Retention of epidermal growth factor receptors in the endoplasmic reticulum of adenovirus-infected cells

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The epidermal growth factor (EGF) receptor is down-regulated during early infection with adenovirus, and this has been attributed to accelerated internalization and degradation of the receptor in the absence of ligand (Carlin, Tollefson, Brady, Hoffman & Wold (1989) *Cell* **57**, 135–144]. Using pulse–chase analysis, we show that loss of functional EGF receptors after infection of human KB and A431 cells with adenovirus type 5 is accompanied by accumulation of a receptor precursor that remains fully sensitive to endoglycosidase H, indicative of retention in the endoplasmic reticulum. A truncated receptor, normally secreted by A431 cells, also accumulates intracellularly as an endoglycosidase H-sensitive precursor. In no case is the block in intracellular transport of EGF receptors complete. We conclude that both stimulation of EGF receptor internalization and degradation and inhibition of intracellular transport of newly synthesized EGF receptors from the endoplasmic reticulum towards the cell surface contribute to EGF receptor down-regulation in adenovirus-infected cells.

INTRODUCTION

The epidermal growth factor (EGF) receptor is a single chain glycoprotein of ~ 170 kDa with ligand-inducible protein tyrosine kinase activity (for review, see Carpenter, 1987). The receptor polypeptide backbone (~ 130 kDa) is glycosylated co-translationally to yield an ~ 160 kDa precursor (Sliker & Lane, 1985; Cummings *et al.*, 1985; Carlin & Knowles, 1986). Addition of core oligosaccharides is essential for both EGF binding capacity and cell surface expression, since both are inhibited by treatment of intact cells with tunicamycin (Soderquist & Carpenter, 1984; Sliker *et al.*, 1986). The endoplasmic reticulum (ER)-resident 160 kDa precursor contains only high-mannose-type oligosaccharides. Oligosaccharide processing of the 160 kDa precursor in the course of transport through the Golgi apparatus results in the mature surface-disposed 170 kDa EGF receptor, which contains mainly complex-type oligosaccharides. Thus endoglycosidase H (endo H) digestion, which distinguishes between high-mannose-type and complex-type oligosaccharides, can be used to follow transport of the newly synthesized EGF receptor. In addition to the 170 kDa EGF receptor, A431 cells secrete a truncated receptor of approx. 110 kDa, consisting of the EGF receptor extracellular domain but lacking both the transmembrane anchor and the cytoplasmic tyrosine kinase domain (Weber & Gill, 1984; Mayes & Waterfield, 1984). Its 95 kDa precursor, containing only high-mannose-type oligosaccharides, can be detected intracellularly in A431 cells (Mayes & Waterfield, 1984).

Several viral proteins have been identified which can modulate EGF receptor expression and function. The vaccinia virus growth factor, an EGF-related polypeptide, binds to the EGF receptor and stimulates its autophosphorylation (Stroobant *et al.*, 1985). The bovine papillomavirus E5 transforming protein was found to inhibit EGF receptor internalization and degradation (Martin *et al.*, 1989). On the other hand, adenovirus infection results in loss of functional EGF receptors (i.e. down-regulation) in the absence of ligand (Carlin *et al.*, 1989). The adenovirus-encoded E3/10.4K protein (Cladaras & Wold, 1985; Tollefson *et al.*, 1990) appears to be responsible for this effect (Hoffman *et al.*,

1990). The observed down-regulation was suggested to be due to accelerated internalization and degradation of the EGF receptor. Since adenovirus infection has been reported to down-regulate major histocompatibility complex MHC class I molecules by abrogating their intracellular transport (Andersson *et al.*, 1985; Burgert & Kvist, 1985; Paabo *et al.*, 1986), we investigated whether a similar mechanism might be involved in EGF receptor down-regulation. The results indicate that, in addition to accelerating EGF receptor internalization and degradation, adenovirus causes retention of newly synthesized EGF receptors in the ER.

