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Recycling of transferrin receptors in A431 cells is inhibited during mitosis

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There is a marked reduction in the number of surface transferrin receptors as A431 cells enter mitosis which persists until telophase when receptors reappear to a level that exceeds the original interphase value. This is most simply explained by assuming that recycling of receptors back to the cell surface is inhibited as cells enter mitosis but that internalisation continues for a short while, causing surface receptor depletion. In telophase recycling would resume before internalisation giving a temporary excess of surface transferrin receptors.

Key words: endocytosis/membrane traffic/mitosis/receptor recycling/transferrin receptor

Introduction

There is a general cessation of vesicle-mediated transport during mitosis in animal cells (Fawcett, 1965; Quintart and Baudhuin, 1976; Berlin *et al.*, 1978; Warren *et al.*, 1983; Hesketh *et al.*, 1984). The inhibition of intracellular transport (Warren *et al.*, 1983) is accompanied by the fragmentation of the Golgi complex (see Wilson, 1925; Burke *et al.*, 1982) which gives rise to tens of thousands of small vesicles (see Zeligs and Wollman, 1979). These disperse throughout the mitotic cell cytoplasm and thereby ensure that, during telophase, each daughter cell will receive an equal complement of Golgi membranes. We have argued (Hesketh *et al.*, 1984) that the fragmentation of the Golgi complex is causally related to the inhibition of transport through it. Normally vesicles are thought to bud from the transitional elements of the endoplasmic reticulum carrying newly-synthesised proteins destined for various parts of the cell (Palade, 1975) and these vesicles appear to fuse with the *cis* Golgi cisternae (Griffiths *et al.*, 1982). Vesicles then appear to bud from the dilated rims of this cisterna and to fuse with the next cisterna in the stack towards the *trans* side, and so on (Rothman *et al.*, 1984a, 1984b). If, at the onset of mitosis, budding of vesicles were to continue but fusion were to be inhibited, then the entire Golgi stack would be rapidly converted into vesicles and transport through the Golgi stack would cease. During telophase, reassembly of the Golgi stack in each daughter cell would occur spontaneously once vesicle fusion resumed.

Consistent with this model is our observation that fusion of secretory granules with the plasma membrane is inhibited during mitosis (Hesketh *et al.*, 1984). Other workers, however, have provided evidence that budding of vesicles from the cell surface is also inhibited at the onset of mitosis (Fawcett, 1965; Berlin *et al.*, 1978). If both of these observations apply at the level of the Golgi complex, and vesicles can neither bud from nor fuse with cisternae, then an alternative mechanism for fragmentation would have to be proposed. One way around this problem would be to suggest that both vesicle budding and fusion are inhibited at the onset of mitosis but that fusion is inhibited first followed, after fragmentation of the Golgi complex, by budding.

To test this possibility we have studied the recycling of the transferrin receptor (Bleil and Bretscher, 1982; Newman *et al.*, 1982). These receptors, together with bound transferrin, are internalised *via* coated pits (Harding *et al.*, 1983; Hopkins and Trowbridge, 1983) and coated vesicles (Booth and Wilson, 1981; Pearse, 1982) and delivered to endosomes (Van Renswoude *et al.*, 1982; Lamb *et al.*, 1983; Harding *et al.*, 1983; Hopkins and Trowbridge, 1983) where the low pH (Tycko and Maxfield, 1982; Helenius *et al.*, 1983) causes the iron to be released from the transferrin (Aisen, 1980). Apotransferrin remains bound to the receptor and is recycled back to the cell surface where it dissociates (Klausner *et al.*, 1983; Dautry-Varsat *et al.*, 1983) to be replaced by more transferrin which continues the cycle bringing iron into the cell (Karin and Mintz, 1981; Octave *et al.*, 1981).

Receptor internalisation involves vesicle budding whereas the last stage of recycling must involve the fusion of vesicles containing receptors with the plasma membrane. If, at the onset of mitosis, vesicle fusion were to be inhibited before vesicle budding, then receptors would no longer be recycled back to the cell surface but internalisation would continue causing a decrease in the number of cell surface receptors. Here we present data showing such a decrease in surface transferrin receptors on A431 cells.

Results

Reduction in surface transferrin receptors on mitotic A431 cells

Cells were fixed and surface-labelled with the B3/25 monoclonal antibody to the transferrin receptor (Trowbridge and Omary, 1981), followed by a rabbit anti-mouse antibody and a rhodamine-conjugated anti-rabbit antibody. A fluorescent DNA stain (Hoechst 33258) was used to locate mitotic cells rapidly and to determine accurately their phase.

As shown in Figure 1, mitotic A431 cells had far less surface fluorescence and hence far fewer transferrin receptors than neighbouring interphase cells. This was true for cells in prophase through to anaphase, but not telophase, which, if anything, had more surface fluorescence than interphase cells. It was often striking that a mitotic cell, outlined so clearly by Nomarski optics, was almost invisible when surface transferrin receptors were being viewed, and all that was visible were brightly-labelled protruberances, from neighbouring interphase cells, growing under the mitotic cells.

The loss of surface fluorescence, as A431 cells entered mitosis, was not simply the result of a modification or redistribution of surface receptors. The epitope recognised by the monoclonal antibody might have been modified at the onset of mitosis so as to bind less antibody but a polyclonal antibody to the receptor gave essentially the same result (Figure 2). The surface receptors might have been redistributed to

