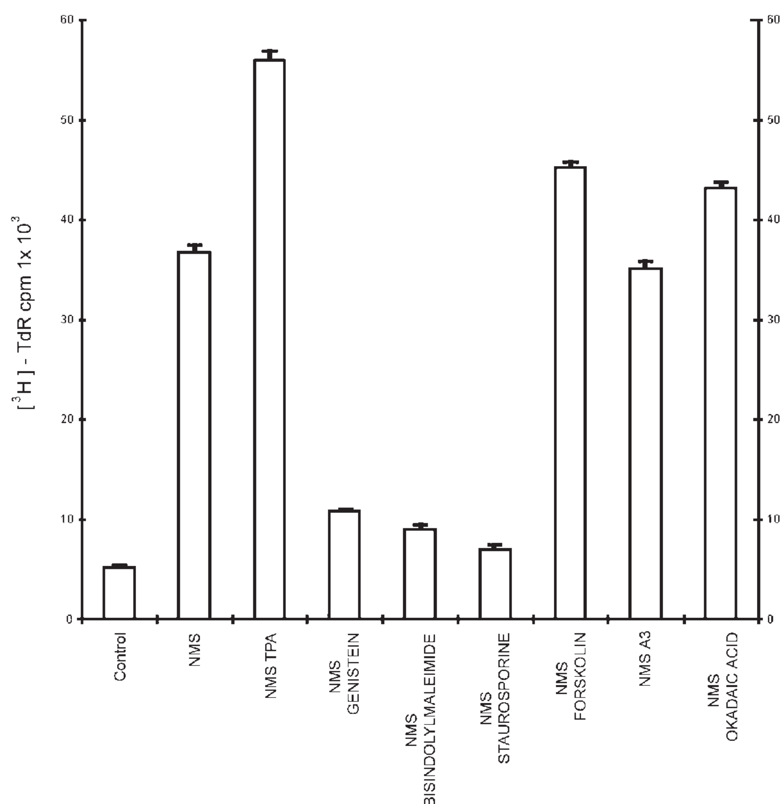

Fig. 4. C3 stimulated proliferation of 3LL cells is mediated by the activity of PKC, but not PKA. 3LL cells were incubated with medium alone and treated with NMS for 15′ in the presence or absence of several agents. Cell proliferation was measured as reported in ''Materials and Methods.''

okadaic acid, the extent of cell proliferation was not altered.

All results obtained with $^{[3H]}$-Tdr incorporation analysis were confirmed by direct counting techniques and by the trypan blue exclusion test.

Subcellular Localisation of C3

Details of C3 subcellular distribution and traffic have been obtained by immunoelectron microscopic techniques. As previously shown [Di Renzo et al., 1999] surface particles were not clustered as in classical coated pits, but they were adherent to and scattered along the plasma membrane. When studying the cytoplasm (Fig. 5B–D), gold particles appeared free in the cytosol and not sequestered in small or large vesicles. Sometimes they were associated with the cytosolic side of a vesicular membrane (Fig. 5D) or arranged in small clusters (Fig. 5C,E). Finally, a few particles were also detected in association with the nuclear membrane and in the nucleoplasm (Fig. 5C,E,F).

DISCUSSION

We have shown that 3LL cells, which do not express complement receptors and do not produce C3 [Di Renzo et al., 1999], are able to internalise the C3 molecules from the external compartment. We have also provided evidence that C3 is translocated by a mechanism that is not cytoskeleton- and/or vesicle-dependent but depends on PKC-specific phosphorylation. In 3LL cells, PKC seems able to modulate proliferation through signals that are dependent on C3 internalisation.

Protein kinases C consists of a family of Ca^{++} and phospholipid-dependent serine-threonine protein kinases [Liu et al., 1992; Mellor and Parker, 1998; Nakashima, 2002]. These enzymes play an important role in cell-signal transduction during cell growth and differentiation as well as in tumour promotion, oncogene activation, and carcinogenesis. There are several reports that correlate PKC activity and cell growth rate [Nishizuka, 1984; Clemons et al., 1992; Matsuzaki et al., 2004]. The involvement