MATERIALS AND METHODS

Cell cultures and virus stocks

Human epidermoid carcinoma KB and A431 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS). Adenovirus type 5 (kindly provided by Roel Schiphof and Peter van der Vliet) was grown in KB cells. Virus-containing medium was harvested immediately before each experiment. 1- β -D-Arabinofuranosylcytosine (ara-C, 20 μ g/ml) was added and infections with about 50 plaque-forming units (p.f.u.) per cell were performed as indicated in the Figure legends. For incubations of control cells, culture supernatants of uninfected KB cells were used.

Antibodies

PTyr8 is a rabbit polyclonal anti-phosphotyrosine antibody, prepared essentially as described (Kamps & Sefton, 1988). For the first immunization, phosphotyrosine (Sigma) was coupled to BSA using the cross-linking agent EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide] (Pierce). Subsequent immunizations were done with phosphotyrosine coupled to keyhole-limpet haemocyanin. Anti-phosphotyrosine antibodies were affinity-purified using phosphotyrosine coupled to Affi-gel P15 (Bio-Rad). After incubation of total serum, the column was washed with sodium phosphate, phosphoserine and phosphothreonine to remove antibodies of undesired specificity. Bound antibodies were eluted with phenyl phosphatase and concentrated and dialysed

Abbreviations used: EGF, epidermal growth factor; ER, endoplasmic reticulum; endo H, endoglycosidase H; MHC, major histocompatibility complex; p.f.u., plaque-forming units; FCS, fetal calf serum; ara-C, 1- β -D-arabinofuranosylcytosine; HLA, human leucocyte antigen; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; NP-40, Nonidet P-40; PDGF, platelet-derived growth factor.

against 50 mM-Tris (pH 7.2)/150 mM-NaCl using Sartorius pressure dialysis equipment. The immunoreactivity of PTyr8 against phosphoproteins was completely blocked by phosphotyrosine and *p*-nitrophenyl phosphate, but was not affected by either phosphoserine or phosphothreonine, indicating that the purified PTyr8 is specific for phosphotyrosine. MoAb 108, directed against the extracellular peptide backbone of the human EGF receptor (Bellot *et al.*, 1990), and polyclonal rabbit anti-(synthetic peptide) antibodies, recognizing the EGF receptor C-terminus, were kindly provided by Joseph Schlessinger (N.Y.U. Medical School, New York, NY, U.S.A.). MoAb 66lg10 is directed against the human transferrin receptor (Van de Rijn *et al.*, 1983). MoAb W6/32 recognizes human leucocyte antigen (HLA)-A, -B, and -C locus products in their properly assembled form (Parham *et al.*, 1979).

Immunoblotting

Cells were cultured in 35 mm diameter dishes. After infection with adenovirus, cells were washed twice with ice-cold phosphate-buffered saline (PBS: 137 mM-NaCl, 3 mM-KCl, 8.1 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄, pH 7.4) and incubated in the absence or the presence of EGF (300 ng/ml) on ice. After 10 min, cells were washed twice with cold PBS, and cell lysates were prepared for immunoblotting essentially as described (Verheijden *et al.*, 1990). Briefly, cells were lysed in SDS sample buffer [3% SDS, 10% glycerol, 4 mM-EDTA, 50 μ M-sodium vanadate, 10 mM-*p*-nitrophenyl phosphate, 5% (v/v) β -mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride (PMSF), aprotinin (3 units/ml) and 10 μ g of leupeptin/ml]. Samples were boiled for 5 min and sonicated for 8 s, and equal amounts of protein were loaded on to a 10% SDS/PAGE gel. Following electrophoresis, proteins were transferred to nitrocellulose. The filters were blocked with milk powder (5%) in TBST (10 mM-Tris, pH 8.0, 150 mM-NaCl, 0.05% Tween-20, 0.01% BSA) and blots were probed with anti-phosphotyrosine antibody PTyr8. Immunodetection was done using peroxidase-conjugated second antibodies [goat anti-(rabbit-IgG); Nordic, Tilburg, The Netherlands] and enhanced chemiluminescence (ECL, Amersham). A Kodak X-OMAT-S film was exposed for 5 min. After exposure, the antibodies were removed by washing the nitrocellulose filter in 67 mM-Tris, pH 6.7, 2% SDS and 5% β -mercaptoethanol for 30 min at 50 °C followed by several washes in TBST. The blot was reblocked and then reprobed with the anti-(EGF receptor C-terminus) antibodies, and immunoreactivity was determined as described for PTyr8.

