Farnesylation of CaaX-Tagged Diphtheria Toxin A-Fragment as a Measure of Transfer to the Cytosol†

Pål Ø. Falnes, Antoni Więdłocha, Andrzej Rapak,‡ and Sjur Olsnes*

Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway

Received February 3, 1995; Revised Manuscript Received June 2, 1995⊗

ABSTRACT: Diphtheria toxin binds to receptor-positive cells through its B-fragment, the toxin is then endocytosed, and the low pH in endosomes triggers the translocation of the enzymatically active A-fragment to the cytosol. A synchronous release of A-fragments into the cytosol can be induced by exposing cells with surface-bound toxin to low pH. We have used this protein translocation system to develop a novel method to study whether or not a protein is exposed to the cytosol. Protein farnesylation is a cytosolic modification signaled by a C-terminal CaaX motif, and to visualize the translocation process, we added a farnesylation signal to the toxin A-fragment. The A-fragment with an added CaaX motif was farnesylated within 1 h after exposure of cells with surface-bound toxin to low pH, and also A-fragment translocated from endosomes was quantitatively farnesylated. The results indicate that all cell-mediated reduction of the toxin implicates translocation of the A-fragment to the cytosol. The farnesylation was inhibited by lovastatin, the alkylating agent NEM, and the peptidomimetic farnesylation inhibitor B581. Farnesylated A-fragment partitioned preferentially into the detergent phase upon extraction with Triton X-114. Our data suggest that farnesylation of a CaaX tag is generally applicable as a cytosolic marker, and this strategy for monitoring protein transfer to the cytosol may have considerable potential for studying the transport to the cytosol of proteins added externally to cells.

While the cell has developed an efficient system for translocation of newly synthesized proteins across cellular membranes, transport of externally added proteins into the cytosol is believed to occur only in a few exceptional cases. Best studied are certain protein toxins that are able to gain entry to the cytosol of mammalian cells where they act on their targets in an enzymatic fashion [for reviews see London (1992) and Olsnes *et al.* (1993)].

Diphtheria toxin is secreted from *Corynebacterium diphtheriae* as a single polypeptide chain of 58 kDa (Greenfield *et al.*, 1983) that can be cleaved by low concentrations of trypsin into two fragments, A (21 kDa) and B (38 kDa), held together by a disulfide bond (Pappenheimer, 1977). The toxin binds to cell surface receptors through its B-fragment (Uchida *et al*, 1972), it is then endocytosed, and the low endosomal pH induces translocation of the A-fragment to the cytosol (Sandvig *et al.*, 1984). Once in the cytosol the A-fragment inactivates EF-2 by ADP-ribosylation (Collier, 1975), leading to inhibition of protein synthesis. The A-fragment can also be translocated directly from the plasma membrane to the cytosol by briefly exposing cells with surface-bound toxin to low pH medium, thereby mimicking the conditions in the endosomes (Draper & Simon, 1980; Sandvig & Olsnes, 1980).

To facilitate the decision whether a protein added extracellularly has reached the cytosol, we set out to develop a general marker for translocation of an external protein to the cytosol. Farnesylation of proteins is an enzymatic modification considered to take place only in the cytosol and not at the cell surface or in the endocytic pathway, and we therefore reasoned that it might be a good candidate for a translocation marker. The process of translocation of diphtheria toxin from the plasma membrane to the cytosol has been well characterized, and we therefore chose this toxin as a model protein to study the potential usefulness of farnesylation as a marker for translocation to the cytosol.

Several proteins are posttranslationally modified by the attachment of prenyl groups, either farnesyl (C15) or geranylgeranyl (C20) groups. In the case of protein farnesylation the lipid group is transferred from farnesyl pyrophosphate to the cysteine residue in a C-terminal CaaX (C = Cys, a = usually aliphatic amino acids, X = S, M, C, A, Q) motif. This tetrapeptide has been shown to be necessary and sufficient for protein farnesylation. After farnesylation the tripeptide −aaX is proteolytically cleaved off, and the now C-terminal cysteine is carboxymethylated [for reviews on protein prenylation, see Clarke (1992) and Cox and Der (1992)]. Usually the farnesylation of a protein leads to an altered mobility in SDS−PAGE,[1] and an increased migration rate is observed in the case of Ras proteins (Kinsella *et al.*, 1991; Kato *et al.*, 1992) and nuclear lamin B2 (Vorburger *et al.*, 1989). Furthermore, when phase separation in Triton X-114 is carried out, prenylation leads to partitioning of proteins into the detergent phase (Gutierrez *et al.*, 1989). Thus, it is easy to follow the farnesylation of a protein as a

† This work was supported by The Norwegian Cancer Society, The Novo Nordic Fund, The Norwegian Research Council for Science and Humanities, Blix legat, Otto and Rachel Bruun's legat, and The Jahre Foundation.

* To whom correspondence should be addressed.

‡ On leave of absence from The Institute of Immunology and Experimental Therapy of The Polish Academy of Sciences, Wroclaw, Poland.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; MES, 2-(*N*-morpholino)-ethanesulfonic acid; FPP, farnesyl pyrophosphate; PBS, phosphate-buffered saline.

0006-2960/95/0434-11152$09.00/0

function of time. We therefore reasoned that, similarly to how protein glycosylation is used to localize proteins throughout the secretory pathway, farnesylation of a C-terminally added CaaX motif might be used as an indicator of whether or not an externally added protein is capable of reaching the cytosol.

The entry of diphtheria toxin A-fragment into the cytosol can be followed by measuring inhibition of cellular protein synthesis, but such experiments do not give direct information about the amount of A-fragment translocated to the cytosol. Also, toxin translocation is accompanied by the reduction of the interfragment disulfide bond (Moskaug *et al.*, 1987; Papini *et al.*, 1992), but it has not been clear if all of the reduced A-fragment has entered the cytosol. In the present study we have constructed a mutant diphtheria toxin A-fragment with the C-terminal sequence Cys-Val-Ile-Met, identical to the farnesylation signal of the K-Ras-4B protein. We have studied the *in vivo* farnesylation of this "CaaX-tagged" A-fragment, and our data show that A-fragment originating from cell-mediated reduction of full-length toxin is quantitatively farnesylated, suggesting that the bulk of reduced A-fragment observed has actually gained access to the cytosol. Also, our data indicate that the farnesylation of a CaaX tag may be generally useful as a tool to follow the entry of externally added proteins into the cytosol.