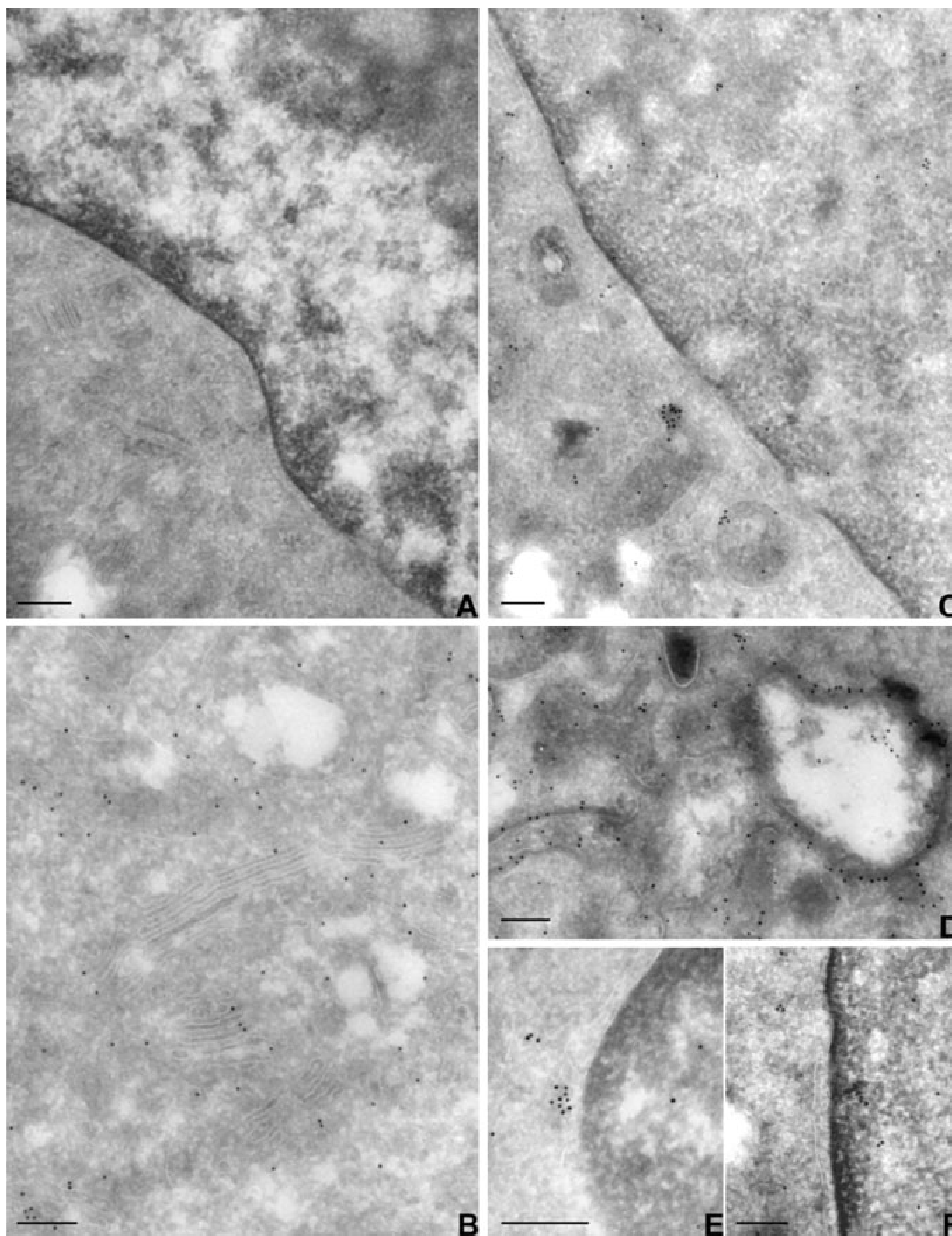

Fig. 5. Subcellular C3 localisation as determined by immunogold labelling. 3LL cells were either left untreated or treated with NMS. Electron microscopy analysis was performed as in "Materials and Methods." **A**: Control cells (3LL − no NMS) (×47,000). **B**: Cytoplasmic C3 distribution in 3LL cells + NMS (×52,000). **C**: Small cytoplasmic clusters and nuclear localisation in 3LL cells + NMS (×41,000). **D**: Vesicle and membrane associated localisation in 3LL cells + NMS (×43,000). **E**: Nuclear membrane (pore) associated localisation in 3LL cells + NMS (×78,000). **F**: Intranuclear cluster of particles in 3LL cells + NMS (×49,000). Bar represent 2 μm.

of PKC activity in cell functions has been demonstrated with inhibitors, that sometimes are not specific and work only at concentrations that may interfere with other biological processes [O'Brian and Ward, 1990; Minana et al., 1991]. For this reason we have used more than one PKC inhibitor and at the lowest efficient dose. In our hands, the PKC inhibitors

prevented the proliferation of 3LL cells induced by C3. Another strategy for demonstrating the PKC involvement in C3-induced cell proliferation was its stimulation by TPA. PKC acts as receptor for phorbol esters and other tumour promoters [Blood and Zetter, 1989]. This is one of the mechanisms by which PKC is thought to interact in the cellular response to growth factors [Castagna et al., 1982; Nishizuka, 1984; Blood and Zetter, 1989]. In fact, when 3LL cells were treated with TPA in the presence of C3, we observed a significant stimulation of cell proliferation.

In contrast, neither selective activation nor inhibition of PKA as used by Mueller et al. [1997] resulted in significant changes of C3 internalisation and proliferation of 3LL cells. Similarly protein phosphatases [Young et al., 1991; Lucocq, 1992] did not seem to transduce biochemical signals required for C3 internalisation and proliferation.

Our results establish that, while PKC is obligatory for C3-induced proliferation of 3LL cells, PKA activation might not be necessary.

The mechanism of C3 internalisation was investigated using inhibitors of the processes that are normally involved in it. The persistent internalisation after treatment of cells with cytochalasin and colchicine demonstrated that the internalisation process does not require a dynamic reorganization of the actin and tubulin cytoskeleton. On the other hand there are conflicting reports on the extent to which these agents inhibit endocytosis [Qualman et al., 2000].

An interesting point in the C3 endocytosis is that a certain amount of these molecules appears to be recycled by the cell and released in an active form in the medium. The rapid C3 exocytosis in an intact active form suggests that C3 recycling does not involve the lysosomal hydrolase-containing compartment: this finding suggests that cells can promptly use C3 again.

The role of C3 in enhancing growth has been demonstrated in normal lymphocytes, in Raji cells and in many other cell lines [Kuraya et al., 1990]. We have shown here that C3 internalisation is an essential step for mediating this important function and that PKC mediates this event.

A final interesting observation may be worth of noting: the translocation and clustering of C3 into the nucleus, which seems to be stimulated by PKC activity. At present, we don't have a plausible explanation for this phenomenon that could be either related to the signalling for proliferation gene activation, or be just a fortuity and anecdotic event.

In conclusion, we have confirmed that C3 is able to stimulate 3LL cell proliferation, and we have provided evidences for its signalling within the cell. First, its rapid traffic seems to be dependent on PKC and independent on the cytoskeleton; second, the increase in cell growth and internalisation are coupled and dependent on PKC, but not on PKA.

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