Radioiodination of cell surface molecules

KB cells were cultured to near confluency in 25 cm² tissue culture bottles. Surface labelling was performed at room temperature with 2 mCi of ¹²⁵I/30 ml by lactoperoxidase-catalysed iodination. Cells were washed three times with ice-cold PBS and either directly lysed or cultured in the presence of ara-C (20 μ g/ml). After culturing without or with adenovirus for different times as indicated in the Figure legends, cells were washed twice with ice-cold PBS and lysed in Nonidet P-40 (NP-40) lysis mix [0.05% (v/v) NP-40, 50 mM-Tris/HCl, pH 7.5, 5 mM-MgCl₂, 1 mM-EGTA, 1 mM-PMSF, 3 units of aprotinin/ml and 10 μ g of leupeptin/ml]. Lysates were precleared with normal mouse serum and normal rabbit serum using fixed *Staphylococcus aureus*. Transferrin receptors were immunoprecipitated with moAb 66lg10. EGF receptors were immunoprecipitated with anti-(EGF receptor C-terminus) antibodies.

Immunofluorescence

KB cells, grown on cover slips, were infected with adenovirus, and after 14 h cells were washed twice with ice-cold PBS. Cells

were permeabilized with cold methanol (−20 °C) for 5 min followed by fixation with cold acetone (−20 °C) for 5 min. Cells were air-dried and incubated with moAb 108 in ice-cold PBS containing 1% (v/v) normal rabbit serum. After 1 h, cells were washed twice with PBS and incubated with fluorescein-conjugated anti-(mouse Ig) for 1 h. After washing with PBS, immunofluorescence was performed using a Leitz microscope equipped with fluorescence optics.

Pulse-chase experiments

KB cells were grown to confluency in 25 cm² tissue culture bottles. After 2 days, cells were incubated for 30 min in methionine-free RPMI medium containing 10% FCS. Cells were pulse-labelled for 15 min with 50 μ Ci of [³⁵S]methionine/ml and chased for the indicated times in the presence of excess (1.5 mM) non-radioactive methionine. Cells were washed twice with ice-cold PBS and lysed in NP-40 lysis mix. Transferrin receptor was immunoprecipitated with moAb 66lg10. The EGF receptor was immunoprecipitated with the anti-(EGF receptor C-terminus) antibodies. MHC class I molecules were immunoprecipitated with moAb W6/32. Immunoprecipitates were washed extensively and boiled for 3 min in endo H digestion buffer (50 mM-sodium citrate, pH 5.5, 0.2% SDS, 1 mM-PMSF, 3 units of aprotinin/ml, 10 μ g of leupeptin/ml). After cooling, 2 units of endo H (Boehringer Mannheim Diagnostics, Houston, TX, U.S.A.) was added where required. Digestion was carried out for 20–24 h at 37 °C. The reactions were stopped by boiling in SDS sample buffer. Molecular masses were determined using calibrated prestained high-molecular-mass markers (BRL) as reference.

