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Regulation of macropinocytosis in v-Src-transformed fibroblasts: cyclic AMP selectively promotes regurgitation of macropinosomes

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SUMMARY

Stable transformation of Rat-1 fibroblasts by the v-Src oncoprotein results into the constitutive formation of macropinosomes. In the present report, we found that macropinosomes do not fuse with transferrin-containing endosomes and investigated the effects of cyclic AMP as a regulator of macropinocytosis in this cell system. The permeant analogs dibutyryl cyclic AMP and 8-bromo-cyclic AMP, as well as the pharmacological activator of adenylate cyclase forskolin, similarly decreased by about 35% the net endocytic accumulation of the fluid-phase tracer horseradish peroxidase at intervals >5 minutes in v-Src-transformed cells but not in the non-transformed parental Rat-1 cell line. However, and in contrast to the phospholipase C inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate or the phosphatidylinositol 3-kinase inhibitor wortmannin, dibutyryl cyclic AMP neither returned the peroxidase accumulation rate of v-Src-transformed cells to that of parental Rat-1/control cells, nor prevented macropinosome formation, as shown by confocal microscopy. Detailed analysis of the kinetics of tracer entry and efflux in transformed cells revealed that dibutyryl cyclic AMP inhibited peroxidase accumulation only after intervals >5 minutes, due to accelerated peroxidase regurgitation, but did not alter the rate of transferrin recycling. Taken together, these data indicate that, in v-Src-transformed fibroblasts, macropinocytosis and micropinocytosis serve different pathways and that cyclic AMP affects neither micropinocytosis nor the formation of macropinosomes, but selectively promotes regurgitation therefrom.

Key words: Macropinocytosis, v-Src, cAMP, Fluid-phase endocytosis, Regurgitation

INTRODUCTION

Macropinocytosis designates the formation of large primary endocytic vesicles, upon closure of membrane ruffles (reviewed by Swanson and Watts, 1995). Whereas macropinosomes are constitutively formed in a limited number of cells, such as dendritic cells (Sallusto et al., 1995; Norbury et al., 1997) or oncogene-transformed fibroblasts (Bar-Sagi and Feramisco, 1986; Veithen et al., 1996), their occurence is dramatically but transiently stimulated in a large variety of cell types by phorbol esters (Swanson, 1989; Keller and Niggli, 1993), growth factors (Racoosin and Swanson, 1989, 1992; Dowrick et al., 1993; Hewlett et al., 1994), thyroid-stimulating hormone (Deery and Heath, 1993), and the chemotactic peptide fMLP (Carpentier et al., 1991; Keller, 1990). The fate of macropinosomes is also variable. They readily fuse with endosomes in macrophages (Racoosin and Swanson, 1993), but follow a distinct route in EGF-stimulated A431 cells (Hewlett et al., 1994).

After having long been regarded as a curiosity of unknown significance, macropinocytosis is receiving a growing interest for at least three reasons. First, recent reports have evidenced a key role for macropinocytosis in antigen presentation by class II and even class I MHC complexes in dendritic cells (Sallusto et al., 1995; Robinson et al., 1996; Norbury et al., 1997). Second, *Salmonella typhi* and *Shigella flexneri* trigger the formation of macropinosomes so as to promote their internalisation (Francis et al., 1993; Alpuche-Arranda et al., 1994, 1995; Mills and Finlay, 1994; Dehio et al., 1995), a phenomenon underlining the importance of macropinocytosis as a gateway for pathogen invasion (reviewed by Swanson and Bear, 1995). Third, macropinocytosis could be involved in the endocytic withdrawal of large structured membrane domains, such as hemi-desmosomes (Holm et al., 1993; Poumay et al., 1994).

In parallel, the molecular mechanisms that regulate macropinocytosis are being unravelled. In particular, signal-transducing proteins such as phosphatidylinositol 3-kinase (Kotani et al., 1994, 1995; Wennström et al., 1994; Araki et al., 1996), the small GTPases Rac (Ridley et al., 1992; Kotani et al., 1995), Ras (Bar-Sagi and Feramisco, 1986) and Arf-6 (Radhakrishna et al., 1996), as well as the heat shock protein 27 (Lavoie et al., 1993), can all induce membrane ruffling and macropinocytosis. Although interactions between these various regulators are poorly understood, they are regarded to reorganise cortical actin and thereby promote the formation of

membrane ruffles that fuse to generate macropinocytic vesicles. A repressor of this regulatory machinery presumably exists, since macropinocytosis induced by growth factors is rapidly down-regulated. Interestingly, elevation of the intracellular adenosine 3′:5′-cyclic monophosphate (cAMP) concentration prevents the stimulation of membrane ruffling and pinocytosis induced by growth factors (Miyata et al., 1989).