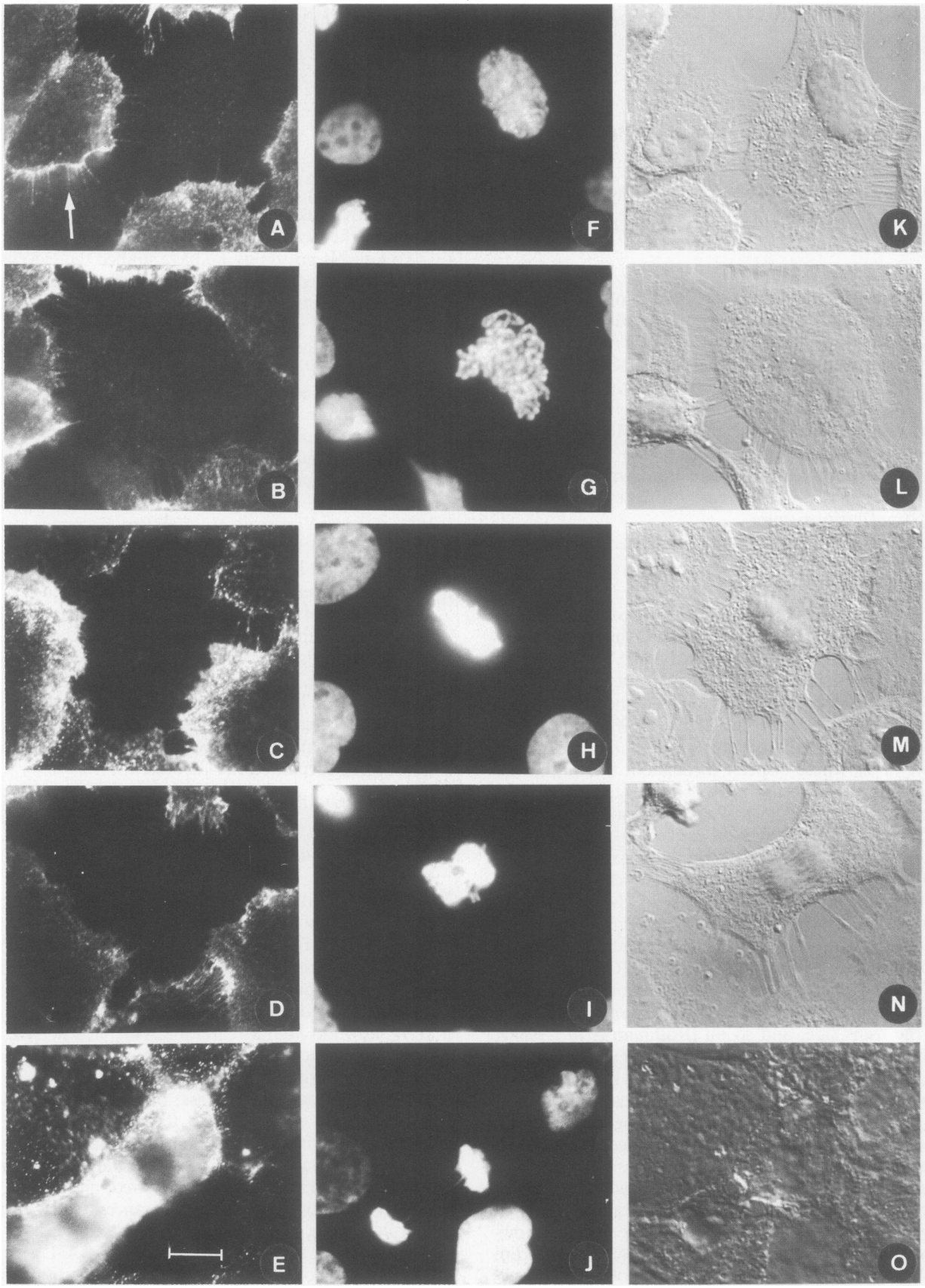

Fig. 1. Surface transferrin receptors labelled with a monoclonal antibody. **(A – E)** Fixed cells were labelled with B3/25 followed by a rabbit anti-mouse IgG and a rhodamine-conjugated, sheep anti-rabbit IgG; **(F – J)** corresponding Hoechst fluorescence showing mitotic cells in **(F)** prophase, **(G)** pro-metaphase, **(F** lower left, **H)** metaphase, **(I)** anaphase and **(J)** telophase; **(K – O)** corresponding Nomarski images. Note that mitotic cells had fewer surface transferrin receptors than neighbouring cells in interphase (e.g., arrow in **A)** with the exception of telophase cells which appeared to have more. Bar 10 μm.