Metabolic labelling of adenovirus-infected cells

KB cells were grown to near confluency in 25 cm² tissue culture bottles. Medium was replaced by a medium containing ara-C and infections were started at different time points. At 5 h before lysis, cells were washed and incubated with methionine-free medium containing 10% dialysed FCS. Cells were then labelled with 100 μ Ci of [³⁵S]methionine/ml of medium for 15 min and chased by replacing the medium with fresh medium containing excess (1.5 mM) non-radioactive methionine for 4 h in the presence of virus (except for control cells, which were not incubated with virus, and cells prepared for lanes 3 in Fig. 5, which were incubated with virus for 3 h before lysis) and ara-C. After the chase, the cells were washed with ice-cold PBS and immunoprecipitations were performed as described above. For metabolic labelling with methionine and cysteine (see Fig. 6), cells were starved in methionine-free and cysteine-free medium for 30 min, pulse-labelled in 100 μ Ci of [³⁵S]methionine plus 100 μ Ci of [³⁵S]cysteine/ml for 30 min, and chased by replacing the medium with fresh medium, containing excess (1.5 mM) non-radioactive methionine and cysteine. A431 cells were labelled with [³⁵S]methionine for 30 min and chased in 2 ml of medium per 25 cm² bottle for 4 or 5 h. Immunoprecipitations were carried out as described above. The truncated EGF receptor secreted by A431 cells was immunoprecipitated from the medium after removing cellular debris by centrifugation and addition of 20 μ l of lysis mix/ml of conditioned medium.

RESULTS

Adenovirus stimulates EGF receptor internalization and degradation

We investigated the effect of adenovirus infection on EGF receptor function by monitoring EGF-induced receptor autophosphorylation *in vivo*. Human KB cells were infected with adenovirus in the presence of ara-C, which inhibits viral DNA

Fig. 1. Effect of adenovirus infection on EGF receptor tyrosine kinase activity in KB cells

KB cells were infected with adenovirus type 5 (~50 p.f.u. per cell). At the indicated times after starting the infection (h; p.i. indicates post-infection), cells were washed and incubated with (C-12) or without (–) EGF (300 ng/ml) for 10 min at 4 °C. Cells were lysed and prepared for immunoblotting as described in the Materials and methods section. (a) Nitrocellulose filter probed with affinity-purified anti-phosphotyrosine antibody PTyr8. (b) The same filter reprobed with antibodies recognizing the EGF receptor C terminus. The first two lanes contain cell lysates from uninfected cells. The decrease in electrophoretic mobility of receptors from EGF-treated cells compared with control cells is attributable to receptor autophosphorylation.

Fig. 2. Effect of adenovirus infection on the half-life of surface-disposed EGF receptors

¹²⁵I surface labelling of KB cells was done as described in the Materials and methods section. After three washes with PBS, cells were either directly lysed (0 h) or cultured in the absence (control) or presence of adenovirus for 4–12 h. Next, receptors were immunoprecipitated with (a) moAb 108 (against EGF receptor) and (b) 66lg10 (against transferrin receptor) as described. NRS indicates immunoprecipitation with normal rabbit serum. EGF indicates iodinated cells cultured in the presence of EGF (200 ng/ml) for 4 h and then lysed.

replication and expression of late viral genes. After infection for 3–12 h, cells were briefly exposed to EGF at 4 °C to stimulate receptor tyrosine kinase activity, while preventing ligand-induced receptor internalization and degradation. Cell lysates were analysed by immunoblotting using the anti-phosphotyrosine antibody PTyr8. As shown in Fig. 1, ligand-induced receptor tyrosine kinase activity was almost completely abolished after 12 h of infection. Reprobing the same blot with antibodies against the EGF receptor C-terminus demonstrated that the

Fig. 3. Effect of adenovirus infection on EGF receptor distribution in KB cells analysed by immunofluorescence

KB cells were grown on cover slips and infected for 14 h with adenovirus. Cells were prepared for immunofluorescence using anti-(EGF receptor) antibody moAb 108 as described in the Materials and methods section. (a) Non-infected cells, (b) infected cells. Magnification ×280.

amount of mature receptors was dramatically decreased after 12 h of infection (Fig. 1b, compare lane 12 with lane C).