In this work, we have investigated whether cAMP, a general second messenger, could indeed play the role of a physiological repressor of macropinocytosis in v-Src-transformed fibroblasts, a convenient model of constitutive macropinocytosis (Veithen et al., 1996). Surprisingly, experimental manipulations that elevated intracellular cAMP did not prevent the formation of macropinosomes in these cells, but decreased the accumulation of the fluid-phase tracer horseradish peroxidase (HRP), due to a selective acceleration of regurgitation from macropinosomes. Macropinosomes did not intermix content with transferrin-containing endosomes and the transferrin recycling rate was not affected by cAMP. These observations show that, in oncogene-transformed fibroblasts, micropinocytosis and macropinocytosis serve distinct pathways, governed by different regulatory machineries.

MATERIALS AND METHODS

Cell culture

Parental Rat-1 cells (Rat-1/control) and Rat-1 cells transformed by the B77 subclone of Rous Sarcoma Virus (Rat-1/BB16), kindly provided by Dr G. Rousseau (ICP, Belgium), were grown at 37°C in Dulbecco's modified Eagle's medium (Gibco BRL, MD, USA) supplemented by 20 mM glucose, 4 mM glutamine, 10 mM $NaHCO_3$, 10 mM Hepes, 10 μg/ml streptomycin, 66 μg/ml penicillin and 10% (v/v) foetal calf serum (FCS, Gibco) under 8% CO_2 and usually seeded at ~10^6 cells on 9.6 cm^2 Petri dishes (for biochemical experiments), or at ~50,000 cells on 1.1 cm^2 glass coverslips (for confocal microscopy). Before each experiment, cells were rinsed twice with FCS-free DMEM and preincubated therein for 30 minutes before the addition of endocytic tracers, to avoid interference by short-lived growth factor effects.

Confocal microscopy

Cells were incubated for 7 minutes in DMEM containing 1 mg/ml Texas Red-neutral dextran 70,000 (Molecular Probes, OR, USA) alone, or combined with 10 μg/ml fluorescein-transferrin (Molecular Probes), as indicated. After extensive washing at 4°C, cells were fixed by 4% formaldehyde (w/v) in 0.1 M phosphate buffer, pH 7.4, for 15 minutes. Coverslips mounted in Mowiol were examined by fluorescence and by interferential contrast microscopy with an Axiovert microscope (Zeiss, Germany) coupled to MRC 1024 confocal scanning equipment (Bio-Rad, CA, USA).

Alternatively, cells were plated in 8-well Labtek coverglass-based culture chambers (Nalgene-Nunc, IL, USA), immersed in 1 mg/ml fluorescein-dextran 70,000 (Molecular Probes) and immediately examined without washing by time-lapse recording at 15 second intervals, so that movement of individual macropinosomes could be followed. This approach is similar to that used to follow macropinosome formation in *Dictyostelium* by Hacker et al. (1997).

Fluid-phase endocytosis of peroxidase

Cells were incubated in DMEM supplemented with 1-4 mg/ml HRP (type 2, Boehringer Mannheim, Germany). After the indicated intervals, cells were transferred to 4°C and extensively washed: 3 times 30 seconds with 3 ml of PBS-Ca^{2+} (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 3.6 mM $CaCl_2$ and 0.74 mM $MgSO_4$, pH 6.5), once for 5 minutes in DMEM containing 10% outdated FCS and 3 times with PBS-Ca^{2+}. Cells were surface-digested at 4°C for 1 hour in DMEM supplemented with 0.3% (w/v) pronase and pelleted in a benchtop microfuge (Beckman, CA, USA) for 1 minute at 4°C. The pellet was rinsed twice with PBS-Ca^{2+} and lysed in 0.01% (v/v) Triton X-100 (Serva Biochemicals, NJ, USA). HRP activity in the lysate was measured by the stopped colorimetric assay using ortho-dianisidine as a substrate as described by Cupers et al. (1994), normalized to a 1 mg/ml extracellular concentration and expressed with respect to cell protein (Smith et al., 1985) using bovine serum albumin as a standard.

For experiments using 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC) or wortmannin, stock solutions were made in dimethylsulfoxide. The final concentration in dimethylsulfoxide never exceeded 0.1% (v/v), at which level there was no effect on basal uptake of HRP.