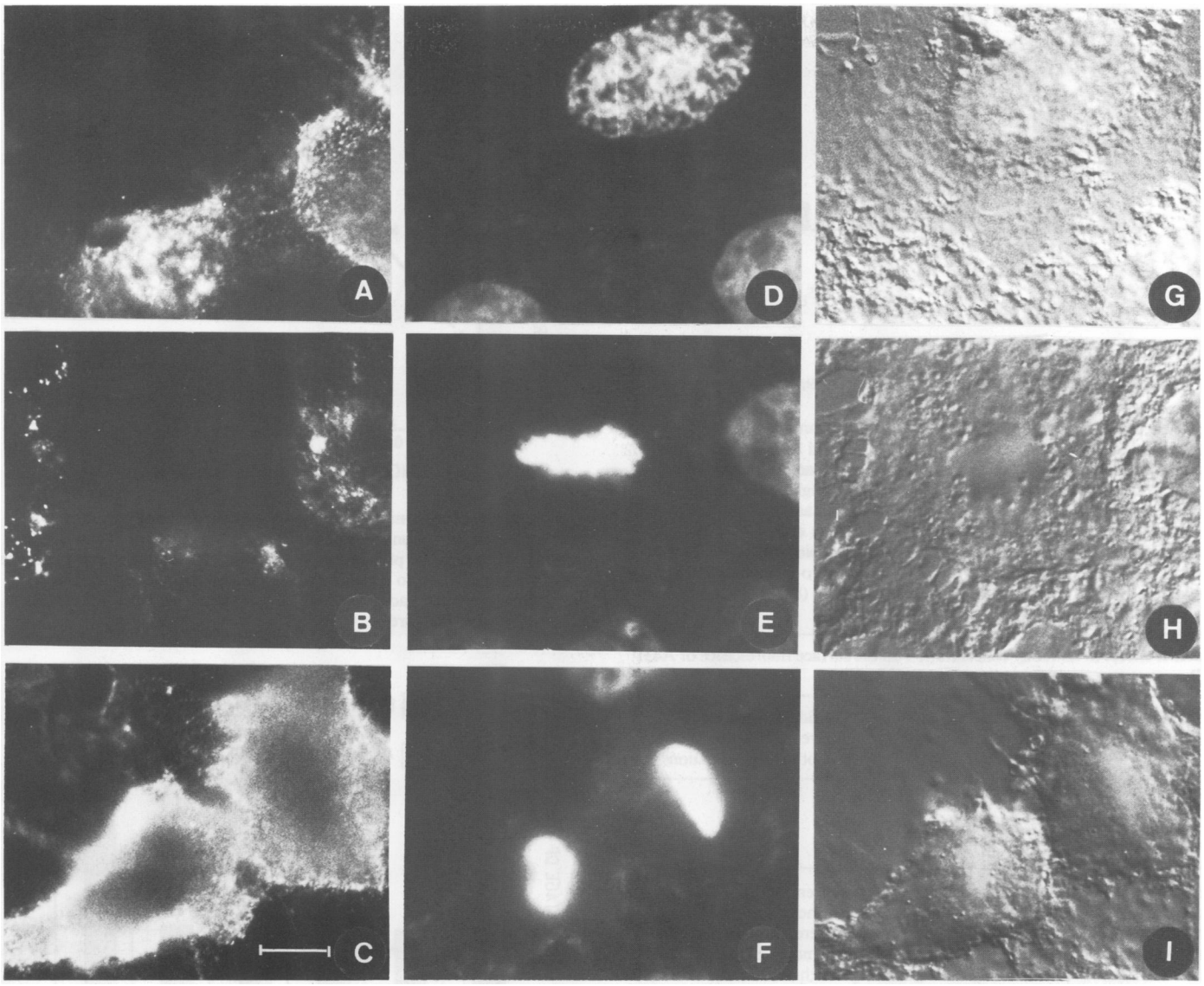

Fig. 2. Surface transferrin receptors labelled with a polyclonal antibody. **(A–C)** Fixed cells were labelled with a polyclonal antiserum to the transferrin receptor followed by a rhodamine-conjugated, sheep anti-rabbit IgG; **(D–F)** corresponding Hoechst fluorescence showing mitotic cells in **(D)** prophase, **(E)** metaphase and **(F)** telophase; **(G–I)** corresponding Nomarski images. Note that the results are comparable to those in Figure 1 using a monoclonal antibody. Magnification x1000; bar 10 μm.

the underside of mitotic cells, nearest to the coverslip, where they might have been inaccessible to antibody labelling. However, it was clear not only that interphase cells were labelled on their undersurface (by focusing up and down), but also that mitotic cells, being rounder, should have had more of their surface accessible to antibody, not less. The loss can also not be explained by changes in receptor turnover since pretreatment for 1 h with inhibitors of protein synthesis (10 μg/ml cycloheximide) or degradation (0.1 mM chloroquine) had no effect on the surface pattern (data not shown).

Not all mitotic cells had as few surface transferrin receptors as those presented in Figures 1 and 2. A431 cells were synchronised using a double-thymidine block before antibody labelling so as to maximise, for purposes of scoring, the number of mitotic cells. The surface fluorescence of mitotic and interphase (mostly G2, see below) cells was scored by eye and the results are presented in Figure 3. Approximately 95% of the interphase cells had medium or light surface fluorescence and 3% were dark. Upon entry into prophase the number of dark cells increased ~20-fold so that >65% of prophase cells were dark and this level was more or less maintained through pro-metaphase and metaphase, dropping to 13% in anaphase. Telophase cells displayed more surface fluorescence than interphase cells with the category of very bright cells comprising 17% of the total compared with <2% in interphase cells.

These results, though striking, were necessarily qualitative so the relative number of transferrin receptors in interphase and mitotic A431 cells was quantitated. Efforts to obtain pure populations of mitotic cells by 'shake-off' (Klevecz, 1975) were hampered by the epithelial nature of these cells which tended to come off in clumps contaminated by interphase cells. Synchronisation using a double-thymidine block gave a maximum mitotic index of only 17% (cf. Petersen and Anderson, 1964) so we therefore looked at individual cells in synchronised populations and measured the fluorescence from the bound antibodies. To use the fluorescence as a measure of the number of transferrin receptors, it was im-

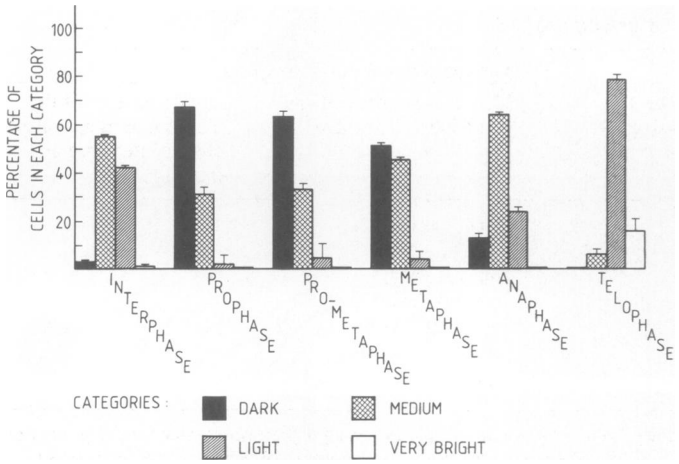

Fig. 3. Scoring the surface transferrin receptors on mitotic and interphase A431 cells. Synchronised cells were fixed and labelled as described in the legend to Figure 1 and the surface fluorescence for each cell phase was categorised by eye as dark, medium, light or very bright. More than 100 cells of each phase were scored over six experiments and the results for each phase are presented as the percentage of cells in each category ± standard error of the mean. Mitosis occupied 1 h of the 24 h cell cycle and was divided into prophase (13 min), pro-metaphase (16 min), metaphase (16 min), anaphase (7 min) and telophase (8 min).

Table I. Effect of antibody concentration on the surface fluorescence of A431 cells

Antibody concentration	Silver extracted as % of 'normal' value	Confidence level of the difference between normal and other concentrations
Normal	100 ± 12 (29)[a]	
Half-normal	67 ± 8 (42)	98%
Twice-normal	115 ± 20 (29)	–

Fixed cells were labelled either with the normal concentration of B3/25, rabbit anti-mouse IgG and rhodamine-conjugated, sheep anti-rabbit IgG, or with half or double these concentrations. Cells were photographed and the silver assayed after extraction from the cut-out images of interphase cells. The number of cells analysed is given in parentheses and the results are presented as the percentage of the mean value for normal antibody concentration. The errors represent the standard error of the mean. Doubling the concentration of the three antibodies did not significantly increase the surface fluorescence.
[a]Silver equivalent to 0.40 A_{500} (after subtraction of background) extracted from 23.4 mg photographic negative.

portant to ensure that each step in the labelling procedure was being carried out using saturating concentrations of antibody. The normal concentration of all antibodies used for labelling was therefore either halved or doubled. Halving the concentrations gave a significant reduction in cell fluorescence whereas doubling the concentrations had no significant effect (Table I).