To investigate the mechanism(s) involved in the loss of EGF receptor expression, receptors at the cell surface were radioiodinated and the cells were cultured in the presence or the absence of adenovirus. At different time points, cells were lysed and immunoprecipitations were carried out. The fate of receptors already present at the cell surface at the start of infection could thus be monitored. After 10–12 h of infection, the amount of surface-labelled EGF receptors in infected cells was considerably less than in control cells, indicating that the half-life of surface-disposed EGF receptors was decreased after adenovirus infection (Fig. 2a). Virus infection did not detectably affect recovery of labelled transferrin receptor (Fig. 2b). Immunofluorescence analysis of infected and uninfected cells revealed that adenovirus caused a dramatic redistribution of EGF receptors (Fig. 3). Cell surface staining in control cells had changed into intracellular punctate staining, probably including multivesicular bodies (J. Calafat, unpublished work).

These results support the view that in adenovirus-infected cells the basal rate of EGF receptor internalization and degradation (i.e. in the absence of ligand) is increased when compared with control cells (Carlin *et al.*, 1989), resulting in abolition of EGF receptor signalling in living cells (Fig. 1).

Biosynthesis of EGF receptor, MHC class I molecules and transferrin receptor in uninfected KB cells

Adenovirus infection might equally well inhibit transport of the EGF receptor from the site of synthesis to the plasma membrane, as has been demonstrated for MHC class I molecules (Andersson *et al.*, 1985; Burgert & Kvist, 1985; Paabo *et al.*, 1987). To investigate this possibility, we performed pulse-chase analysis in combination with endo H digestions. KB cells were pulse-labelled with [³⁵S]methionine for 15 min and chased for the

Fig. 4. Biosynthesis of EGF receptor (a), MHC class I molecules (b) and transferrin receptor (c) in KB cells

KB cells were pulse-labelled for 15 min with [35 S]methionine and chased for the indicated times. Immunoprecipitations with antibodies against transferrin receptor (c), EGF receptor (a) and MHC class I molecules (b), and endoglycosidase H (Endo H) digestions, were performed as described in the Materials and methods section. The open arrow indicates the 160 kDa EGF receptor precursor; the filled arrow indicates the 140 kDa EGF receptor precursor after endo H digestion. The doublet in the anti-(MHC class I) precipitates most probably represents the HLA-a and HLA-b locus products.

Fig. 5. Effect of adenovirus infection on biosynthesis of EGF receptor (a), MHC class I molecules (b) and transferrin receptor (c) in KB cells

KB cells were incubated with ara-C for 10 h before lysis. At 5 h before lysis, cells were incubated in methionine-free medium, pulse-labelled for 15 min with [35 S]methionine and chased for 4 h. Then cells were lysed, and immunoprecipitations and endo H digestions were performed as described in the Materials and methods section. Time p.i. denotes the time between start of the infections and lysis of cells (h post-infection). C, non-infected control cells; open arrow, ~160 kDa EGF receptor precursor; filled arrow, ~140 kDa EGF receptor precursor after endo H digestion.

times indicated in Fig. 4. At zero time of chase the EGF receptor appeared as an ~160 kDa precursor which was completely sensitive to digestion with endo H. It took about 3–4 h before almost all EGF receptors were processed into the mature 170 kDa form, which contains mainly complex-type oligosaccharides and is therefore largely resistant to endo H digestion (Fig. 4a). For comparative purposes, the biosynthesis of two other well-characterized glycoproteins, MHC class I molecules (Fig. 4b) and transferrin receptor (Fig. 4c) was analysed. MHC class I molecules and transferrin receptors were processed to endo H-resistant molecules in less than 2 h (the mature transferrin receptor remains partially endo H-sensitive; see Fig. 5). To allow

complete processing of the EGF receptor, a chase of at least 4 h was performed in subsequent experiments.

