Okadaic acid treatment leads to a fragmentation of the *trans*-Golgi network and an increase in expression of TGN38 at the cell surface

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Okadaic acid (OA) is a protein phosphatase inhibitor which has, among other properties, previously been shown to induce a fragmentation of the cisternae of the Golgi stack [for review, see Lucocq (1992) J. Cell Sci. **103**, 875–880]. The effects of OA are reversible and mimic intracellular events which occur during mitosis. To date, due to a lack of endogenous marker proteins, the effects of OA on the *trans*-Golgi network (TGN) have not been studied. Certain drugs, e.g. Brefeldin A (BFA), have different effects on the morphology of the Golgi stack and the TGN; it is therefore relevant to ask what effect(s) OA has on the TGN. We now present data from a study in which we have used antibodies to TGN38, an integral membrane protein predominantly localized to the TGN of rat NRK cells [Luzio, Brake, Banting, Howell, Braghetta and Stanley (1990) Biochem. J. **270**, 97–102], to investigate the effects of OA on this organelle. OA induces a reversible fragmentation of the TGN. This fragmentation occurs with similar kinetics to that observed within the Golgi stack, and is independent of protein synthesis. The sensitivity of the TGN to OA is similar to that of the Golgi stack. The fragmentation of the TGN induced by OA also leads to a 10-fold increase in the level of TGN38 expressed at the plasma membrane.

INTRODUCTION

The classical secretory pathway in higher eukaryotic cells involves the passage of newly secreted proteins through a discrete set of intracellular compartments before sorting and targeting to the appropriate membrane. The organelle from which proteins are delivered to the appropriate membrane has been termed the *trans*-Golgi network (TGN) (Griffiths and Simons, 1986), and has been shown to be an organelle independent of the Golgi stack (Doms et al., 1989; Chege and Pfeffer, 1990; Reaves and Banting, 1992). It is well documented that the Golgi apparatus disassembles during mitosis, and that this phenomenon can be mimicked by incubation of cells in okadaic acid (OA) (for review see Lucocq, 1992). OA is a protein phosphatase inhibitor with a specificity for protein phosphatase 1 and protein phosphatase 2A (Takai et al., 1987; Bialojan and Takai, 1988). It has been shown previously that incubation of interphase cells with OA induces disassembly of the cisternae of the Golgi stack in a process analogous to that which occurs during mitosis (Yamashita et al., 1990; Lucocq et al., 1991; Thyberg and Moskalewski, 1992). OA treatment also has the effects of (i) stopping transport from the endoplasmic reticulum to the Golgi apparatus (Lucocq et al., 1991; Davidson et al., 1992), (ii) inhibiting regulated secretion (Churcher et al., 1990; Gomperts et al., 1990; Takuma and Ichida, 1991; Whalley et al., 1991; Yanagihara et al., 1991), and (iii) inhibiting fluid-phase endocytosis (Lucocq et al., 1991). All of these phenomena have been observed in mitotic cells (Berlin and Oliver, 1980; Hesketh et al., 1984; Warren et al., 1984; Featherstone et al., 1985). The effects of OA on cells are reversible, and the reassembly of the Golgi apparatus which occurs on OA wash-out is reminiscent of that which occurs during telophase (Yamashita et al., 1990; Lucocq et al., 1991; Thyberg and Moskalewski, 1992). Morphological studies on the effects of OA on the Golgi apparatus have relied on the use of antibodies which recognize mannosidase II and galactosyltransferase, proteins restricted to specific cisternae of the Golgi stack (for review see Lucocq, 1992). Since the TGN is an independent organelle which behaves differently from the cisternae of the Golgi stack in response to certain drugs, e.g. Brefeldin A (BFA) (Lippincott-Schwartz et al., 1991; Reaves and Banting, 1992), it is pertinent to assess the effects of OA on the TGN. A suitable marker protein for the TGN (TGN38) has now been identified, and antibodies to TGN38 have been raised (Luzio et al., 1990; Wilde et al., 1992). These provide a tool for monitoring the effects of OA on the TGN. We now present data which (i) compare the gross effects of OA on the cisternae of the Golgi stack and on the TGN, and (ii) monitor the effects of OA on the cell surface expression of TGN38.