The fluorescence from individual cells was then measured either directly, using, as a light detector, a photomultiplier tube, or by extracting and assaying the silver from a photographic negative of the fluorescent cell. Both methods are described in more detail in Materials and methods. For the silver extraction procedure the response of the photographic film to increasing fluorescence was determined by photography of a trapped solution of rhodamine-antibody (Figure 4). The amount of silver extracted/mg negative was linear up to 1 μg/ml rhodamine and all results presented here were within this linear portion of the response.

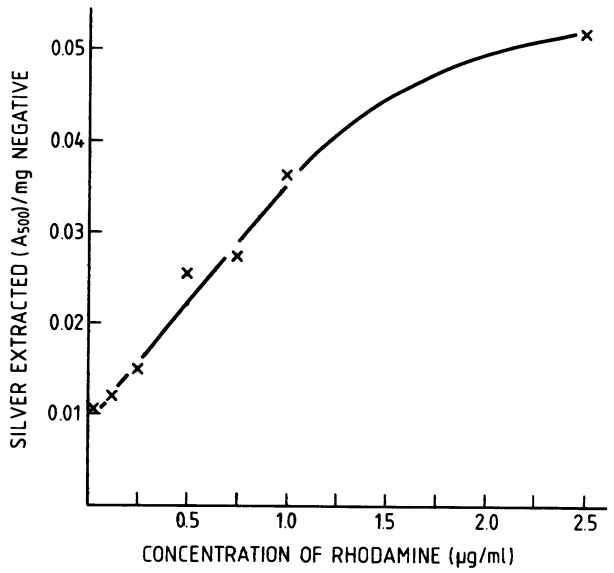

Fig. 4. Effect of increasing concentrations of rhodamine (present as rhodamine-antibody) on the amount of silver (measured at 500 nm) extracted/mg negative prepared as described in Materials and methods. The linearity extended up to 1 μg/ml rhodamine and the intercept on the ordinate represented background which was subtracted when fluorescent cells were being measured.

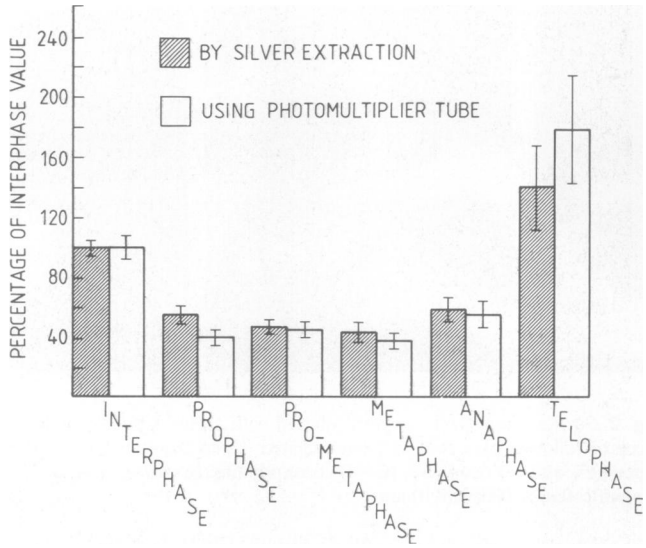

Fig. 5. Quantitation of surface transferrin receptors on mitotic and interphase A431 cells. Fixed cells were labelled as described in the legend to Figure 1 and surface fluorescence was assayed either by silver extraction or by using a photomultiplier tube as described in Materials and methods. Results are expressed as the percentage of the mean interphase value and error bars represent the standard error of the mean. The 100% interphase value for the silver extraction procedure represents silver equivalent to 0.55 A_{500} (after subtraction of background) extracted from 34.2 mg photographic negative.

The two methods give similar results (Figure 5) which confirmed those presented in Figure 3 where the surface fluorescence was scored by eye. The data in Figure 5 obtained using the photomultiplier tube are also presented in Table II together with the confidence levels for the difference between interphase and mitotic cells. Mitotic cells, with the exception of telophase, had approximately half the number of surface receptors found on interphase cells and this difference was significant at a confidence level >99.9%. Telophase cells had

Table II. Quantitation of surface and internal transferrin receptors on mitotic and interphase A431 cells

Phase	Surface fluorescence		Surface and internal fluorescence	
	Fluorescence measured as % of interphase value	Confidence level of the difference in mean values between interphase and other phases	Fluorescence measured as % of interphase value	Confidence level of the difference in mean values between interphase and other phases
Interphase	100 ± 8 (30)[a]		100 ± 8 (50)[a]	
G2	90 ± 9 (30)	–		
Prophase	40 ± 5 (9)	>99.9%	144 ± 17 (9)	90%
Pro-metaphase	45 ± 5 (9)	>99.9%	107 ± 16 (13)	–
Metaphase	38 ± 5 (8)	>99.9%	112 ± 14 (15)	–
Anaphase	55 ± 9 (7)	>99.9%	85 ± 14 (5)	–
Telophase	179 ± 36 (5)	97.5%	90 ± 23 (7)	–

Synchronised cells were fixed and labelled as in the legends to Figures 1 and 5 and the fluorescence was quantitated using a photomultiplier tube. Results are expressed as the percentage of the mean interphase value and errors are given as the standard error of the mean. The number of cells analysed is given in parentheses. There was a significant reduction in the number of surface transferrin receptors during mitosis but not in the total number.
[a]The ratio of total to surface fluorescence was ~1.7.