Peroxidase regurgitation

For pulse-chase experiments, cells were incubated at 37°C in DMEM supplemented by HRP for 5 or 10 minutes, washed at 4°C as described above, and reincubated at 37°C for the indicated intervals. Thereafter, cells were rapidly washed 3 times with PBS-Ca^{2+} at 4°C, pronase-digested, lysed and assayed for HRP activity and protein content. Results were expressed as the residual fraction of the intracellular amount of HRP at the initial chase time and were adjusted by least-squares fitting with SYSTAT 5.2. software (SYSTAT Inc., USA) to an exponential decay equation as previously described:

$$Q_t/Q_o = 1 - Q_r/Q_o(1 - e^{-kt}),$$

where Q_t represents the intracellular amount of HRP at chase time t, Q_o the amount of HRP at the initiation of chase, Q_r the amount of regurgitable HRP, and k the rate constant of regurgitation (Cupers et al., 1994).

Transferrin recycling

Iron-saturated transferrin (Sigma, MO, USA) was labelled by ^{125}I iodine (100 mCi/ml, Amersham, UK) as described by McFarlane (1958) to a specific radioactivity of ~250 cpm/ng protein. Cells were incubated for 10 minutes in DMEM containing 50 nM ^{125}I-transferrin, washed extensively at 4°C and reincubated at 37°C for the indicated intervals. Thereafter, cells were rapidly washed 3 times with PBS-Ca^{2+} at 4°C, pronase-digested and lysed in Triton X-100 as described above. The radioactivity associated with the pellet and the supernatant were taken as measures of intracellular and surface-bound transferrin, respectively. Results were expressed as the residual fraction of intracellular transferrin at the initial chase time and were adjusted to the same exponential decay equation as described for fluid regurgitation.

RESULTS

In v-Src-transformed fibroblasts, macropinosomes are preferentially formed at the tip of peripheral extensions and do not readily fuse with endosomes

We recently reported that fluid-phase endocytosis, monitored in biochemistry and morphology by HRP uptake, is stimulated upon v-Src-transformation of Rat-1 fibroblasts, due to the constitutive formation of macropinocytic vesicles (Veithen et al., 1996). First, we examined whether macropinocytosis was affected by cell confluence. The level of intracellular HRP accumulation per cell after 30 minutes of uptake at 4 mg/ml HRP in the medium was constant between 30,000 and 150,000 cells/cm^2, both in control Rat-1 fibroblasts (272±8 ng HRP/mg

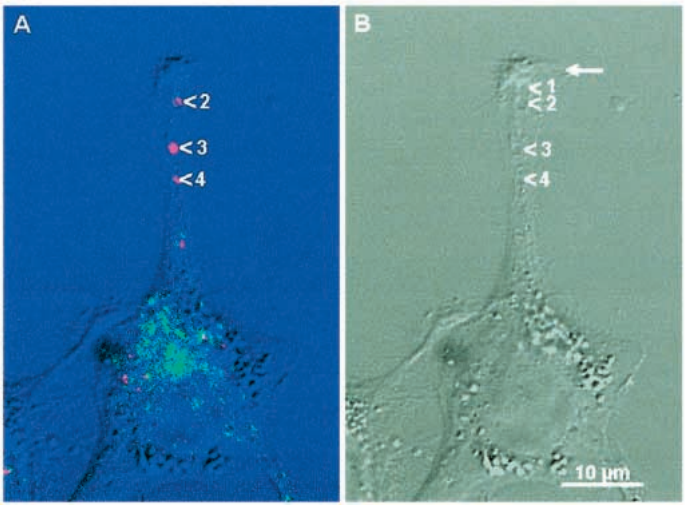

Fig. 1. Double labelling of macropinosomes and endosomes in v-Src-transformed fibroblasts. Rat-1/BB16 cells were incubated at 37°C for 7 minutes in DMEM containing 1 mg/ml Texas Red-dextran and 10 μg/ml fluorescein-transferrin, washed, fixed and examined by fluorescence (A) and interferential contrast microscopy (B). Macropinosomes, numbered from 1 to 4, are formed at the tip of a peripheral extension showing extensive ruffling (arrow) and migrate slowly towards the cell body. Macropinosomes numbered 2, 3 and 4 are labelled by Texas Red-dextran, while number 1 is not, presumably because it was formed during the short interval between tracer removal and complete cell cooling. Endosomes containing fluorescein-transferrin are concentrated near the nucleus. Notice the absence of detectable double labelling of macropinosomes and endosomes.

cell protein; mean ± s.e.m. of 12 dishes) and v-Src-transformed Rat-1 fibroblasts (540±6 ng HRP/mg cell protein), and corresponded to clearance values of 136±4 and 270±3 nl/mg cell protein/hour, respectively. Thus, micropinocytosis and macropinocytosis were both independent of cell confluence in our experimental conditions.