EXPERIMENTAL PROCEDURES

Buffers and Media. Dialysis buffer: 140 mM NaCl, 20 mM HEPES, and 2 mM $CaCl_2$, adjusted to pH 7.0 with NaOH. HEPES medium: bicarbonate- and serum-free Eagle's minimal essential medium buffered with HEPES to pH 7.4. Lysis buffer: 0.1 M NaCl, 20 mM NaH_2PO_4, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1 mM NEM, pH 7.4. MES−gluconate buffer: 140 mM NaCl, 5 mM sodium gluconate, and 20 mM MES, adjusted with Tris to pH 4.8. PBS: 140 mM NaCl and 10 mM NaH_2PO_4, pH 7.4.

Cell Cultures. Vero cells were propagated as earlier described (Sandvig & Olsnes, 1980). For experiments where [^{35}S]methionine-labeled toxin was used, cells were seeded into 12-well Costar (Cambridge, MA) microtiter plates at a density of 2×10^5 cells/well on the day preceding the experiments. For toxicity experiments, cells were transferred to 24-well plates at a density of 5×10^4 cells/well 1 day prior to the experiments.

Plasmid Construction. Escherichia coli strain DH5α was used in the cloning procedures. The plasmid encoding dtA-CaaX was constructed by insertion of the linker

CCTGTGTTATTATGTAGTGCA
GGACACAATAATACATC

between the *Stu*I and *Nsi*I restriction sites of the plasmid pKD-25 (Ariansen *et al.*, 1993), thereby destroying the *Nsi*I site. The sequence of the resulting plasmid was verified by dideoxy sequencing. The plasmid pKD-52 (Ariansen *et al.*, 1993) encodes the wild-type diphtheria toxin A-fragment, whereas the plasmid pBD-23 (Stenmark *et al.*, 1992) encodes the B-fragment.

In Vitro Transcription and Translation. Plasmid DNA was linearized downstream of the encoding gene and transcribed in a 20 μL reaction mixture with T3 RNA polymerase as described (McGill *et al.*, 1989). The mRNA was precipitated with ethanol and dissolved in 10 μL of H_2O containing 10 mM DTT and 0.1 unit/μL RNasin. The translation was performed for 1 h at 30 °C in micrococcal nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI) using 5 μL of the dissolved mRNA/100 μL of lysate. Radioactive proteins were made with lysates containing 1 μM [^{35}S]-methionine and 25 μM each of the 19 other amino acids. Labeled methionine was replaced by 25 μM unlabeled methionine when making nonradioactive proteins. The amount of protein in the nonlabeled lysates was estimated as earlier described (Stenmark *et al.*, 1992) by translating in parallel a 10 μL aliquot of the lysate in the presence of 5 μM [^{35}S]methionine.

In Vitro Farnesylation. Twenty microliters of translation mixture as described above (without labeled methionine) was added to 0.2 μCi of [^{3}H]farnesyl pyrophosphate (Du Pont-New England Nuclear, Boston, MA); then mRNA was added, and the translation was carried out for 1 h at 30 °C. To the reaction mixture was then added $MgCl_2$ to a final concentration of 5 mM and in some cases 1 μL of canine pancreatic microsomes (Promega, Madison, WI), and the mixture was further incubated 30 min at 37 °C. Labeled protein was recovered from the reaction mixture by immunoprecipitation with anti-diphtheria toxin serum and analyzed by SDS−PAGE and fluorography.

In Vitro Reconstitution of Active Toxin. When A-fragment and B-fragment are mixed under reducing conditions, and the reducing agent is subsequently removed by dialysis, active heterodimeric toxin will form quantitatively (Stenmark *et al.*, 1992). A- and B-fragments were made separately by *in vitro* transcription and translation, mixed together, and then allowed to associate by overnight dialysis against dialysis buffer.

SDS−PAGE. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in 12% gels as described by Laemmli (1970). After electrophoresis the gel was fixed for 30 min in 27% methanol/4% acetic acid and then incubated for 30 min in 1 M sodium salicylate/2% glycerol, pH 5.8. Kodak XAR-5 film was exposed to the dried gel in the absence of an intensifying screen at −80 °C.

Assay for Translocation and Cellular Processing of A-Fragments. All experiments were started by incubating Vero cells with reticulocyte lysate containing [^{35}S]methionine-labeled reconstituted toxin (1 nM) and 1 mM unlabeled methionine for 1 h at 4 °C, followed by four washes in ice-cold PBS to remove unbound toxin. In some experiments the translocation of the toxin was then induced by incubating the cells for 2 min at 37 °C in MES−gluconate buffer (pH 4.8), and the cells were further incubated at 37 °C in HEPES medium containing 10 μM monensin to allow time for cellular processing. In other experiments the PBS washes were directly followed by incubation at 37 °C in HEPES medium, allowing entry of toxin by endocytosis. Both kinds of experiments were usually terminated by lysing the cells in lysis buffer for 10 min on ice. The cell lysate was transferred to an Eppendorf tube, nuclei were removed by centrifugation, and cellular proteins were precipitated for 30 min on ice in the presence of 5% TCA. After centrifugation, the pellet was washed twice with ether and subjected to nonreducing SDS−PAGE. In a few experiments the cells were not lysed directly on the plate but taken off by incubation for 5 min at 37 °C in HEPES medium containing

5 mg/mL Pronase and 10 μM monensin. The cells were then transferred to an Eppendorf tube, washed in HEPES containing 1 mM NEM and 1 mM PMSF, lysed, and further treated as described above.

Triton X-114 Partitioning of Farnesylated Proteins. Triton X-114 was equilibrated with PBS and diluted to a 20% stock solution as previously described (Madshus *et al.*, 1984). PBS (300 μL) and Triton X-114 stock solution (100 μL) were mixed on ice, *in vitro* made protein was added, and the mixture was incubated at 0 °C for 15 min. Separation of the phases was induced by 15 min incubation at 37 °C, followed by 2 min centrifugation at room temperature in an Eppendorf centrifuge. The water (upper) phase was transferred to a new tube, and the Triton (lower) phase was washed at 37 °C with 500 μL of PBS. The two phases were each diluted to 1 mL with PBS (0 °C), followed by immunoprecipitation with anti-diphtheria toxin serum, SDS−PAGE, and fluorography. Partitioning of cellular proteins was done by lysing the cells directly in the Triton/PBS mixture in the presence of 1 mM NEM and 1 mM PMSF, and after removal of nuclei by centrifugation, the procedure for phase separation described above was followed.