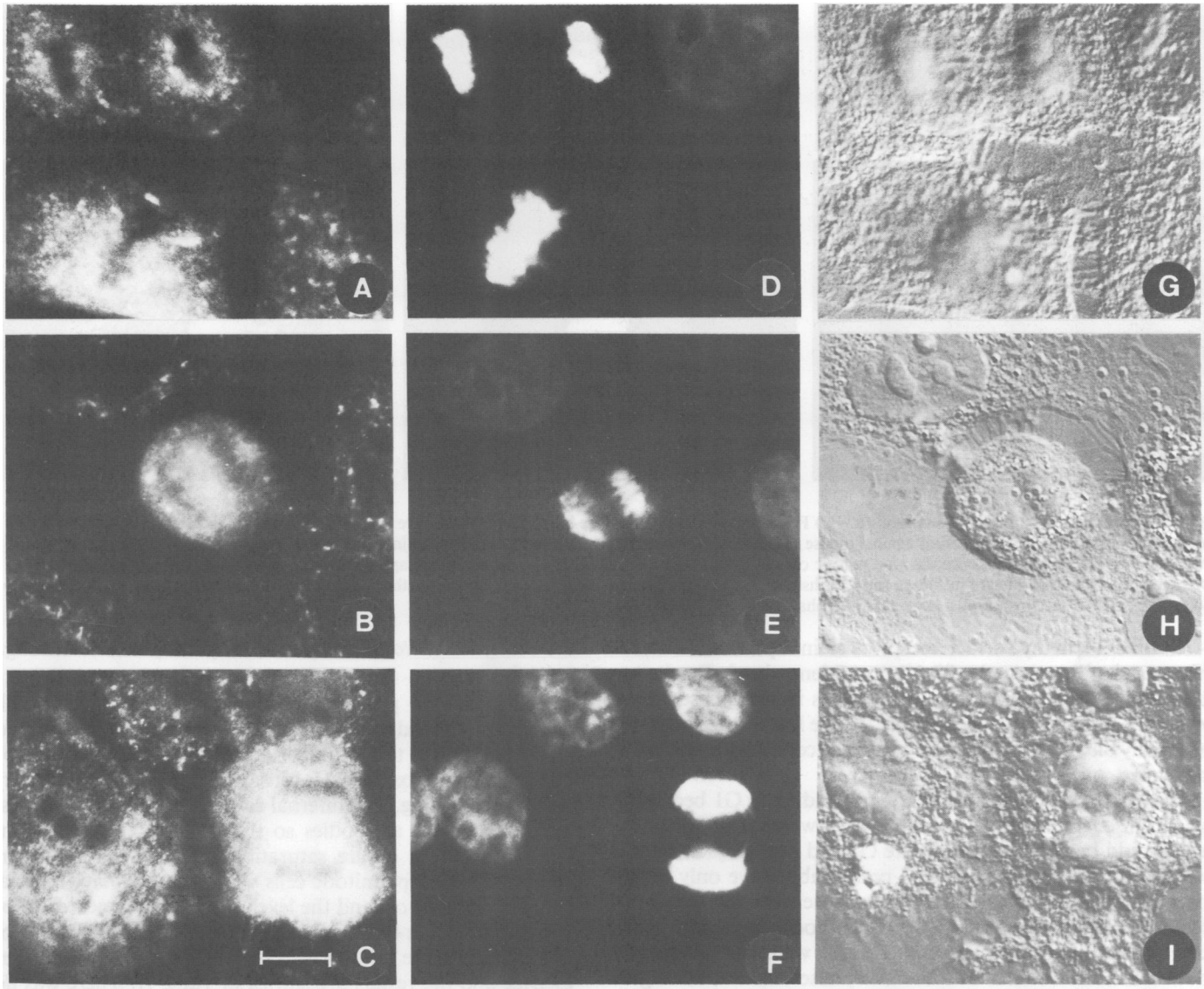

Fig. 6. Surface and internal transferrin receptors labelled with polyclonal or monoclonal antibodies. **(A–C)** Fixed cells were permeabilised using Triton X-100 before labelling with B3/25 **(A,B)** or a polyclonal antiserum to the transferrin receptor **(C)**; **(D–F)** corresponding Hoechst fluorescence showing mitotic cells in **(D** lower left) metaphase; **(E–F)** anaphase, and **(D** upper left) telophase; **(G–I)** corresponding Nomarski images. Note that mitotic cells have internal transferrin receptors. Magnification x1000; bar 10 μm.