To examine whether macropinosomes would readily fuse with endosomes in v-Src-transformed fibroblasts as they do in macrophages, fibroblasts were pulsed for 7 minutes with 1 mg/ml Texas Red-dextran, as a tracer of fluid-phase endocytosis, together with 10 μg/ml transferrin, as a tracer for receptor-mediated endocytosis. Large profiles that were strongly and homogeneously labelled by Texas Red-dextran (Fig. 1A), and corresponding by interferential contrast imaging as light-lucent cytoplasmic vesicles with a diameter >1 μm (Fig. 1B), were frequently observed in v-Src-transformed cells. These macropinosomes were preferentially formed at the ruffling end of long cellular extensions where they remained poorly mobile over a few minutes time-scale, but after ~30 minutes of chase, had either disappeared, or had slowly migrated towards the cell body (data not shown). Thus, their motility contrasted with the irregular saltatory motion that is typical of endosomes. Moreover, transferrin-containing endosomes, appearing as multiple small dots, were not detected in the peripheral extensions but were concentrated in the perinuclear region of the cell body. There was no labelling of these endosomes by 1 mg/ml Texas Red-dextran at the detection level used. However, if cells were doubly incubated with 1 mg/ml of both Texas Red-dextran and fluorescein-transferrin, so that the latter would also behave as a fluid-phase tracer, all macropinosomes appeared yellow, indicating that they contained both probes (data not shown). When macropinosomes were loaded with Texas Red-dextran for 5 minutes, then chased for 20 minutes in tracer-free medium, and cells incubated for 5 further minutes with fluorescent-transferrin, remaining macropinosomes had largely moved towards the cell body, but still did not merge with transferrin-labelled endosomes (data not shown).

In v-Src-transformed fibroblasts, cAMP decreases peroxidase accumulation but does not prevent the formation of macropinosomes

To investigate whether cAMP affects macropinocytosis, we first examined the effects of its permeant analogs 2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (db-cAMP) and 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), as well as of the adenylate cyclase activator forskolin, on the net accumulation of the fluid-phase tracer HRP in normal and v-Src-transformed fibroblasts (Fig. 2A). Whereas treatment of transformed cells with the usual concentrations of these three agents caused a comparable ~35% decrease of HRP accumulation after a 30 minute pulse, it had no effect on non-transformed fibroblasts. That an elevation of intracellular cAMP decreased accumulation of an endocytic fluid-phase tracer selectively in v-Src-transformed fibroblasts was suggestive of an interference with macropinocytosis.

Although clearly significant (P<0.001), inhibition of HRP accumulation in the transformed cells was only partial, as it did not return to the level of non-transformed cells. This contrasts with the full abrogation of the difference between transformed and non-transformed cells by 100 μM NCDC or by 100 nM wortmannin (Fig. 2A). These drugs, respectively, inhibit phospholipase C and phosphatidylinositol 3-kinase, which are potentially implicated in the stimulation of macropinocytosis (Veithen et al., 1996; Araki et al., 1996). To exclude the possibility that the partial inhibition reflected an incomplete effect, cells were preincubated with higher concentrations of db-cAMP (Fig. 2B), or for a longer interval (data not shown). Inhibition of HRP accumulation induced by db-cAMP became detectable at 0.1 mM and reached its maximal from 0.5 to 2 mM, never exceeding the level intermediate between transformed and non-transformed cells.

One simple interpretation of these results would be that high intracellular concentrations of cAMP reduce by half the formation rate or the average size of macropinosomes in v-Src-transformed cells. To test this hypothesis, the effects of db-cAMP on the formation of macropinosomes was analysed by confocal microscopy, using Texas Red-dextran as a fluid-phase tracer (Fig. 3). When fibroblasts had been pretreated with 1 mM db-cAMP, the location, abundance and size of macropinosomes appeared comparable to those of non-treated cells (Fig. 3A and B vs Fig. 1). The abundance of macropinosomes in v-Src transformed fibroblasts was further estimated by counting large (>0.5 μm) fluorescent circles after a 30 minute uptake of the fluid-phase tracer Texas Red-dextran in five fields, each showing about 50 cells. The analysis disclosed no significant difference in the absence of db-cAMP treatment (4.66±0.49 macropinosomes/cell) and after such treatment (4.39±0.23 macropinosomes/cell). In contrast,