MATERIALS AND METHODS

Materials

The rabbit anti-(rat TGN38) polyclonal antiserum has been described previously (antibody 3 in Wilde et al., 1992). The monoclonal antibody 2F7.1 was produced by standard procedures, essentially as described by Kohler and Milstein (1975), after immunization of a Balb/c female mouse with the synthetic peptide LPSASKPNNTSSENNPPC (corresponding to the N-terminus of the lumenal domain of mature TGN38) conjugated to thyroglobulin via its N-terminal cysteine residue and a MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) bifunctional cross-linker (Sigma Chemical Co., Poole, Dorset, U.K.). The immunization procedure followed was: day 1, intraperitoneal injection with 100 μl of thyroglobulin–peptide conjugate emulsified in 100 μl of complete Freund's adjuvant; day 15, intraperitoneal injection with 100 μl of thyroglobulin–peptide

Abbreviations used: BFA, Brefeldin A; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; M6PR, mannose 6-phosphate receptor; NRK, normal rat kidney; OA, okadaic acid; TfR, transferrin receptor; TGN, *trans*-Golgi network; TRITC, tetramethylrhodamine isothiocyanate isomer R.

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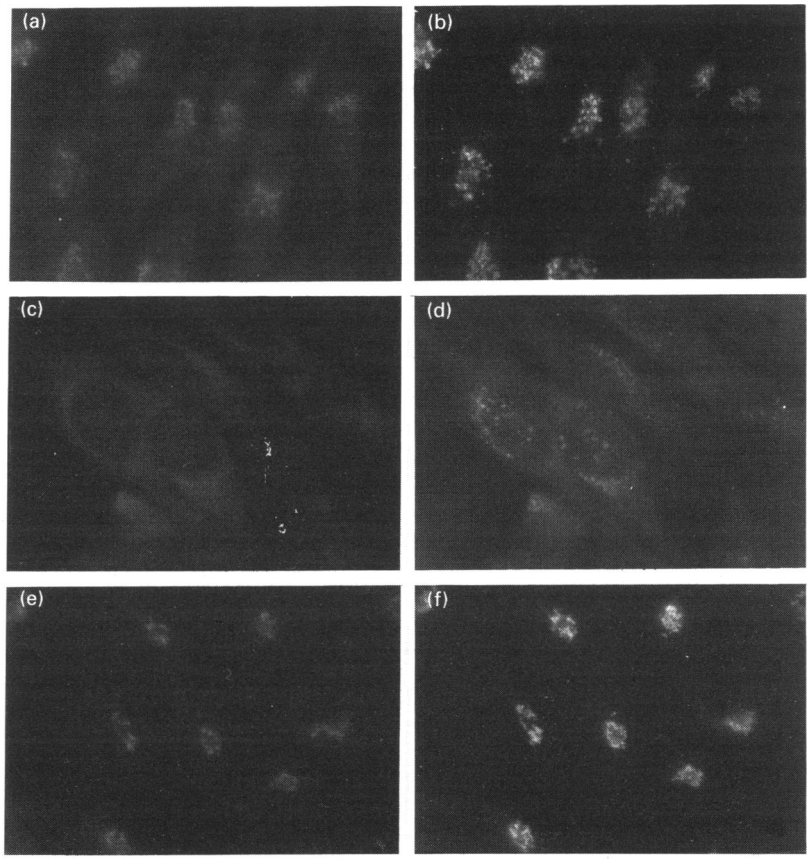