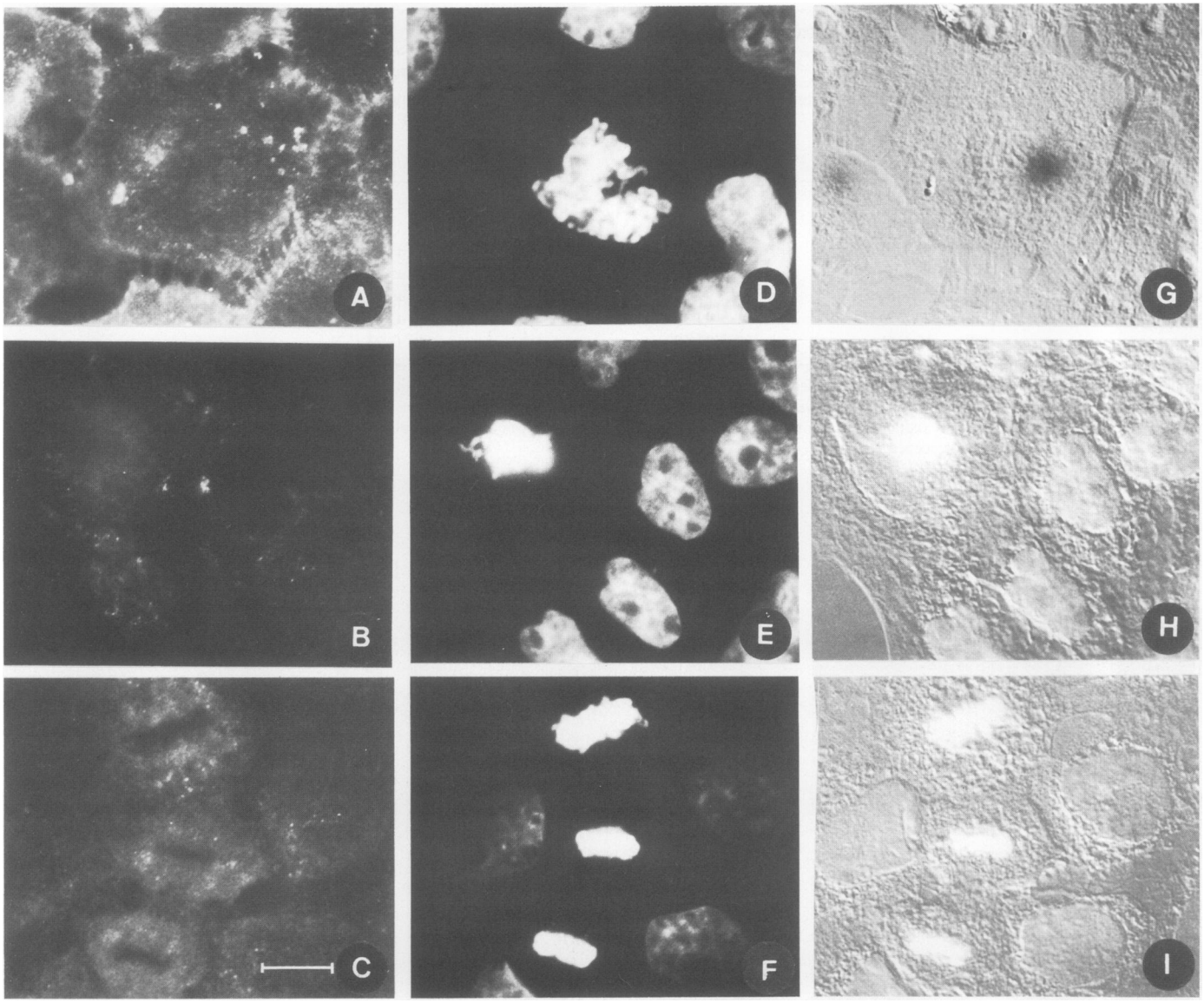

Fig. 7. Surface and internal HLA receptors. **(A–C)** Fixed cells were **(A,B)** surface-labelled, or **(C)** surface and internally labelled after Triton X-100 treatment, with a polyclonal antiserum against mouse H-2 which recognises HLA, followed by a rhodamine-conjugated, sheep anti-rabbit IgG; **(D–F)** corresponding Hoechst fluorescence showing mitotic cells in **(D)** pro-metaphase, **(E, F** upper middle) metaphase and **(F** lower middle) telophase; **(G–I)** corresponding Nomarski images including the Hoechst fluorescence in **H** and **I**. Note that, in contrast with the transferrin receptor, HLA is found roughly to the same extent on the surface of mitotic and interphase cells. Magnification x1000; bar 10 μm.

~1.8 times as many surface receptors as interphase cells and this was significant at the 97.5% confidence level. Furthermore, since the total number of receptors in interphase cells was ~1.7 times the surface number (Table II) this means that essentially all of the receptors in telophase cells were at the cell surface.

Interphase cells were mostly late G2 and early G1 because synchronised populations of A431 cells were used. Mitotic cells should be compared with late G2 and not early G1 cells which are only half the size and presumably have only half the number of receptors. The times chosen for the double-thymidine block (see Materials and methods) seemed to ensure that the majority of interphase cells were late G2 since the surface fluorescence of these interphase cells was not significantly different from that of late G2 cells prepared by adding nocodazole to prevent entry into G1 (Table II). In this and all subsequent experiments the interphase cells can therefore be considered as equivalent to late G2 cells.

No reduction in total transferrin receptors from G2 through mitosis

To eliminate the possibility that, early in mitosis, the cells were simply shedding plasma membrane containing transferrin receptors, the total number of receptors was estimated by permeabilising fixed A431 cells with Triton X-100 before antibody labelling. The internal receptors appeared to be less accessible to the antibodies so the antibody concentrations were doubled to ensure saturation of receptors. By fluorescence microscopy mitotic cells were found to have internal transferrin receptors and the level of labelling appeared to be equivalent to that of interphase cells (Figure 6). This was confirmed using the photomultiplier tube to measure fluorescence and the results are shown in Table II. There was no significant reduction in total transferrin receptors as interphase (G2) cells traversed mitosis. The increase noted for prophase cells was not found in other experiments using the silver extraction procedure (data not shown).

Table III. Quantitation of surface and internal HLA on mitotic and interphase A431 cells

Phase	Surface fluorescence		Surface and internal fluorescence	
	Silver extracted as % of interphase value	Confidence level of the difference in mean values between interphase and other phases	Silver extracted as % of interphase value	Confidence level of the difference in mean values between interphase and other phases
Interphase (G2)	100 ± 14 (13)[a]		100 ± 7 (22)[b]	
Prophase	83 ± 18 (3)	–	114 ± 21 (3)	–
Pro-metaphase	96 ± 10 (7)	–	93 ± 10 (7)	–
Metaphase	94 ± 20 (7)	–	83 ± 7 (8)	90%
Anaphase	103 ± 21 (5)	–	93 ± 21 (5)	–
Telophase	87 ± 21 (5)	–	108 ± 10 (4)	–

Synchronised cells were fixed and labelled as in the legend to Figure 7, photographed and the silver assayed after extraction from the cut-out images of mitotic and interphase cells. Results are expressed as a percentage of the mean interphase value and errors are given as the standard error of the mean. The number of cells analysed is given in parentheses. With one exception, at the 90% confidence level, there was no significant difference between interphase and mitotic cells with respect to either surface or total HLA.

[a]Silver equivalent to 0.12 A_{500} (after subtraction of background) extracted from 25.5 mg photographic negative.

[b]Silver equivalent to 0.17 A_{500} (after subtraction of background) extracted from 20.3 mg photographic negative.

Surface and total HLA unchanged from G2 through mitosis

The behaviour of the transferrin receptor on A431 cells was not shared by all other cell surface proteins. Mitotic A431 cells had surface and internal histocompatability antigens (HLA) similar to that of interphase cells (Figure 7). The amounts of cell HLA, measured by silver extraction and presented in Table III, show that there was no significant difference in surface or total HLA between interphase (G2) and mitotic cells. The one possible exception (total HLA in metaphase cells) was not a consistent finding.

Inhibition of FITC-dextran uptake in mitotic A431 cells.

Synchronised A431 cells were allowed to endocytose FITC-dextran for 3 min and were then fixed, washed and stained with Hoechst dye 33258. When scored for the presence or absence of internal fluorescence, ≤1% of pro-metaphase, metaphase and anaphase cells had taken up FITC-dextran, whereas 100% of the interphase cells were positive (Figure 8). The number of positive telophase cells (27%) was consistent with the resumption of endocytosis at the end of telophase as reported by Berlin and Oliver (1980). The large number of labelled prophase cells was presumably derived from late G2 cells which had taken up FITC-dextran and then entered prophase. This was corroborated by allowing endocytosis for only 30 s which reduced the number of labelled prophase cells by more than half without affecting the percentage of labelled interphase cells. This also showed that endocytosis was inhibited very early in prophase.