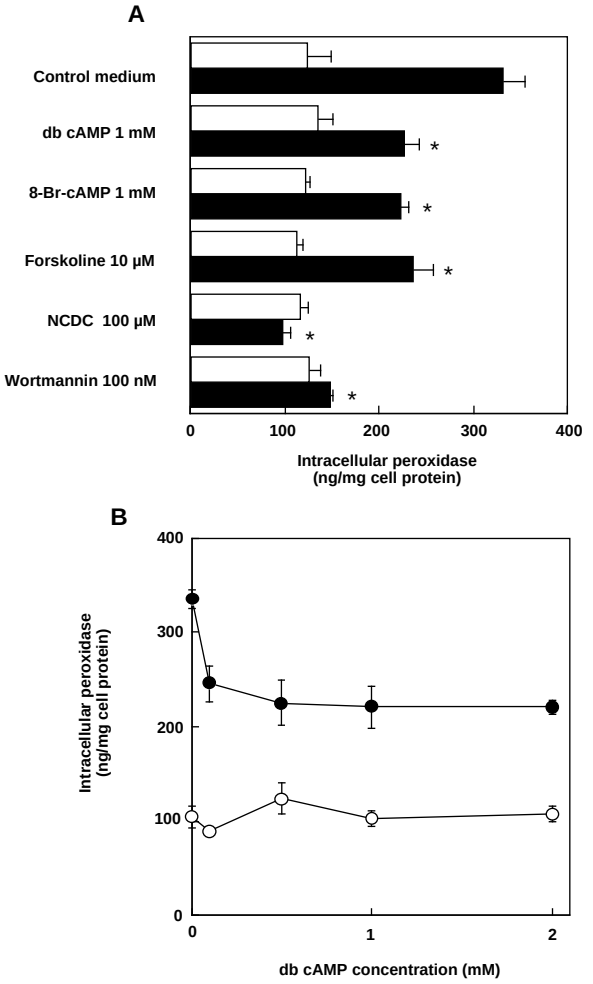

Fig. 2. cAMP partially inhibits peroxidase accumulation in v-Src-transformed fibroblasts. Rat-1/control cells (open symbols) and Rat-1/BB16 cells (filled symbols) were first incubated at 37°C for 30 minutes in DMEM containing the various indicated agents (A) or the indicated db-cAMP concentrations (B). They were then incubated in the same media supplemented with 4 mg/ml of HRP for 30 minutes. Cells were cooled down to 4°C, washed extensively (see Materials and Methods) and their content in HRP (normalized to 1 mg/ml) and protein were determined. Values are means ± s.d. of 4-12 culture dishes, pooled form 2-4 experiments. *P<0.001, as compared to control medium by Student's t-test.

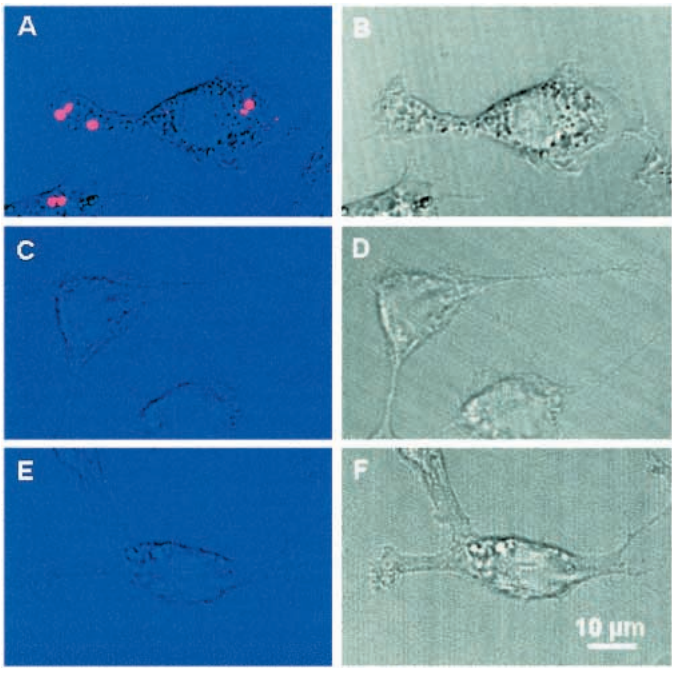

Fig. 3. cAMP does not abolish the formation of macropinocytic vesicles in v-Src-transformed fibroblasts. Rat-1/BB16 cells were incubated at 37°C for 30 minutes in DMEM containing 1 mM db-cAMP (A,B), 100 µM NCDC (C,D) or 100 nM wortmannin (E,F) and then for 7 minutes in the same media supplemented with 1 mg/ml of Texas Red-dextran. Cells were washed at 4°C and fixed in formaldehyde. The same optical fields are shown either by confocal fluorescence microscopy (A,C,E) or by interferential contrast microscopy (B,D,F). This experiment has been reproduced 3 times.

dextran-labelled vacuoles could only exceptionally be observed after treatment by NCDC (Fig. 3C and D) or wortmannin (Fig. 3E and F), demonstrating that these drugs had essentially abolished the formation of macropinosomes. These results led us to examine the possibility that cAMP affected macropinocytosis at a stage distinct from the formation of macropinosomes.