Figure 1 OA effects on the TGN are reversible

NRK cells were incubated in the presence of 0 μM (**a**, **b**) or 0.3 μM OA (**c**, **d**) at 37 °C for 3 h before fixation and processing for double immunofluorescence analysis as described in the Materials and methods section. NRK cells in (**e**) and (**f**) were incubated in 0.3 μM OA at 37 °C for 3 h, washed four times in PBS, then incubated in the absence of OA for 4 h before fixation and processing for double immunofluorescence analysis. Bound rabbit polyclonal antiserum to TGN38 was detected with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (**a**, **c** and **e**), and bound monoclonal anti-mannosidase II was detected with a tetramethylrhodamine isothiocyanate isomer R (TRITC)-conjugated goat anti-mouse IgG (**b**, **d** and **f**). (No differences were observed between control cells incubated in the absence and presence of 0.5% dimethyl sulphoxide).

conjugate emulsified in 100 μl of incomplete Freund's adjuvant; day 35, as day 15; day 49, intravenous injection with 100 μl of thyroglobulin–peptide conjugate in PBS; day 52, mouse killed and spleen removed for fusion. Isolated spleen cells were fused to Sp2/0-Ag14 cells (Shulman et al., 1978) using poly(ethylene glycol) 4000 (Goodfellow et al., 1988). Hybridoma supernatants were initially screened in e.l.i.s.a.s against peptide conjugated to BSA via SMCC [sulphosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate] (Sigma). Positive supernatants were further screened (i) in immunoblots of recombinant TGN38 protein expressed in bacteria (produced as previously described; Luzio et al., 1990), and (ii) in immunofluorescence analysis of methanol-fixed cells, by previously described procedures (Reaves and Banting, 1992). One positive hybridoma was subjected to three rounds of cloning by limiting dilution, and designated 2F7.1. Isotype analysis of this antibody showed it to be an IgG1. The coupling of peptide to carrier proteins used previously published procedures (Green et al., 1982). The peptide was synthesized within the SERC Molecular Recognition Centre, University of Bristol. Fluorescein-labelled goat anti-rabbit IgG was from Sigma, and Texas-Red-labelled goat anti-mouse IgG and enzyme-conjugated antibodies for use in e.l.i.s.a.s and immunoblots were from Dako (High Wycombe, Bucks., U.K.).

OA was purchased from Calbiochem. (U.S.A.) and prepared as a 200 mM stock in 50 % dimethyl sulphoxide. Cycloheximide (Sigma) was prepared immediately before use as a 700 μM stock in double-distilled water.

Methods

Cell culture and immunofluorescence microscopy were performed as previously described (Reaves and Banting, 1992) as was the TGN38 internalization experiment (Reaves et al., 1993), with the minor modifications that the antibody bound to the cell surface was the monoclonal antibody 2F7.1 and the antibody used to detect the total cellular pool of TGN38 was a rabbit polyclonal antiserum (antibody 3 in Wilde et al., 1992). Epifluorescence microscopy was performed on a Zeiss Universal microscope with an $\times 63$ 1.4 oil-immersion objective. Fluorescence-activated cell sorting (FACS) analysis was performed by using standard procedures on a Becton Dickinson FACScan.

RESULTS

Effect of OA on the TGN

It has previously been shown that concentrations of OA as low as 0.25 μM can lead to the fragmentation of the cisternae of the Golgi stack within 1 h (Thyberg and Moskalewski, 1992). In order to compare the effects of OA on the cisternae of the Golgi stack and on the TGN, NRK cells were incubated with 0 μM,