Adenovirus infection inhibits oligosaccharide maturation of the EGF receptor

Infection of KB cells with adenovirus was started at different time points before or after pulse-labelling with [35 S]methionine. After chase for 4 h, cells were lysed and immunoprecipitations were performed sequentially with anti-(transferrin receptor), anti-(EGF receptor) and anti-(MHC class I) antibodies (Fig. 5). Adenovirus infection resulted in a decrease in the amount of the 170 kDa endo H-resistant mature EGF receptor, and simultaneously in a small but significant increase in the amount of an endo H-sensitive 160 kDa receptor precursor. Inhibition of EGF receptor maturation was detectable only if infection was started ≥ 2 h before pulse-labelling, suggesting a lag time of 1–2 h, which is presumably required for virus entry and synthesis of viral products. Inhibition of EGF receptor maturation was less pronounced than that of the MHC class I molecules, which also became progressively less resistant to digestion with endo H, in agreement with published reports (Burgert & Kvist, 1985; Andersson *et al.*, 1985; Paabo *et al.*, 1986). In contrast, there was no decrease in the endo H-resistance of the transferrin receptor at any time after infection (Fig. 5c), indicating that infection did not cause general impairment of terminal glycosylation. As the EGF receptor precursor immunoprecipitated from infected cells comigrated with the EGF receptor immunoprecipitated from non-infected cells that were directly lysed after pulse-labelling and was equally sensitive to endo H digestion (Fig. 6a), it most likely represents the ER-resident receptor. Although retention of EGF receptors was still incomplete after prolonged infection (until 14 h before lysis; Fig. 6a), our results suggest that EGF receptor down-regulation is due in part to inhibition of transport of newly synthesized receptors from the ER to the cell surface.

The adenovirus E3/10.4K protein has been reported to induce EGF receptor down-regulation (Hoffman *et al.*, 1990). In our immunoprecipitation experiments we have not been able to detect a direct association between E3/10.4K and the EGF receptor. On the other hand, the adenovirus E3/19K protein, which is responsible for retention of MHC class I molecules in the ER, was clearly co-immunoprecipitated with anti-(MHC class I) antibodies (Fig. 6a). The E3/19K glycoprotein was sensitive to endo H digestion, as expected, since it contains an ER retention signal (Gabathuler & Kvist, 1990; Paabo *et al.*, 1987). After overexposure of the autoradiographs, minor amounts of E3/19K were visible in immunoprecipitations with two different anti-(EGF receptor) antibodies, but also with anti-(transferrin receptor) antibody (Fig. 6b) and normal mouse serum or normal rabbit serum (results not shown). Therefore the possible involvement of E3/19K in the retention of EGF receptors in the ER of infected cells is questionable.

Adenovirus infection inhibits secretion of a 110 kDa truncated EGF receptor

To further substantiate that adenovirus infection inhibits intracellular transport of newly synthesized EGF receptors, we took advantage of the fact that A431 cells secrete a truncated form of the EGF receptor of approx. 110 kDa. This secreted form of the receptor, which lacks the transmembrane and cytoplasmic sequences, is fully glycosylated and is able to bind EGF (Mayes & Waterfield, 1984; Carpenter, 1987). Infection of A431 cells with adenovirus, which we achieved with variable success, resulted in accumulation not only of the 160 kDa precursor of the full-length EGF receptor but also of the 95 kDa precursor of the truncated receptor. This latter precursor most likely represents the ER-resident form of the otherwise secreted