cAMP stimulates fluid regurgitation but not receptor recycling in v-Src-transformed fibroblasts

Since the level of inhibition by cAMP of HRP accumulation after a 30 minute pulse appeared not to result from a comparable decrease of its sequestration into macropinosomes, we characterized cAMP effects on the kinetics of endocytic processing of HRP. First, a detailed analysis of the kinetics of uptake revealed that the effect of db-cAMP varied with the interval of HRP accumulation: inhibition was obvious after pulses of >5 minutes (Fig. 4A), but cAMP had no effect for pulses of <5 minutes (Fig. 4B). This discrepancy cannot be accounted for by a delay in the increase of intracellular cAMP concentration since, in all cases, cells were preincubated for at least 30 minutes with db-cAMP before HRP addition to the incubation medium. In non-transformed Rat-1 fibroblasts, db-cAMP had no effect on the level of HRP accumulation from 5 to 30 minutes of pulse, which was about 2.5 lower than in their v-Src-transformed counterparts.

Thus, the possibility was considered that a decrease by db-cAMP of HRP accumulation in v-Src-transformed fibroblasts after a lag of ~5 minutes could reflect a stimulation of regurgitation, as previously observed in primary cultures of rat foetal fibroblasts upon potassium depletion, incubation in hypertonic media (Cupers et al., 1994) or equilibration with 2 µM monensin (Cupers et al., 1997). We therefore compared by pulse-chase experiments the kinetics of regurgitation in v-Src-transformed cells that were treated or not by db-cAMP (Fig. 5A). After a 5 minute pulse, the regurgitation rate, estimated by the k parameter of the adjustment equation, was about doubled after treatment with db-cAMP (0.19±0.02 minute^{-1} vs 0.11±0.02 min^{-1} in untreated conditions). The effect on the regurgitable fraction was less important (40±1% after treatment with db-cAMP vs 32±2% in control cells). Similar observations were made for regurgitation analyses performed

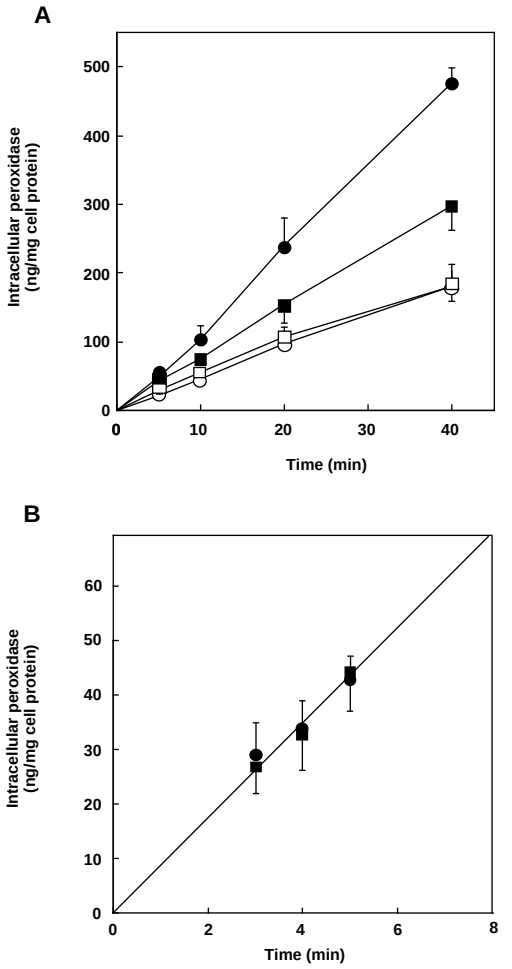

Fig. 4. Effects of db-cAMP on the kinetics of peroxidase accumulation in v-Src-transformed fibroblasts. Rat-1/control cells (○,□) and Rat-1/BB16 cells (●,■) were incubated at 37°C for 30 minutes in DMEM with (□,■) or without (○,●) 1 mM db-cAMP and then for the indicated times in the same media supplemented with 1 mg/ml of HRP. (A) Long-term pulses; (B) short-term pulses. Values are means ± s.d. of 5-6 dishes, pooled from 2 separated experiments.