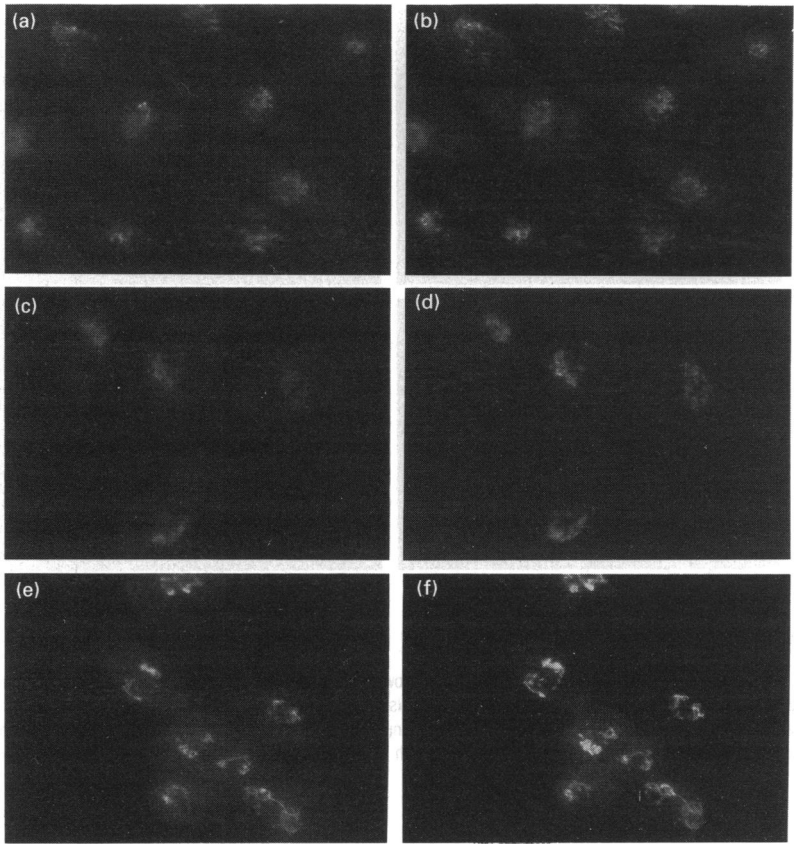

Figure 2 The effects of OA on the TGN are independent of protein synthesis

NRK cells were incubated in the presence of 70 μM cycloheximide alone for 3 h at 37 °C (**a** and **b**), 70 μM cycloheximide and 0.3 μM OA for 3 h at 37 °C (**c** and **d**) or 70 μM cycloheximide and 0.3 μM OA for 3 h at 37 °C, followed by four washes in PBS and a further incubation for 4 h at 37 °C in 70 μM cycloheximide (**e** and **f**) before fixation and processing for double immunofluorescence analysis as described in the Materials and methods section. Bound rabbit polyclonal antiserum to TGN38 was detected with a FITC-conjugated goat anti-rabbit IgG (**a**, **c** and **e**), and bound monoclonal anti-mannosidase II was detected with a TRITC-conjugated goat anti-mouse IgG (**b**, **d** and **f**).

0.05 μM or 0.3 μM OA for either 1 or 3 h. They were then fixed and processed for double immunofluorescence analysis, using a rabbit polyclonal antiserum to TGN38 to decorate the TGN and a mouse monoclonal antibody to mannosidase II to decorate the cisternae of the Golgi stack. The TGN and the cisternae of the Golgi stack both have a juxtanuclear localization in the absence of OA (Figures 1a and 1b). Pretreatment with 0.05 μM OA induces little, if any, detectable fragmentation of either the TGN or the cisternae of the Golgi stack after 1 h, but does lead to some disassembly of both organelles by 3 h (results not shown). However, pretreatment for 1 h with 0.3 μM OA induces clearly discernible fragmentation of both organelles, an effect which is more marked after 3 h (Figures 1c and 1d). The concentration of OA used in all subsequent experiments was 0.3 μM, since this gave a clear fragmentation of the TGN and the cisternae of the Golgi stack within 3 h, and is well below the concentration (0.5 μM) previously shown to be partially toxic to cells (Thyberg and Moskalewski, 1992).

The effects of OA on the TGN are reversible

It has been shown previously that, following OA treatment, the fragmented cisternae of the Golgi stack can reassemble once the OA has been removed (Thyberg and Moskalewski, 1992; Lucocq et al., 1991). Is the same true for the TGN, and are the kinetics of reassembly similar? NRK cells were incubated in 0.3 μM OA at 37 °C for 3 h (Figures 1c and 1d) or incubated in the presence of 0.3 μM OA for 3 h at 37 °C, then washed free of OA (by four washes in PBS) and incubated for various times at 37 °C before fixation and processing for double immunofluorescence analysis, using a rabbit polyclonal antiserum to TGN38 to decorate the TGN and a mouse monoclonal antibody to mannosidase II to decorate the cisternae of the Golgi stack. The kinetics of reassembly were similar for both the TGN and the cisternae of the Golgi stack (results not shown), the process being complete by 4 h (Figures 1e and 1f). This time course of reassembly is similar to that previously described for the cisternae of the Golgi stack in CHO and HeLa cells (Thyberg and Moskalewski, 1992; Lucocq et al., 1991).