Fig. 6. Co-immunoprecipitation of adenovirus proteins

(a) Infected or control KB cells were pulse-labelled with [35 S]methionine and [35 S]cysteine for 30 min and chased for 4 h. After immunoprecipitation of transferrin receptor (Tf-R), samples were split into two and EGF receptor was immunoprecipitated with antibodies against the C-terminus (EGF-R) or with moAb 108 against the extracellular domain (b, middle panel). The corresponding samples were pooled again and precipitation was continued with anti-MHC class I antibody (MHC I). (b) Over-exposures of the autoradiograms: upper panel, moAb 108; middle panel, antibody against EGF-R C-terminus. Lane C contains non-infected control cells; time p.i. denotes the time between start of the infection and lysis of the cells. P, non-infected but pulse-labelled cells; β_2m , β_2 -microglobulin (the light chain of MHC class I molecules). The

Fig. 7. Effect of adenovirus infection on biosynthesis of the truncated EGF receptor in A431 cells

(a) A431 cells were cultured in the presence of adenovirus plus ara-C (i) or ara-C only (C) for 13 h. Then cells were pulse-labelled for 30 min and chased for 5 h. Cells were lysed and immunoprecipitations of transferrin receptor (Tf-R), EGF receptor (EGF-R) and MHC class I molecules (MHC I), and endo H digestions, were performed as described in the Materials and methods section. EGF receptors were immunoprecipitated with moAb 108. C, non-infected cells; i, infection started 18.5 h before lysis. Open arrowhead, 160 kDa EGF receptor precursor; filled arrowhead, 140 kDa EGF receptor precursor after endo H digestion; open arrow, 95 kDa truncated EGF receptor precursor; filled arrow, 68 kDa truncated EGF receptor precursor after endo H digestion. t-EGF-R is the truncated EGF receptor, immunoprecipitated from the medium. (b) A431 cells were cultured in the presence of adenovirus plus ara-C (i) or ara-C only (C,P). After 8 h cells were pulse-labelled and directly lysed (P) or chased for 4 h (C,i). EGF receptor was immunoprecipitated with moAb 108. Arrows and arrowheads are as in (a).

EGF receptor, as it comigrates with the truncated precursor precipitated from uninfected cells that were directly lysed after pulse-labelling (Fig. 7b). Decreased amounts of the truncated EGF receptor were immunoprecipitated from the medium of infected cells when compared with control cell medium, which is in accordance with the observed retention in the ER of the receptor precursor (Fig. 7a).

DISCUSSION

Various cellular proteins can modulate EGF receptor function, by regulating transcription of the EGF receptor gene (Thompson *et al.*, 1988; Hudson *et al.*, 1990), by inducing post-transcriptional

banding pattern that we believe to represent E3/19K is only observed in immunoprecipitates from infected cells and is identical to that of immunoprecipitated E3/19K using anti-E3/19K antibodies (results not shown).

modifications (Collins *et al.*, 1983; Cochet *et al.*, 1984; Bird & Saklatvala, 1989; Donato *et al.*, 1989) or by interfering with receptor-mediated signal transduction pathways (Ullrich & Schlessinger, 1990; Iwashita *et al.*, 1990; Verheijden *et al.*, 1990). Several viral proteins are also capable of modulating the function of growth factor receptors. For example, the bovine papilloma-virus E5 transforming protein may interact, either directly or indirectly, with the receptors for EGF, colony-stimulating factor-1 and platelet-derived growth factor (PDGF), thereby affecting their activity and metabolic fate (Martin *et al.*, 1989; Petti *et al.*, 1991). The vaccinia virus growth factor (Stroobant *et al.*, 1985) and the simian sarcomavirus protein v-sis (Huang *et al.*, 1984) are ligands for the EGF receptor and the PDGF receptor respectively, and the Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the ER and affects its metabolism (Yoshimura *et al.*, 1990). Furthermore, adenovirus E1a gene products, which cause transcriptional repression of the EGF receptor-related *HER-2/c-erbB-2* and *neu* proto-oncogenes, act as transforming suppressors of the *neu* oncogene (Yu *et al.*, 1990, 1991; Yan *et al.*, 1991).