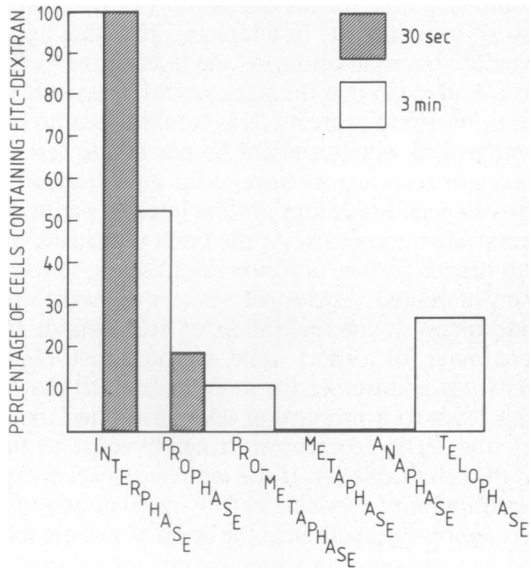

Fig. 8. Uptake of FITC-dextran by mitotic and interphase A431 cells. A431 cells were incubated for 3 min (□) or 30 s (□) with 50 mg/ml FITC-dextran, fixed, washed and labelled with Hoechst dye 33258. 20–50 cells of each phase were scored for the presence or absence of internal FITC-dextran.

Discussion

The reduction in cell surface transferrin receptors during mitosis in A431 cells occurred without a significant reduction in the total number of receptors and was not observed for HLA, a protein which functions at the cell surface. The reduction is therefore most easily explained as an imbalance in receptor recycling that occurs at the onset of mitosis. Recycling of receptors back to the cell surface would be inhibited first and internalisation would continue for a short time, sufficient for half of the receptors to be internalised. This should take ~2.5 min (Hopkins and Trowbridge, 1983) and internalisation would then cease, consistent with the observed inhibition of FITC-dextran uptake, early in prophase, in this (Figure 8) and other (Berlin *et al.*, 1978; Berlin and Oliver, 1980) cell lines. For a macrophage cell line (Berlin and Oliver, 1980), endocytosis ceased within 0.5 min of the cells entering prophase and if this holds true for A431 cells then recycling would have to be inhibited a few minutes before cells enter prophase.

If internalisation continues for only 2.5 min after the inhibition of recycling then only a few percent of the plasma membrane surface will have been internalised (Silverstein *et al.*, 1977). Coated pits comprise a few percent of the plasma membrane surface (Goldstein *et al.*, 1979) so there would only be enough time for a single round of coated vesicle formation from coated pits. Since about half of the surface transferrin receptors are internalised, at least half of them must therefore be present in coated pits at any given time in late G2. Hopkins and Trowbridge (1983) have shown that >75% of the receptors are in fact coated in pits in A431 cells. The corresponding figure for HeLa cells is ~10% (Bretscher, 1984) which would explain why we have not seen as marked a reduction in surface transferrin receptors on mitotic HeLa

cells (data not shown). In general, for other receptors, we might therefore expect a significant reduction during mitosis only if most of the receptors are normally present in coated pits. During telophase, transferrin receptors re-appear on the cell surface to a level higher than that during interphase (G2). This might be explained by the fact that endocytosis does not resume until the end of telophase (see Figure 8 and Berlin and Oliver, 1980) so that receptors recycled back to the cell surface accumulate until internalisation resumes and restores the steady state. Overall these data would argue that recycling stops before internalisation at the onset of mitosis and resumes before internalisation at the end of mitosis.

If these interpretations apply at the level of the Golgi complex they would support our model for its fragmentation. The time taken for complete fragmentation in Normal Rat Kidney cells (Burke *et al.*, 1982) is 10–15 min which is significantly longer than the 2.5 min between inhibition of receptor recycling and internalisation. There could be many reasons for this, the most important point being, however, that inhibition of fusion (recycling) would precede that of budding (internalisation) allowing time for the disassembly of the Golgi stack. It is also of interest that, in telophase, recycling appears to resume before internalisation. At the level of the Golgi complex this would mean that the stack would re-assemble before it started to transport proteins. This would appear to be a sensible arrangement which it might be possible to test.

For receptor recycling we have so far only discussed events at the plasma membrane and not the internal pathway taken by the transferrin receptors. At the onset of mitosis when recycling to the cell surface (a fusion event) stops, so too should the fusion of coated vesicles (or vesicles derived therefrom) with endosomes. If internalisation of receptors (a budding event) continues for a short while, so too should removal of receptors from endosomes for delivery back to the cell surface. This leads to a problem at telophase when the surface levels of transferrin receptor are roughly equal to the total levels in the cell (Table II). If the telophase level is explained as the resumption of recycling before internalisation then the surface receptors internalised at the onset of mitosis should be delivered to endosomes in telophase but they should not be able to leave the endosome and appear on the cell surface until budding resumes. The simplest way to explain how this internal pool could appear on the surface of telophase cells is to suggest that the vesicles, instead of fusing with endosomes, can fuse directly with the cell surface in a process analogous to that observed for other internalised molecules (Tietze *et al.*, 1982; Weigel and Oka, 1984; Townsend *et al.*, 1984). Clearly more work will be needed to clarify this point.

There are other conditions which lead to a reduction in cell surface transferrin receptors during interphase but none can explain our observations on mitotic cells because all involve changing some component in the medium bathing the cells. One condition, however, deserves further comment. Addition of phorbol esters can cause a rapid and reversible internalisation of transferrin receptors (Rovera *et al.*, 1982) and this is accompanied by hyperphosphorylation of the receptor (May *et al.*, 1984). Since changes in phosphorylation are known to initiate certain processes early in mitosis (e.g., disassembly of the nuclear lamins, Gerace and Blobel, 1980) it would be of interest to know whether the transferrin receptor becomes hyperphosphorylated as cells enter mitosis. Such a phosphorylation might help provide a molecular explanation for the inhibition of vesicle fusion which stops receptor recycling.

In conclusion, we suggest that the inhibition of vesicle-mediated traffic during mitosis occurs in two temporally distinct steps with the inhibition of vesicle fusion preceding that of vesicle budding. Cell-free systems are now needed to determine the molecular basis of this inhibition.