The effects of OA on the TGN are independent of protein synthesis

The OA-induced disassembly of the cisternae of the Golgi stack is independent of protein synthesis (Yamashita et al., 1990; Lucocq et al., 1991; Thyberg and Moskalewski, 1992). Is the same true for the OA-induced fragmentation of the TGN? The protein-synthesis inhibitor cycloheximide was chosen to address this question. NRK cells were incubated in media containing 70 μM cycloheximide (Figures 2a and 2b), or 0.3 μM OA and

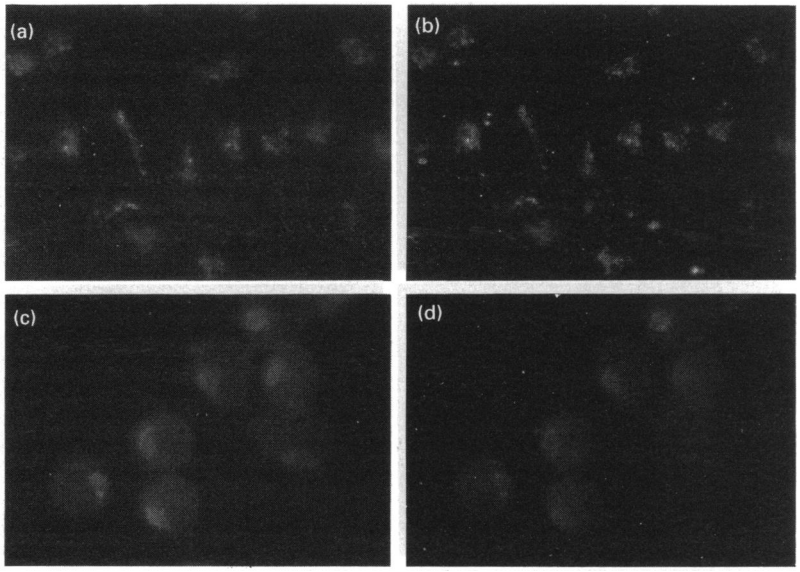

Figure 3 TGN38 is internalized and routed to the TGN in OA-treated cells

Control intact NRK cells were incubated with monoclonal antibody 2F7.1 for 1 h at 4 °C (to allow antibody binding to cell-surface TGN38) and a further 3 h at 37 °C (to allow internalization) (**a** and **b**). A second set of intact NRK cells were incubated with 0.3 μM OA for 3 h at 37 °C to disrupt the TGN. The monoclonal antibody 2F7.1 was then added for 1 h at 4 °C (to allow antibody binding to cell-surface TGN38) and the cells were incubated at 37 °C for a further 3 h in the continued presence of 0.3 μM OA (to allow internalization) (**c** and **d**). Cells were then fixed and incubated with rabbit polyclonal anti-TGN38 antibody. Bound 2F7.1 (internalized TGN38) was detected with a TRITC-conjugated goat anti-mouse IgG (**a** and **c**); bound rabbit polyclonal antiserum (total cell-associated TGN38) was detected with a FITC-conjugated goat anti-rabbit IgG (**b** and **d**).