Measurement of Cytotoxicity of Endocytosed Toxin. Vero cells were incubated overnight in growth medium with increasing amounts of toxin reconstituted from *in vitro* translated A- and B-fragments. The cells were allowed to incorporate radioactivity for 30 min at 37 °C in HEPES medium with 4 μCi/mL [^{3}H]leucine and no unlabeled leucine. The cells were washed with 5% TCA for 10 min, followed by a brief wash in 5% TCA, and then dissolved in 0.1 M KOH, and the cell-associated radioactivity was measured.

RESULTS

Formation of Diphtheria Toxin with a CaaX Box in the A-Fragment. Cleavage of diphtheria toxin between the A- and the B-fragments is necessary to activate the toxin, and the cleavage normally occurs next to either of three closely spaced arginine residues, leading to a mixed population of toxin molecules (DeLange *et al.*, 1976). However, active diphtheria toxin can also be prepared by formation of an interfragment disulfide bridge between separately expressed A- and B-fragments (Stenmark *et al.*, 1992), allowing the construction of a heterodimeric toxin molecule with a well-defined sequence at the C-terminus of the A-fragment. We have earlier constructed an A-fragment with the wild-type C-terminal sequence Cys-Ala-Gly-Asn (Ariansen *et al.*, 1993), in the following denoted dtA-wt (Figure 1A). In the present work we have constructed a mutant A-fragment, denoted dtA-CaaX (Figure 1A), where the three C-terminal amino acids were changed such that the resulting protein terminated in the tetrapeptide Cys-Val-Ile-Met. This sequence is identical to the C-terminal farnesylation signal of the K-Ras-4B protein and has been shown to be sufficient for farnesylation both *in vitro* (Goldstein *et al.*, 1991) and *in vivo* (Hancock *et al.*, 1991a).

Conceivably, the changes in the C-terminal sequence of the A-fragment could interfere with its ability to form a disulfide bond with the B-fragment. To test this, the A- and B-fragments were separately expressed by *in vitro* transcription from a T3 promoter, followed by *in vitro* translation in a reticulocyte lysate in the presence of [^{35}S]methionine, and translation mixtures containing A- and B-fragments were

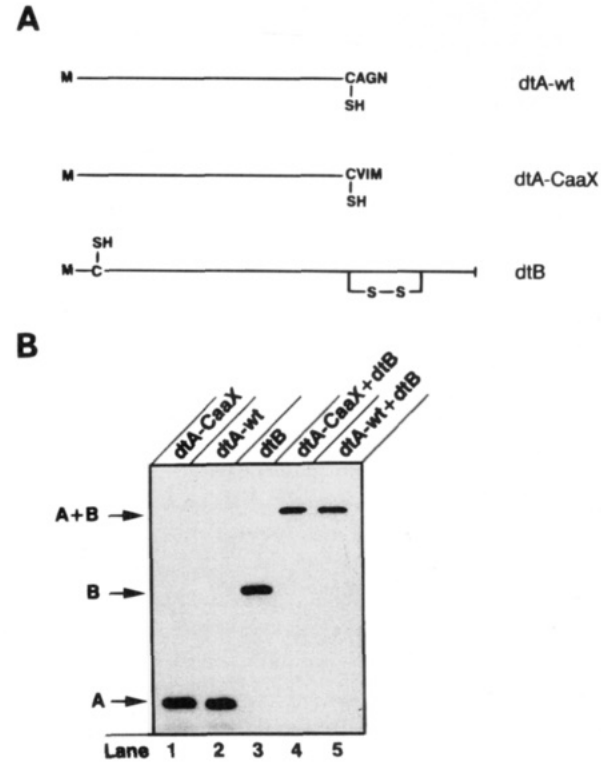

FIGURE 1: Description of constructs used and their *in vitro* translation products. (A) The A-fragment part of wild-type diphtheria toxin is indicated as dtA-wt. In dtA-CaaX the three C-terminal amino acids of dtA-wt have been replaced, generating a C-terminal farnesylation signal. dtA-wt and dtA-CaaX can associate with the diphtheria toxin B-fragment, dtB, through disulfide bond formation, generating [dtA-wt + dtB] and [dtA-CaaX + dtB], respectively. (B) Radiolabeled proteins were expressed by *in vitro* transcription followed by *in vitro* translation in a reticulocyte lysate in the presence of [^{35}S]methionine. Separately expressed, [^{35}S]methionine-labeled A- and B-fragments were dialyzed together, allowing formation of an interfragment disulfide bond. The proteins were analyzed by nonreducing SDS−PAGE and fluorography.

mixed together and dialyzed overnight to remove reducing agents, allowing the formation of the interfragment disulfide bond. The results (Figure 1B) showed that dtA-CaaX was equally efficient as the wild-type A-fragment in forming a disulfide bond with the B-fragment. Also, it appears that the majority of *in vitro* expressed dtA-CaaX was in the nonfarnesylated form, since farnesylation of the single cysteine residue of the A-fragment would prevent the formation of a disulfide bond with the B-fragment. This is in accordance with previous studies reporting that farnesylation is inefficient in a reticulocyte lysate unless mevalonate, a precursor of farnesyl pyrophosphate, is added (Hancock *et al.*, 1991b; Sanford *et al.*, 1991).

In Vitro Farnesylation of dtA-CaaX. When the K-Ras-4B protein is translated in a reticulocyte lysate in the presence of radiolabeled farnesyl pyrophosphate or mevalonate, the farnesylated protein can be detected after SDS−PAGE and fluorography, and it displays a higher migration rate than the corresponding nonfarnesylated, [^{35}S]methionine-labeled protein (Hancock *et al.*, 1991b; Kato *et al.*, 1992). Furthermore, when microsomes are added, proteolytic cleavage and methylation take place (Hancock *et al.*, 1991b), and this can be observed as a further increase in migration rate on SDS−PAGE (Kato *et al.*, 1992). To study if dtA-wt and dtA-CaaX could be farnesylated *in vitro*, the proteins were