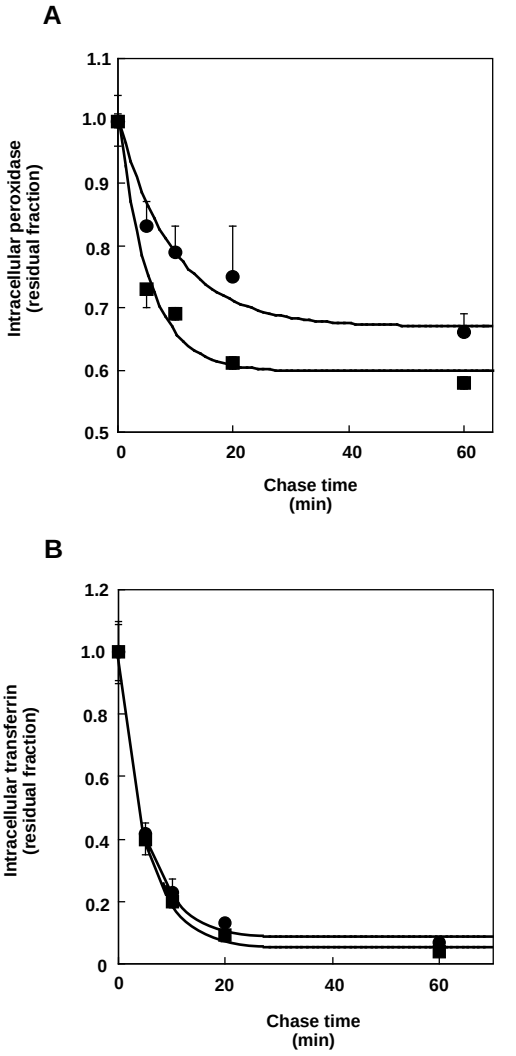

Fig. 5. db-cAMP stimulates peroxidase regurgitation but not transferrin recycling in v-Src-transformed fibroblasts. Rat-1/BB16 cells were incubated at 37°C for 30 minutes in DMEM with (■) or without (●) 1 mM db-cAMP and pulsed in the same media containing 4 mg/ml of HRP for 5 minutes (A) or 50 nM ^{125}I-transferrin for 10 minutes. Cells were rapidly washed at 4°C and chased for the indicated times at 37°C in DMEM containing or not 1 mM db-cAMP. Values are means ± s.d. of 4 dishes, expressed as the residual fraction of intracellular amount of tracer at the end of the pulse and were fitted to an exponential decay. Absolute 100% values for control and db-cAMP-treated cells, respectively, were 50±1 and 45±2 ng HRP/mg of cell protein (after normalization to 1 mg/ml extracellular concentration); and 420±40 and 340±30 fmoles of intracellular transferrin/mg of cell protein; surface-bound transferrin was 227±32 and 199±36 fmoles/mg of cell protein.

after a 10 minute pulse (data not shown). These results directly demonstrate that cAMP selectively interferes with fluid-phase regurgitation from macropinosomes in v-Src-transformed cells.

In contrast, pulse-chase analysis using transferrin as a tracer of receptor-mediated endocytosis revealed that cAMP did not influence its recycling (Fig. 5B). Both in db-cAMP-treated and non-treated v-Src-transformed fibroblasts, >90% of the transferrin taken up during a 10 minute pulse was released within a 1-hour chase, with a similar efflux rate of 0.20 minute^{-1}.

DISCUSSION

In v-Src-transformed fibroblasts, macropinosomes are continuously generated at ruffling zones, which are preferentially located at the tip of long cellular extensions (Veithen et al., 1996). This structural feature, combined with the use of fluorescent probes coupled to interferential contrast imaging, makes it convenient to follow their fate in living or fixed cells. Despite the fact that, by definition, fluid-phase tracers label both macro- and micropinocytic routes, a formal distinction between macropinosomes (about 1 micrometer in diameter) and primary micropinocytic vesicles (about 100

nanometers in diameter) using short-pulses of tracer is generally easy, based on the principle that a 10-fold difference in size will result in a 1,000-fold difference in tracer load. Accordingly, an appropriate setting of detection level allows one to selectively extract the former and ignore the latter, which require much higher concentrations of the fluid-phase tracer to be detected (Hewlett et al., 1994). When using longer pulses of fluid-phase tracer, however, the distinction between macropinosomes and vesicular endosomes (about 300-500 nm in diameter) filled by the tracer brought by multiple primary micropinocytic vesicles is more difficult. The difference may even be further attenuated if maturing macropinosomes shrink (Swanson and Watts, 1995).

Recently formed macropinosomes are rather immobile over a few minutes time-scale, and either disappear or exhibit a slow net centripetal movement, but do not appear to release their content into perinuclear transferrin-containing endosomes. Their origin, dynamics and fate are thus distinct from the micropinocytic apparatus, which can be demonstrated using low concentrations of transferrin, indicating that they depend on different molecular machineries.