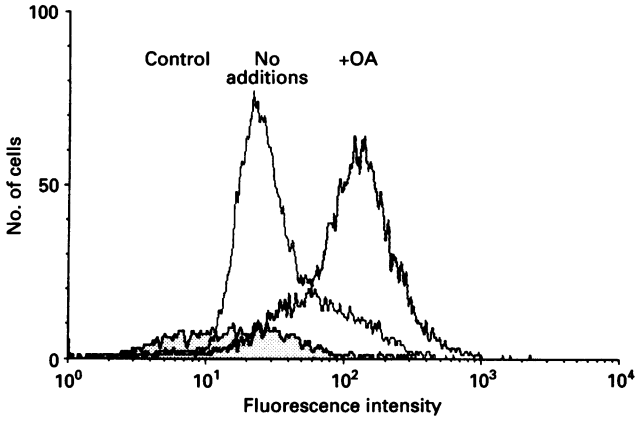

Figure 4 OA treatment induces an elevated level of TGN38 expression at the plasma membrane

NRK cells which had received no additives (no additions) and NRK cells which had been incubated in 0.1 μM OA for 3 h (+ OA) were processed for FACS analysis as described in the Materials and methods section. Mouse monoclonal anti-TGN38 antibody bound to the surface of intact cells was recognized by a FITC-conjugated goat anti-mouse IgG and detected by FACS analysis. The 'control' cells were not incubated with a primary antibody, and their fluorescence intensity (shaded area) represents non-specific binding of the FITC-conjugated antibody. This experiment was repeated three times with similar results. A representative result is presented.

70 μM cycloheximide (Figures 2c and 2d) for 3 h at 37 °C before fixation and processing for double immunofluorescence analysis, using a rabbit polyclonal antiserum to TGN38 to decorate the TGN (Figures 2a and 2c) and a mouse monoclonal antibody to mannosidase II to decorate the cisternae of the Golgi stack (Figures 2b and 2d). Cycloheximide treatment does not affect the pattern of staining observed with either antibody (compare Figure 1a with Figure 2a, and Figure 1b with Figure 2b). Neither does it protect either the TGN or the cisternae of the Golgi stack from the effects of OA (compare Figure 1c with Figure 2c, and Figure 1d with Figure 2d). The same result was obtained when cells were incubated in cycloheximide for 3 h before the addition of OA for a further 3 h in the continued presence of cycloheximide (results not shown). Thus protein synthesis is not required for the OA to exert its effect on the morphology of the TGN. A further experiment demonstrated that protein synthesis is not required for recovery from the effects of OA. NRK cells were incubated in medium containing 0.3 μM OA for 3 h at 37 °C, washed four times in PBS and then incubated for a further 3 h at 37 °C in medium containing 70 μM cycloheximide before fixation and processing for double immunofluorescence analysis as described above. Both the TGN (Figure 2e) and the Golgi stacks (Figure 2f) can be seen to be capable of reassembly in the absence of protein synthesis.

OA treatment leads to an increase in cell surface expression of TGN38

TGN38 has previously been shown to recycle between the TGN and the plasma membrane, with little of the molecule at the plasma membrane at any one time (Ladinsky and Howell, 1992; Reaves et al., 1993; Bos et al., 1993). OA has been shown to inhibit fluid-phase endocytosis (Lucocq et al., 1991), but its effect on the endocytosis of integral membrane proteins varies according to the protein. The amount of mannose 6-phosphate receptor (M6PR) present at the cell surface decreases upon OA treatment, although its rate of internalization is unaffected, whereas the amount of transferrin receptor (TfR) at the cell surface is unaltered by OA treatment (Braulke and Mieskes, 1992). In light of these data we chose (i) to ask whether TGN38

was still internalized from the cell surface and returned to the TGN in OA-treated cells, and (ii) to assess the level of expression of TGN38 at the cell surface of control and OA-treated cells. Internalization of TGN38 from the cell surface and its intracellular targeting were assayed as previously described (Reaves et al., 1993), by using a mouse monoclonal antibody (2F7.1, raised against the extreme N-terminus of TGN38) to monitor internalized TGN38 (Figures 3a and 3c) and a rabbit polyclonal antibody to TGN38 (antibody 3 in Wilde et al., 1992) to detect the total intracellular pool of the molecule (Figures 3b and 3d). Internalized TGN38 is returned to the TGN of cells treated with OA (Figure 3c and 3d), as it is in control cells (Figure 3a and 3b).