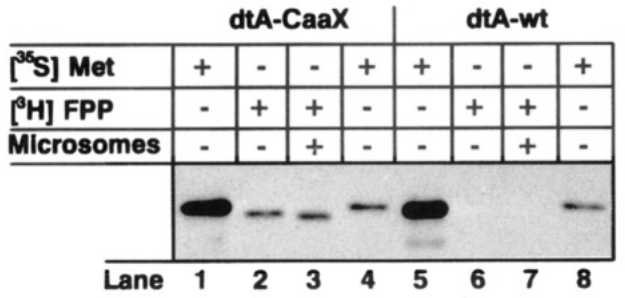

FIGURE 2: *In vitro* farnesylation of dtA-CaaX. (Lanes 1, 4, 5, 8) A-Fragments were translated *in vitro* for 1 h at 30 °C in the presence of [^{35}S]methionine, and 0.2 μL (lanes 1 and 5) or 0.05 μL (lanes 4 and 8) of the translation mix was subjected to SDS−PAGE and fluorography. (Lanes 2, 3, 6, 7) The translation was carried out in the presence of [^{3}H]farnesyl pyrophosphate and followed by addition of $MgCl_2$ to a final concentration of 5 mM and 30 min incubation at 37 °C in the absence (lanes 2 and 6) or presence (lanes 3 and 7) of microsomes. A-Fragment was immunoprecipitated from 20 μL of translation mix and analyzed by SDS−PAGE and fluorography.

translated in a rabbit reticulocyte lysate containing [^{3}H]-farnesyl pyrophosphate or [^{35}S]methionine, and the labeled proteins were analyzed by SDS−PAGE and fluorography. The results (Figure 2) showed that dtA-CaaX (lane 2), but not dtA-wt (lane 6), was labeled by [^{3}H]farnesyl pyrophosphate and that the farnesylated protein migrated faster than the corresponding [^{35}S]methionine-labeled protein (lanes 1, 4). Also, when microsomes were added (lane 3), the migration rate was further increased, similarly to what has been observed with the K-Ras-4B protein. Thus, the farnesylation signal of dtA-CaaX appears to be fully functional. The C-terminal sequence CAGN of dtA-wt ressembles a CaaX motif, but since a glycine in the penultimate position appears to be detrimental for the function of the CaaX motif (Reiss *et al.*, 1991; Kato *et al.*, 1992), it is not surprising that dtA-wt was not farnesylated.

Translocation Competence of Diphtheria Toxin Containing dtA-CaaX. When diphtheria toxin is bound to cell-surface receptors and the cells are exposed to low pH, the A-fragment is translocated to the cytosol, and this can be observed on SDS−PAGE as a reduction of the interfragment disulfide bond (Moskaug *et al.*, 1987). Also, if the cells are subsequently treated with Pronase to remove nontranslocated material, the A-fragment, but not the remaining full-length toxin, is protected against digestion by externally added Pronase (Moskaug *et al.*, 1988). To study if the mutant A-fragment, dtA-CaaX, was translocation competent, the protein was expressed in a reticulocyte lysate in the presence of [^{35}S]methionine and reassociated with unlabeled B-fragment. When the mutant toxin was bound to Vero cells, the interfragment disulfide bond was reduced in response to low-pH treatment (Figure 3, lane 2), and similar amounts of reduced A-fragment were observed as in the case of wild-type toxin (lane 5). Furthermore, the A-fragment was in both cases protected from Pronase digestion (lanes 3, 6). The data indicate that the translocating ability of the A-fragment was not impaired by the mutations introduced in the dtA-CaaX protein.

In Vivo Farnesylation of dtA-CaaX. To investigate whether translocated dtA-CaaX is also farnesylated *in vivo*, we carried out an experiment where the translocation of surface-bound toxin was induced by low pH, followed by further incubation at 37 °C to allow time for processing.

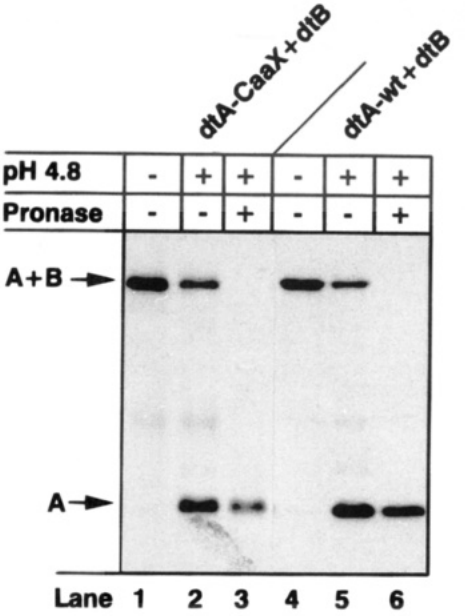

FIGURE 3: Translocation of dtA-CaaX and dtA-wt to the cytosol of Vero cells. Vero cells were incubated for 1 h at 4 °C with diphtheria toxin (1 nM) that had been reconstituted from unlabeled B-fragment and [^{35}S]methionine-labeled A-fragment, unbound toxin was removed by washing four times with PBS, and the cells were incubated for 2 min at 37 °C in MES−gluconate buffer of pH 7.0 (lanes 1 and 4) or pH 4.8 (lanes 2, 3, 5, 6). In some cases (lanes 1, 2, 4, 5) the cells were subsequently lysed in lysis buffer for 10 min at 0 °C. Alternatively (lanes 3 and 6), the cells were incubated for 5 min at 37 °C with HEPES medium containing 10 μM monensin and 5 mg/mL Pronase, and the cells, which during the Pronase treatment had detached from the plastic, were transferred to an Eppendorf tube, collected by centrifugation, washed in HEPES medium containing 1 mM NEM and 1 mM PMSF, and finally lysed. In all cases the nuclei were removed from the cell lysates by centrifugation, and protein in the supernatant was precipitated with TCA and analyzed by nonreducing SDS−PAGE and fluorography.

Monensin was present during the 37 °C incubation to prevent translocation of toxin from endosomes. The results (Figure 4A, lanes 1−6) showed that essentially all dtA-CaaX was converted into a faster migrating species within 1 h, whereas the wild-type A-fragment, dtA-wt (lanes 7−12), did not undergo any conversion. Similar results were obtained when the cells were treated with Pronase before lysis (data not shown), showing that also the farnesylated form of dtA-CaaX is inaccessible to externally added Pronase. When the mutant toxin containing dtA-CaaX was endocytosed, all reduced A-fragment was eventually converted into the faster migrating form (Figure 4B), similarly to the experiments where low pH was used to induce the translocation of the toxin. In these experiments, a small amount of free A-fragment could be observed also at $t = 0$ min. However, control experiments with A-fragment alone revealed that when incubated at 4 °C the A-fragment bound unspecifically to the plastic of the tissue culture plastes and that the majority of A-fragment bound to the plastic in this way disappeared within 5 min of incubation at 37 °C (data not shown). Therefore, we feel confident that the weak A-fragment band observed at $t = 0$ min in Figure 4B is a result of unspecific binding to plastic of traces of free A-fragment not associated with B-fragment and that the faster migrating form of the A-fragment observed at later time points only originates from A-fragments formed by cell-mediated reduction of the full-length toxin. Interestingly, the farnesylated dtA-CaaX appeared to be less stable