Although several studies have reported that cAMP can influence endocytosis, the effects of this second messenger on endocytic processes are pleiotropic and seem to depend on the cellular context. Indeed, an increase in intracellular cAMP stimulates clathrin-independent micropinocytosis at the apical pole of polarized MDCK cells (Eker et al., 1994), inhibits the basal rate of pinocytosis in alveolar macrophages (Pataki et al., 1995) and abrogates the growth factor-induced stimulation of pinocytosis in KB and A431 cells (Miyata et al., 1989). Since it has been clearly demonstrated that stimulation of A431 cells by growth factors selectively induces macropinocytosis in these cells (Hewlett et al., 1994), we hypothesized that the inhibitory effect of cAMP could reflect a repression of this particular endocytic pathway.

In the present work, we have investigated this possibility on macropinocytosis in v-Src-transformed fibroblasts. Although cAMP significantly decreased the net accumulation of the fluid-phase tracer HRP, specifically in these cells and not in their non-transformed counterparts, macropinosome formation was apparently not affected and the effect depended on the interval of tracer accumulation. In addition, treatment with db-cAMP stimulated fluid regurgitation in v-Src-transformed cells, without affecting the rate of transferrin recycling. Taken together, these results lead us to propose that the effect of cAMP on endocytosis in v-Src-transformed cells corresponds mainly, if not exclusively, to a stimulation of fluid regurgitation selectively from macropinosomes. The extent to which regulation of constitutive macropinocytosis in transformed fibroblasts can be extrapolated to constitutive macropinocytosis in dendritic cells, or to transient macropinocytosis induced in other cells by growth factors or phorbol esters, remains to be examined.

Our observations clearly contrast with the reported inhibitory effects of cAMP on endocytosis in growth factor-stimulated cells or in non-stimulated alveolar macrophages, observations that were attributed to a direct inhibition of endocytic vesicle formation. In both growth factor-stimulated A431 and KB cells, the suppression by cAMP of endocytosis stimulation was accompanied by a decrease of membrane ruffling (Miyata et al., 1989), which is regarded as the prerequisite of macropinosome formation (Swanson and Watts, 1995; Araki et al., 1996; Hacker et al., 1997). In addition, in growth factor-stimulated KB cells, cAMP was reported not to increase, but actually decrease fluid regurgitation (Miyata et al., 1989). One could, however, argue that this decrease might correspond to an effect on regurgitation from endosomes rather than from macropinosomes, since cAMP inhibited regurgitation of HRP that had been taken up *before* stimulation of macropinocytosis by growth factors. Similarly, in non-stimulated alveolar macrophages, cAMP decreased by about 50% the accumulation of the fluid-phase tracer FITC-dextran, without detectable effect on regurgitation (Pataki et al., 1995), but whether these observations reflect a decrease of micropinocytosis or a selective inhibition of macropinocytosis in these cells remains to be clarified.

A selective stimulation of regurgitation from macropinosomes in v-Src-transformed fibroblasts raises the questions of the underlying mechanism and, in particular, of the actual fate of macropinosomes in these cells. Whether formed in macrophages upon stimulation by phorbol esters and M-CSF or in A431 cells stimulated by EGF, macropinosomes migrate to the central region of the cell, and concomitantly part of the fluid content taken up by macropinocytosis is released back to the extracellular medium (Swanson, 1989; Racoosin and Swanson, 1993; Hewlett et al., 1994). This regurgitation is believed to be mediated by small vesicles that would bud from macropinosomes and fuse with the pericellular membrane (Swanson, 1989). Alternatively, macropinosomes formed in macrophages can fuse with other organelles such as endosomes, lysosomes and phagolysosomes (Racoosin and Swanson, 1993; Berthiaume et al., 1995). Part of the material ultimately regurgitated by macropinosomes may thus actually transit via other endocytic organelles before release.

Such an indirect regurgitation route appears unlikely in A431 cells or in v-Src-transformed fibroblasts, since macropinosomes formed in these cells do not apparently fuse with endosomes or lysosomes (Hewlett et al., 1994; this report). A simpler mechanism would be the direct fusion of macropinosomes back to the cell surface. Although this mechanism has only seldom been observed in macrophages (Swanson and Watts, 1995), it could play a more important role in other cell types. In HeLa cells overexpressing the GTPase Arf-6, macropinosomes formed upon AlF4 induction remain in the peripheral cytoplasm where membrane ruffling occurs and where they appear to rapidly regurgitate their entire fluid content (Radhakrishna et al., 1996). The absence of significant migration of such macropinosomes and the rapidity of their regurgitation would suggest a direct fusion, without vesicular intermediate, with the cell surface where they originated. The denomination ‘retroendocytosis’, first introduced in another context (Aulinskas et al., 1981), would be best applied to this process. Although the contribution of indirect versus direct regurgitation from macropinosomes in v-Src-transformed fibroblasts remains to be elucidated, we suggest that one of these phenomena is triggered by cAMP.

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