Materials and methods

Cells and cell synchronisation

The human epidermoid carcinoma, A431 (Fabricant *et al.*, 1977), was maintained as described by Anderson *et al.* (1981) and under these conditions the cells doubled every 24 h. Trypsinised cells were seeded at 1 x 10^4 cells/cm^2 on autoclaved glass coverslips (PolyLabo, Paul Block and Cie, Strasbourg, France) and were either used 48 h later, or after 24 h were synchronised using a double-thymidine block (Peterson and Anderson, 1964). Thymidine (2 mM) was present for 19 h, absent for 8 h, and re-added for 19 h. The cells, at the G1/S boundary, were then washed free of thymidine and ~7 h later they reached a mitotic index of ~17%, comparable with that obtained using Chinese hamster ovary cells (Peterson and Anderson, 1964). G2 cells were obtained by adding 0.04 μg/ml nocodazole (Zieve *et al.*, 1980) 2 h after final removal of thymidine for a total of 5 h. Nocodazole prevents mitotic cells from entering G1 so that nearly all of the interphase cells which were then scored for transferrin receptor were in the G2 phase.

Sample preparation for immunofluorescence microscopy

The general procedures described by Wang *et al.* (1982) were followed. Cells on coverslips were rapidly transferred from growth medium at 37°C to 3% (w/v) formaldehyde containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at room temperature. After 20 min incubation the cells were washed [three times using phosphate-buffered saline (PBS) pH 7.4] and quenched by a 10 min incubation with 50 mM NH_4Cl in PBS. After washing, some coverslips were incubated for 3 min with 0.2% (w/v) Triton X-100 in PBS to expose intracellular antigenic sites. The cells were then washed and non-specific binding sites were blocked by a 10 min incubation with PBS containing 0.2% (w/v) gelatin which was used in all subsequent washing and antibody labelling steps. After washing, the edge of the coverslip was touched to filter paper to remove excess liquid and was then inverted on to 100 μl drops of antibody on parafilm in a humid chamber. The first antibody, the B3/25 monoclonal anti-transferrin receptor antibody (Trowbridge and Omary, 1981), was used at 20 μg/ml. Before use, debris was removed from this and all subsequent antibody-containing solutions by a 1 min centrifugatiion in an Eppendorf microfuge. After 20 min incubation, the cells were washed and labelled for 20 min with affinity-purified rabbit anti-mouse IgG (Ternynck and Avrameas, 1976) used at 10 μg/ml followed, after washing, by a 20 min incubation with 4 μg/ml of an affinity-purified sheep anti-rabbit IgG (Ternynck and Avrameas, 1976) coupled to rhodamine. Each IgG molecule was coupled to four molecules of rhodamine and the coupling was carried out as described by Brandtzaeg (1973) except that the fluorochrome was dissolved in dioxane:di-methylformamide (4:1, v:v) instead of acetone.

In other experiments cells were labelled with either rabbit anti-transferrin receptor antiserum (Bleil and Bretscher, 1982) or rabbit anti-H2 antiserum (Kvist *et al.*, 1978) at a 100-fold dilution, and then with the rhodamine-conjugated antibody.

Finally, the cell chromosomes were stained using Hoechst dye 33258 (Berlin *et al.*, 1978), the coverslips rinsed in water, drained and mounted in Mowiol (Heimer and Taylor, 1974).

Immunofluorescence microscopy and fluorescence quantitation

Cell fluorescence was scored in three ways:

By eye. Cells were observed using epi-illumination on a Zeiss Photomicroscope III equipped with a planapo X63 oil immersion objective and appropriate filter sets for rhodamine and Hoechst dye 33258 (Berlin *et al.*, 1978). It proved relatively easy to classify the surface fluorescence of mitotic and interphase cells into four categories: dark, medium, light and very bright.

By silver extraction. The cells viewed by eye were photographed using a 45 s exposure at a magnification of 280x using Kodak Tri-X film rated at 3200 ASA. The images of mitotic and interphase cells were cut out of the negative, particular care being taken to choose cells that did not have neighbouring cells growing over or under them. For mitotic cells this was particularly difficult but in no instance did any overlapping parts of the bright interphase cells constitute >5% of the estimated area of the mitotic cell image. The cut-out images were then weighed and the silver extracted, using 0.5 ml 1 M NaOH, and assayed as described by Suissa (1983). Background was subtracted using an equivalent weight of negative from a region of the slide containing no cells. Cells not labelled with the first antibody gave background levels of extracted silver. A standard curve relating extracted silver to fluorescence was con-

structed by photographing a 25 μm thick film of rhodamine-conjugated antibody diluted to varying extents in PBS.

Using a photomultiplier. This approach was modelled on that taken by Berlin and Oliver (1980). Rhodamine-labelled cells were observed using an inverted Zeiss microscope IM35 equipped with a X63 plan Neofluar objective and the filters specified above. Mitotic and interphase cells were located and centred using the filters suitable for Hoechst dye 33258. A circular field diaphragm (2.3 mm diameter) was then inserted in the image plane prior to the binocular eyepiece and this restricted the observed field to a diameter of 34 μm, sufficient to encompass the largest of the cells being measured. The excitation iris was then closed until as much of the cell of interest as possible was visible with minimal interference from neighbouring cells. The diameter of the observed field varied from 20 to 34 μm and this arrangement ensured that only fluorescence from the cell of interest would be measured (Koppel *et al.*, 1976). The rhodamine filters were then inserted and the light switched from the binocular eyepiece to a photomultiplier tube (Hamamatsu, R647-01) operated at minus 500 volts. The response was linear over the range of fluorescence studied and background was subtracted by focusing on an area of the same size but not containing cells.

For any experiment using silver extraction or a photomultiplier the results for mitotic cells were expressed relative to the average interphase cell set at 100%. Results from several experiments could then be pooled and expressed as the mean ± standard error of the mean. The confidence level of any observed difference was then determined using Student's t test and was considered significant only at the ≥90% level. In some cases, the distribution of all the differences between cells from the two conditions being compared was plotted directly. For each of the Gaussian-like probability histograms, the estimated mean of the differences gave a confidence level identical to that obtained using Student's t test.

Uptake of FITC-dextran

This was performed as described by Berlin and Oliver (1980) using 50 mg/ml FITC-dextran (Sigma) in growth medium.

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