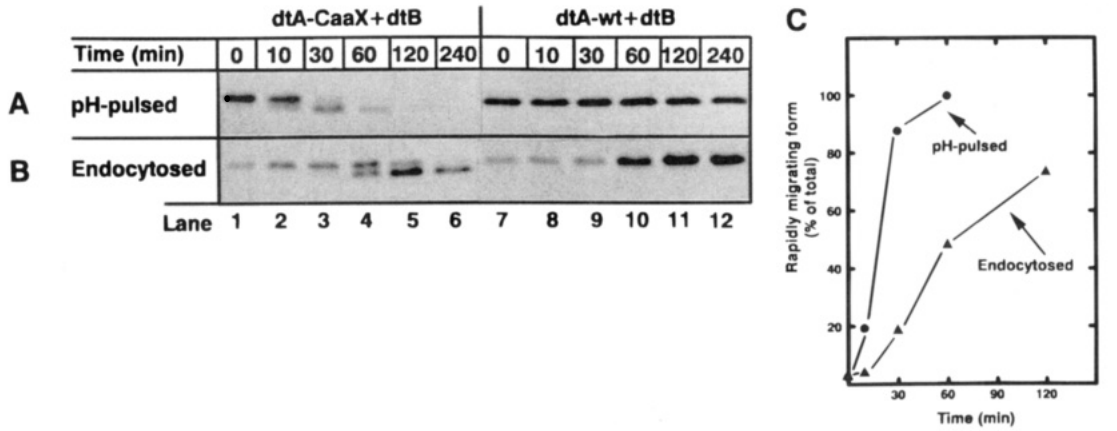

FIGURE 4: *In vivo* processing of A-fragment translocated with low-pH pulse (A) or after endocytotic uptake (B). Reconstituted, [^{35}S]-methionine-labeled toxin (1 nM) containing dtA-CaaX (lanes 1−6) or dtA-wt (lanes 7−12) was bound to Vero cells by 60 min incubation at 4 °C, and unbound toxin was removed by washing four times with PBS. (A) The cells were exposed to MES−gluconate buffer (pH 4.8) for 2 min at 37 °C, then incubated in HEPES medium containing 10 μM monensin for the indicated time periods, and finally lysed. (B) After washing the cells were incubated in HEPES medium for the indicated periods of time before they were lysed. In both cases TCA-precipitable material of the lysed cells was analyzed by nonreducing SDS−PAGE and fluorography. (C) The bands representing the two forms of dtA-CaaX in (A) and (B) were quantitated by densitometric scanning, and the relative amount of the rapidly migrating form has been expressed as the percentage of the sum of the two forms. Since dtA-CaaX was unstable when modified, only the earlier time points have been included. Curves: ●, translocation induced by exposure to low pH; ▲, endocytosis of the toxin.

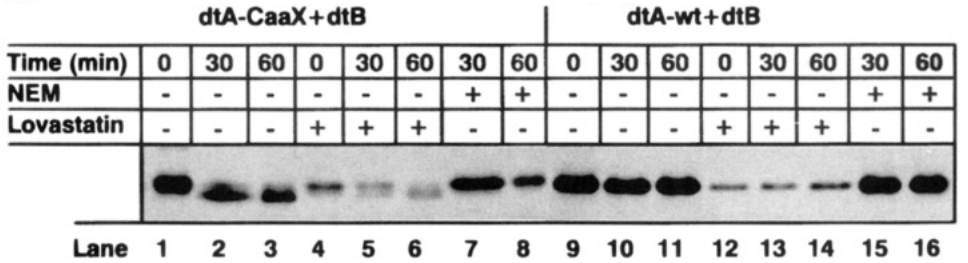

FIGURE 5: Ability of lovastatin and NEM to inhibit processing of translocated dtA-CaaX. Experiments similar to those described in Figure 4A were carried out except that in some cases the cells had been pretreated for 18 h with 5 μg/mL lovastatin (lanes 4, 5, 6, 12, 13, 14), whereas in other cases (lanes 7, 8, 15, 16) 100 μM NEM was present during the incubation after the low-pH treatment.

in the cells than dtA-wt (compare lanes 5 and 6 with lanes 11 and 12).

To address the kinetics of modification of dtA-CaaX in a more quantitative way, the fluorograms in Figure 4A,B were subjected to densitometric scanning, and the results are shown in Figure 4C. The conversion of dtA-CaaX to the faster migrating form was considerably faster when the entry was induced by exposure to low pH ($t_{1/2} \sim 20$ min) than when the toxin was endocytosed ($t_{1/2} \sim 60$ min), in accordance with the observation that exposure of diphtheria toxin to low pH at the level of the plasma membrane leads to the rapid entry of the A-fragment into the cytosol (Draper & Simon, 1980; Sandvig & Olsnes, 1980).

Ability of Lovastatin, N-Ethylmaleimide, and B581 to Inhibit Farnesylation of dtA-CaaX. To test further if the *in vivo* conversion of translocated DTA-CaaX to a faster migrating species is due to farnesylation, we tested if lovastatin, which inhibits the synthesis of mevalonate, a necessary precursor for the prenylation of proteins, would inhibit the conversion of dtA-CaaX into the faster migrating species. The results showed that the conversion of dtA-CaaX into the faster migrating form was moderately delayed when the cells had been pretreated with lovastatin (Figure 5, lanes 4−6) as compared to the untreated control (lanes 1−3).

Since the farnesyl group is attached to a cysteine residue by a thioester bond, the prenylation of translocated DTA-CaaX should be inhibited by *N*-ethylmaleimide, an alkylating agent known to block free sulfhydryl groups. This was indeed found to be the case (Figure 5, lanes 7, 8).