The comparative levels of cell-surface TGN38 expression on NRK cells which had been incubated for 3 h in the absence or presence of OA were assayed by FACS analysis using monoclonal antibody 2F7.1. Cells which had been incubated in as little as 0.1 μM OA for 3 h before FACS analysis showed an approx. 10-fold increase in cell-surface expression of TGN38 (Figure 4).

DISCUSSION

Previous studies have shown that incubation of cells with OA leads to disassembly of the cisternae of the Golgi stack in a process which mimics mitosis (for review see Lucocq, 1992). We have now addressed the question of whether the TGN is also affected by OA. The data that we present demonstrate that the TGN and the cisternae of the Golgi stack behave in a similar fashion in response to OA in terms of (i) gross morphological alterations (as assayed by immunofluorescence analysis)(Figure 1), (ii) dose–response to OA, (iii) time course of response to OA, (iv) recovery after OA treatment (Figure 1), and (v) independence from protein synthesis (Figure 2).

Thyberg and Moskalewski (1992) used an antibody to mannosidase II to monitor the disassembly of the cisternae of the Golgi stack in response to OA. They observed that some of the Golgi-derived vesicles in OA-treated cells were not recognized by the anti-mannosidase II antibody, and postulated that such vesicles may be derived from the *trans* cisternae of the Golgi stack, since mannosidase II is predominantly restricted to the medial cisternae (Dunphy and Rothman, 1983; Goldberg and Kornfeld, 1983). In light of the data that we present, it is also likely that at least some of these mannosidase II-negative structures correspond to TGN-derived vesicles.

Although the effects of OA on many intracellular transport events have been well documented (Churcher et al., 1990; Gomperts et al., 1990; Yamashita et al., 1990; Takuma and Ichida, 1991; Whalley et al., 1991; Yanagihara et al., 1991; Lucocq et al., 1991; Thyberg and Moskalewski, 1992; Davidson et al., 1992), there is a paucity of data concerning its effect on receptor-mediated endocytosis. The amount of M6PR present at the cell surface decreases in OA-treated cells (although its rate of internalization from the cell surface is unaffected), and the amount of TfR at the cell surface does not change in OA-treated cells (Braulke and Mieskes, 1992). In control cells, and also in BFA-treated cells, TGN38 has been shown to recycle between the TGN and the cell surface, with a steady-state distribution where the majority of TGN38 is in the TGN (Ladinsky and Howell, 1992; Reaves et al., 1993; Bos et al., 1993). It has been localized to clathrin-coated endocytic vesicles (Ladinsky and Howell, 1992), and utilizes a tyrosine-containing internalization motif analogous to that used by many integral membrane proteins which are internalized via clathrin-coated vesicles (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). It is now evident that, in contrast with the M6PR and unlike the TfR, the level of cell-surface TGN38 expression can increase on incubation of cells with OA (Figure 4). This might simply be a result of vesiculated fragments of the TGN fusing with the plasma membrane. An alternative explanation is that OA directly affects TGN38. The cytoplasmic domain of the M6PR has been shown to be specifically phosphorylated in the TGN (Meresse and Hoflack, 1993), and OA has been shown to inhibit its dephosphorylation (Braulke and Mieskes, 1992). Thus, in the case of the M6PR, OA may cause a decrease in cell surface expression by 'locking' the protein in its phosphorylated, TGN-associated, form. If the cytoplasmic domain of TGN38 were also phosphorylated, and that phosphorylation were to occur specifically at the plasma membrane, then the inhibition of dephosphorylation by OA might partially 'lock' the protein at the cell surface. To date, however, there are no published results which suggest that TGN38 is phosphorylated at any stage of its recycling pathway.

We are grateful to Dr. G. Warren for provision of the monoclonal antibody to mannosidase II, to Dr. B. Reaves and Dr. A. Wilde for constructive comments on the manuscript, and to the M.R.C. for financial support.

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Received 28 September 1993/17 February 1994; accepted 21 February 1994