Carlin and coworkers have reported that the EGF receptor is down-regulated by the adenovirus E3/10.4K protein (Carlin *et al.*, 1989; Hoffman *et al.*, 1990). We extended their results by showing that adenovirus infection results in the loss of EGF receptor signalling in living cells, as monitored by EGF receptor autophosphorylation. By performing radioiodination of surface-disposed receptors prior to infection of cells, we could directly demonstrate that adenovirus infection reduced the half-life of EGF receptors, but not transferrin receptors, present at the cell surface at the beginning of the infection. Furthermore, our results indicated that adenovirus infection causes retention of newly synthesized receptors in the ER. Strikingly, the soluble truncated 110 kDa form of the EGF receptor, normally secreted by A431 cells, also accumulates in the ER of infected cells. Accumulation of EGF receptor precursors in the ER of adenovirus-infected cells is less prominent than observed with MHC class I molecules (Figs. 5–7). Degradation of EGF receptors retained in the ER might explain why a massive accumulation of the receptor precursor is not observed in virally infected cells.

Decreased surface expression of the EGF receptor thus appears to be the net result of two different mechanisms: (1) internalization and degradation of receptors in the absence of ligand, and (2) inhibition of intracellular transport of newly synthesized receptors from the ER to the plasma membrane. Transcriptional repression is apparently not involved in EGF receptor down-regulation, because receptor synthesis *de novo* is not affected by adenovirus infection (Carlin *et al.*, 1989). How are these two distinct mechanisms explained at the molecular level? From mutational studies and transfection experiments it appears that the adenovirus E3/10.4K protein is responsible for accelerated internalization (Carlin *et al.*, 1989; Hoffman *et al.*, 1990). Since E3/10.4K contains a small domain with sequence similarity to a cytoplasmic EGF sequence, Carlin *et al.* (1989) have postulated that E3/10.4 forms heterodimers with the EGF receptor at the plasma membrane, resulting in receptor internalization. Recent study on the expression of truncated and wild-type EGF receptors suggests that EGF receptor homodimerization may occur intracellularly and may even be essential for transport of the EGF receptor to the cell surface (Lax *et al.*, 1990; G. Verheijden, unpublished work). In such a model, formation of EGF receptor–E3/10.4K heterodimers in the ER may compete for EGF receptor homodimerization and impair subsequent transport of EGF receptors to the cell surface. On the other hand, the truncated EGF receptor is also retained in the ER but lacks the transmembrane and intracellular domains, including the se-

quence similar to part of the E3/10.4K protein. Hence E3/10.4K may facilitate EGF receptor internalization and degradation, without being involved in receptor retention in the ER. Thus far, we have been unable to detect the E3/10.4K protein in any of our immunoprecipitations, probably because the conditions of immunoprecipitation were not compatible with the recovery of an EGF receptor–E3/10.4K complex, if it exists.

Another mechanism that could explain retention of EGF receptors in the ER involves interaction between the receptor and a viral protein which contains an ER retention signal, such as the E3/19K protein (Gabathuler & Kvist, 1990; Paabo *et al.*, 1987). The E3/19K viral protein was found to directly interact with the extracellular domain of MHC class I molecules and to trap them in the ER, while leaving the transferrin receptor and the insulin receptor unaffected (Burgert & Kvist, 1987). Additional experiments should reveal whether E3/19K or another viral protein that may contain an ER retention signal similarly interacts with the EGF receptor extracellular domain, thereby causing the observed retention.

Down-regulation of MHC class I molecules has been suggested to be a means for adenovirus to escape immune surveillance. The biological significance of decreased EGF receptor levels is less obvious. Probably viruses benefit from regulating the proliferative state of the cells by modulating growth factor receptor levels, while leaving receptors essential for cell metabolism (e.g. transferrin, insulin) unaffected. It will be interesting to find out which mechanisms determine specificity for receptor down-regulation, in particular at the level of ER retention.

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