In vitro farnesylation of proteins can be inhibited by tetrapeptides corresponding to the CaaX sequence (Reiss *et al.*, 1990). Such peptides are not very efficient *in vivo*, since they are susceptible to proteolytic degradation and have low membrane permeability, but recently several analogues of CaaX tetrapeptides with increased membrane permeability and protease resistance have been synthesized and shown to inhibit farnesyltransferase *in vivo* (James *et al.*, 1993; Kohl *et al.*, 1993; Nigam *et al.*, 1993; Garcia *et al.*, 1993). The peptidomimetic inhibitor B581 has been constructed on the basis of the structure of the tetrapeptide CVFM (Garcia *et al.*, 1993), and we found that this inhibitor efficiently blocked the *in vitro* farnesylation of dtA-CaaX in a reticulocyte lysate (Figure 6A), although the concentration needed was approximately 100-fold higher than what was sufficient to block the *in vitro* prenylation of H-ras (Garcia *et al.*, 1993). The farnesylation inhibitor B581 also considerably delayed the conversion of dtA-CaaX to a faster migrating species after it had been translocated into the cytosol of Vero cells, both when the translocation was induced at the level of the plasma membrane by low pH (Figure 6B) and when the toxin was translocated from endosomes (Figure 6C). The conversion was not completely blocked, but due to our limited supply of B581 we used a concentration (50 μM) which only

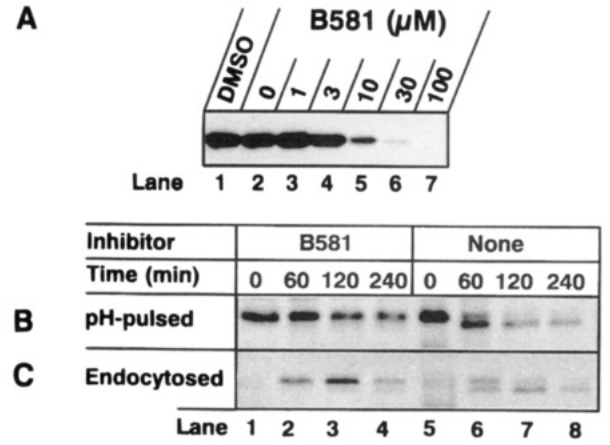

FIGURE 6: Inhibition of farnesylation of dtA-CaaX by the peptidomimetic inhibitor B581. (A) *In vitro* farnesylation reactions as described in the legend to Figure 2 were carried out in the presence of increasing concentrations of B581 (lanes 2−7) or a concentration of DMSO (1%) equal to the highest concentration of DMSO added with B581 (lane 1). (B and C) Similar to the experiments in panels A and B of Figure 4, respectively, but in some cases (lanes 1−4) the cells had been pretreated for 18 h with 50 μM B581 which was also present during the binding of the toxin and during the subsequent incubation at 37 °C.

incompletely blocked also farnesylation of Ras in vivo (Garcia *et al.*, 1993).

Ability of Farnesylated dtA-CaaX To Partition into the Detergent Phase of Triton X-114. Ras proteins need a palmitoylation signal or a polybasic domain of six lysine residues in addition to their CaaX motifs to become membrane associated (Hancock *et al.*, 1990, 1991b). However, farnesylation of a CaaX motif appears to be sufficient to make Ras proteins partition into the detergent phase upon extraction with Triton X-114 (Hancock *et al.*, 1989, 1990). We therefore decided to investigate whether farnesylation is sufficient to make dtA-CaaX partition into the detergent phase. The results in Figure 7A show that while *in vitro* expressed, [^{35}S]methionine-labeled dtA-CaaX, which is not farnesylated, partitioned into the water phase (lanes 1, 2), the [^{3}H]farnesyl-labeled protein partitioned into the detergent phase (lanes 3, 4).

We also carried out experiments where [^{35}S]methionine-labeled dtA-CaaX reconstituted with dtB was bound to Vero cells which were then exposed to pH 4.8 to allow translocation to occur. The cells were then incubated for different time periods, allowing various degrees of farnesylation to take place, and the cells were lysed and the postnuclear supernatant was submitted to Triton X-114 partitioning. As shown in Figure 7B the rapidly migrating farnesylated form of dtA-CaaX partitioned into the detergent phase (lanes 1−4), whereas the nonmodified form was recovered from the aqueous phase (lanes 5−8). The results indicate that when farnesylation is used as a marker for translocation to the cytosol, Triton X-114 partitioning may be a way of separating farnesylated and nonfarnesylated protein. This may prove to be useful when only a small amount of protein is translocated to the cytosol compared to the total amount that is associated with the cells.

Effect of Farnesylation on the Toxic Effect of Diphtheria Toxin. The diphtheria toxin A-fragment kills cells by acting enzymatically on elongation factor 2, and it has been shown that entry of one A-fragment molecule into a cell is actually sufficient to kill the cell (Yamaizumi *et al.*, 1978). Therefore, it is likely that the time the A-fragment prevails in the cytosol influences its toxicity. The mutant A-fragment dtA-CaaX was quite unstable in the cytosol (Figure 4), and we carried out experiments to see how this affected its cytotoxicity. The results showed that toxin consisting of the mutant A-fragment dtA-CaaX was approximately 10 times less toxic than toxin reconstituted from dtA-wt (Figure 8). The cytotoxicity of toxin consisting of dtA-CaaX was somewhat increased when the farnesylation inhibitor B581

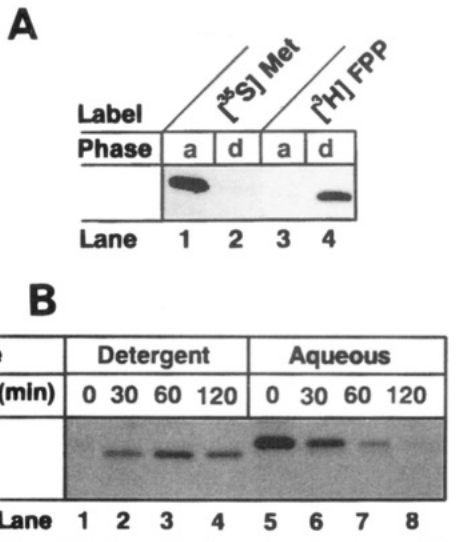

FIGURE 7: Triton X-114 partitioning of *in vitro* (A) and *in vivo* (B) farnesylated dtA-CaaX. (A) [^{35}S]Methionine- and [^{3}H]FPP-labeled dtA-CaaX were prepared as described in the legend to Figure 2. Triton X-114 partitioning into aqueous (a) and detergent (d) phases followed by immunoprecipitation was carried out as described in Experimental Procedures. (B) Same as the experiment in Figure 4A, but the cells were lysed in 5% Triton X-114 in PBS, containing 1 mM PMSF and 1 mM NEM, and phase separation and immunoprecipitation were carried out as described in Experimental Procedures.

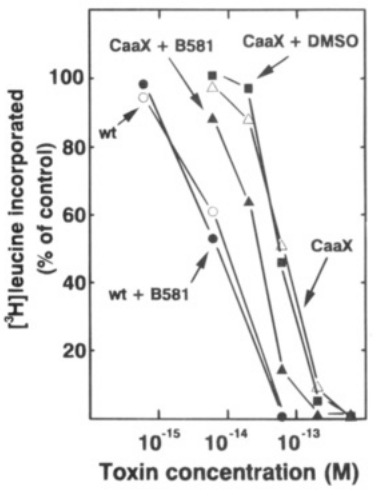

FIGURE 8: Cytotoxicity of dtA-wt and dtA-CaaX in the presence and absence of the peptidomimetic inhibitor B581. Vero cells were preincubated for 18 h at 37 °C without any additions (○, △), in the presence of of 200 μM B581 (●, ▲), or in the presence of a concentration of DMSO (1%) equal to the concentration of DMSO in the B581 treated samples (■). Increasing amounts of unlabeled reconstituted [dtA-wt + dtB] (●, ○) or [dtA-CaaX + B] (△, ▲, ■) were added, and the cells were further incubated for 18 h at 37 °C. The cells were subsequently incubated for 30 min at 37 °C in HEPES medium containing 4 μCi/mL [^{3}H]leucine and no unlabeled leucine. Finally, the radioactivity incorporated into TCA-precipitable material was measured by scintillation counting.

was present, whereas the toxicity of wild-type toxin was not altered by the presence of B581. This shows that the farnesylation of dtA-CaaX is at least partly responsible for the reduced toxicity of the mutant toxin [dtA-CaaX + dtB].

DISCUSSION

Two main conclusions can be drawn from this work. First, the farnesylation of a CaaX motif appears to be generally applicable as a marker to determine whether an externally added protein is able to enter the cytosol. Second, it appears that all the reduced A-fragment observed after the cell-mediated reduction of diphtheria toxin has actually reached the cytosol, since dtA-CaaX became quantitatively farnesylated.

Entry of diphtheria toxin A-fragment into the cytosol is accompanied by the reduction of the disulfide bond between the A- and B-fragment, both when toxin entry is induced by exposing cells with surface-bound toxin to low pH (Moskaug *et al.*, 1987) and when the toxin is endocytosed (Papini *et al.*, 1993). Upon exposure of surface-bound toxin to acidic pH, the A-fragment becomes inaccessible to digestion by externally added Pronase (Moskaug *et al.*, 1988). Furthermore, the A-fragment, but not the membrane-inserted B-fragment, is released into the medium from Pronase-treated cells by applying saponin in the same concentration range as that required to release cytosolic proteins (Moskaug *et al.*, 1988). This indicates that Pronase-protected A-fragment is actually translocated to the cytosol, and the current observation that *all* the reduced dtA-CaaX becomes farnesylated demonstrates that *all* the Pronase-protected A-fragment actually has been translocated to the cytosol.

Once the dtA-CaaX protein had been translocated to the cytosol and converted to a faster migrating form, it was degraded, possibly due to a perturbed conformation of the protein in response to farnesylation. Another possibility is that the proteolytic cleavage following the farnesylation makes the protein susceptible to further proteolysis. Prenylated proteins are generally quite stable; only a 25−30% decline in radioactive labeling of proteins was observed when cells that had been labeled with [^{3}H]mevalonate were chased for 7 h (Repko & Maltese, 1989). However, in some cases prenylated proteins have been shown to undergo further proteolysis. In the case of nuclear lamin A, its farnesylation leads to removal of the 18 C-terminal amino acids, including the farnesyl group itself (Beck *et al.*, 1990). Also, when the γ subunit of transducin is translated *in vitro* in a reticulocyte lysate, farnesylation of the protein leads to its degradation (Sanford *et al.*, 1991).

Pretreatment of the cells with lovastatin delayed the conversion of dtA-CaaX to the faster migrating form, but the conversion was not completely blocked. However, lovastatin was not present during toxin binding and subsequent incubations at 37 °C, and it is therefore likely that the cellular pool of farnesyl pyrophosphate was not completely depleted. Lovastatin has been shown to inhibit the cytotoxicity of diphtheria toxin in Vero cells (Oda & Wu, 1994), and in our experiments where the interfragment disulfide bond was reduced by exposing cells to low pH, lovastatin pretreatment considerably reduced the intensity of the A-fragment band. However, lovastatin pretreatment strongly affected the growth and morphology of the cells, and the amount of TCA-precipitable material from the lovastatin-treated cells was notably smaller. It can therefore not be concluded from our data that lovastatin inhibits the low pH-induced translocation of the A-fragment from the plasma membrane to the cytosol.

The use of farnesylation as a marker for translocation to the cytosol relies on the assumption that protein farnesylation does not take place at the cell surface or in the endocytic or secretory pathways. There are several reasons why we think this assumption is highly likely to hold true. None of the known prenylated proteins in animal cells appear to be export proteins, although they are associated with intracellular membranes through their prenyl groups. Also, farnesyl transferase, the enzyme catalyzing the attachment of farnesyl groups to proteins, has been isolated from the cytosolic fraction of cells (Reiss *et al.*, 1990), and no farnesyl transferase activity was detected in the membrane fraction (Schaber *et al.*, 1990). Export proteins usually contain characteristic N-terminal signal sequences (von Heijne, 1990), and the cloned sequences of the α and β subunits of human farnesyl transferase (Omer *et al.*, 1993) do not contain any typical signal sequences.

In the case of some growth factors and cytokines there are indications that part of their action may be dependent on the transport of the protein into the cytosol and nucleus (Baldin *et al.*, 1990; Imamura *et al.*, 1990; Lobie *et al.*, 1994; Moroianu & Riordan, 1994; Solari *et al.*, 1994; Więdłocha *et al.*, 1994). A major problem in such studies is to demonstrate that the protein in question has really gained access to the cytosol, rather than being present in intracellular vesicles or cisternal compartments that may have a juxtanuclear location. The present study suggests that farnesylation of a CaaX tag may be useful as a marker to determine if an externally added protein binding to cell-surface receptors also is able to enter the cytoplasm. In future work we would like to apply the method described here to investigate whether various proteins that bind to extracellular receptors are capable of reaching the cytosol. By combining the use of farnesyl transferase inhibitors and detergent extraction of farnesylated protein, we expect to be able to detect translocation to the cytosol even if only a small fraction of the surface-bound protein is translocated. In a separate study we have already used this approach to investigate the translocation of acidic fibroblast growth factor to the cytosol (manuscript in preparation). The present method could also be used to test whether bacterial proteins produced upon contact of pathogenic bacteria (*e.g.*, *Yersenia*) with mammalian cells are able to reach the cytosol.

ACKNOWLEDGMENT

We are grateful to Marianne Andreassen for her expert handling of the cell cultures and to Drs. Kirsten Sandvig and Markus Lanzrein for critical reading of the manuscript. The farnesylation inhibitor B581 was a gift from Dr. A. M. Garcia, and lovastatin was a gift from Merck (Rahway, NJ).

REFERENCES

Ariansen, S., Afanasiev, B. N., Moskaug, J. Ø., Stenmark, H., Madshus, I. H., & Olsnes, S. (1993) *Biochemistry 32*, 83−90.

Baldin, V., Roman, A.-M., Bosc-Bierne, I., Amalric, F., & Bouche, G. (1990) *EMBO J. 9*, 1511−1517.

Beck, L. A., Hosick, T. J., & Sinensky, M. (1990) *J. Cell Biol. 110*, 1489−1499.

Clarke, S. (1992) *Annu. Rev. Biochem. 61*, 355−386.

Collier, R. J. (1975) *Bacteriol. Rev. 39*, 54−85.
Cox, A. D., & Der, C. J. (1992) *Curr. Opin. Cell Biol. 4*, 1008−1016.
DeLange, R. J., Drazin, R. E., & Collier, R. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 69−72.
Draper, R. K., & Simon, M. I. (1980) *J. Cell Biol. 87*, 849−854.
Garcia, A. M., Rowell, C., Ackermann, K., Kowalczyk, J. J., & Lewis, M. D. (1993) *J. Biol. Chem. 268*, 18415−18418.
Goldstein, J. L., Brown, M. S., Stradley, S. J., Reiss, Y., & Gierasch, L. M. (1991) *J. Biol. Chem. 266*, 15575−15578.
Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G. A., Collier, R. J., & Caplan, D. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6853−6857.
Gutierrez, L., Magee, A. I., Marshall, C. J., & Hancock, J. F. (1989) *EMBO J. 8*, 1093−1098.
Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989) *Cell 57*, 1167−1177.
Hancock, J. F., Paterson, H., & Marshall, C. J. (1990) *Cell 63*, 133−139.
Hancock, J. F., Cadwallader, K., Paterson, H., & Marshall, C. J. (1991a) *EMBO J. 10*, 4033−4039.
Hancock, J. F., Cadwallader, K., & Marshall, C. J. (1991b) *EMBO J. 10*, 641−646.
Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J. A. M., Hla, T., & Maciag, T. (1990) *Science 249*, 1567−1570.
James, G. L., Goldstein, J. L., Brown, M. S., Rawson, T. E., Somers, T. C., McDowell, R. S., Crowley, C. W., Lucas, B. K., Levinson, A. D., & Marsters, J. C., Jr. (1993) *Science 260*, 1937−1942.
Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., & Der, C. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6403−6407.
Kinsella, B. T., Erdman, R. A., & Maltese, W. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8934−8938.
Kohl, N. E., Mossner, S. D., deSolms, S. J., Guiliani, E. A., Pompliano, D. L., Graham, S. L., Smith, R. L., Scolnick, E. M., Oliff, A., & Gibbs, J. B. (1993) *Science 260*, 1934−1937.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lobie, P. E., Mertani, H., Morel, G., Morales-Bustos, O., Norstedt, G., & Walters, M. J. (1994) *J. Biol. Chem. 269*, 21330−21339.
London, E. (1992) *Mol. Microbiol. 6*, 3277−3282.
Madshus, I. H., Olsnes, S., & Sandvig, K. (1984) *EMBO J. 9*, 1945−1950.
McGill, S., Stenmark, H., Sandvig, K., & Olsnes, S. (1989) *EMBO J. 8*, 2843−2848.
Moroianu, J., & Riordan, J. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1677−1681.
Moskaug, J. Ø., Sandvig, K., & Olsnes, S. (1987) *J. Biol. Chem. 262*, 10339−10345.
Moskaug, J. Ø., Sandvig, K., & Olsnes, S. (1988) *J. Biol. Chem. 263*, 2518−2525.
Nigam, M., Seong, C.-M., Qian, Y., Hamilton, A. D., & Sebti, S. M. (1993) *J. Biol. Chem. 268*, 20695−20698.
Oda, T., & Wu, H. C. (1994) *Exp. Cell Res. 212*, 329−337.
Olsnes, S., van Deurs, B., & Sandvig, K. (1993) *Med. Microbiol. Immunol. 182*, 51−61.
Omer, C. A., Kral, A. M., Diehl, R. E., Prendergast, G. C., Powers, S., Allen, C. M., Gibbs, J. B., & Kohl, N. E. (1993) *Biochemistry 32*, 5167−5176.
Papini, E., Rappuoli, R., Murgia, M., & Montecucco, C. (1993) *J. Biol. Chem. 268*, 1567−1574.
Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem. 46*, 69−94.
Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., & Brown, M. S. (1990) *Cell 62*, 81−88.
Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S., & Goldstein, J. L. (1991) *Proc. Natl. Sci. U.S.A. 88*, 732−736.
Repko, E. M., & Maltese, W. A. (1989) *J. Biol. Chem. 264*, 9945−9952.
Sandvig, K., & Olsnes, S. (1980) *J. Cell Biol. 87*, 828−832.
Sandvig, K., Sundan, A., & Olsnes, S. (1984) *J. Cell Biol. 98*, 963−970.
Sanford, J., Codina, J., & Birnbaumer, L. (1991) *J. Biol. Chem. 266*, 9570−9579.
Schaber, M. D., O'Hara, M. B., Garsky, V. M., Mosser, S. D., Bergstrom, J. D., Moores, S. L., Marshall, M. S., Friedman, P. A., Dixon, R. A. F., & Gibbs, J. B. (1990) *J. Biol. Chem. 265*, 14701−14704.
Solari, R., Smithers, N., Kennard, N., Ray, K., & Grenfell, S. (1994) *Biochem. Pharmacol. 47*, 93−101.
Stenmark, H., Afanasiev, B. N., Ariansen, S., & Olsnes, S. (1992) *Biochem. J. 281*, 619−625.
Uchida, T., Pappenheimer, A. M., Jr., & Harper, A. A. (1972) *Science 175*, 901−903.
von Heijne, G. (1990) *J. Membr. Biol. 115*, 195−201.
Vorburger, K., Kitten, G. T., & Nigg, E. A. (1989) *EMBO J. 8*, 4007−4013.
Więdłocha, A., Falnes, P. Ø., Madshus, I. H., Sandvig, K., & Olsnes, S. (1994) *Cell 76*, 1039−1051.
Yamaizumi, M., Mekada, E., Uchida, T., & Okada, Y. (1978) *Cell 15*, 245−250.

BI9502598