The International Journal of Biochemistry & Cell Biology 43 (2011) 1365–1372

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

On diphtheria toxin fragment A release into the cytosol—Cytochalasin D effect and involvement of actin filaments and eukaryotic elongation factor 2

Muhammet Bektaş*, Ebru Hacıosmanoğlu, Bilge Özerman, Başak Varol, Rüstem Nurten, Engin Bermek

Istanbul University, Istanbul Faculty of Medicine, Department of Biophysics, 34390 Çapa, Istanbul, Türkiye

ARTICLE INFO

Article history:
Received 17 February 2011
Received in revised form 13 May 2011
Accepted 26 May 2011
Available online 12 June 2011

Keywords:
ADP-ribosylation
Cytochalasin D
Diphtheria toxin
Early endosomes
Eukaryotic elongation factor 2
F-actin
Fragment A

ABSTRACT

Diphtheria toxin has been well characterized in terms of its receptor binding and receptor mediated endocytosis. However, the precise mechanism of the cytosolic release of diphtheria toxin fragment A from early endosomes is still unclear. Various reports differ regarding the requirement for cytosolic factors in this process. Here, we present data indicating that the distribution of actin filaments due to cytochalasin D action enhances the retention of diphtheria toxin in early endosomes. Treating cells with cytochalasin D reduces the cytosolic fragment A activity and leads to changes in the intracellular distribution and size of early endosomes with toxin cargo. F-actin and eukaryotic elongation factor 2 can promote fragment A release from toxin-loaded early endosomes in an *in vitro* translocation system. Moreover, these proteins bind to toxin-loaded early endosomes *in vitro* and promote each other's binding. They are thus thought to be involved in the cytosolic release of fragment A. Finally, ADP-ribosylation of eukaryotic elongation factor 2 is shown to inhibit fragment A release and, via a feed-back mechanism, to account for the minute amounts of fragment A normally found in the cytosol.

1. Introduction

Diphtheria toxin (DT) is a well-characterized representative of bacterial protein toxins (Pappenheimer, 1984; Collier, 2001). It is synthesized and released by toxicogenic *Corynebacterium diphtheria* strains as a single polypeptide chain of 58 kDa. After a mild treatment with trypsin and reduction of its disulfide bonds, this polypeptide is cleaved into two fragments: fragment A (FA) of 21 kDa and fragment B (FB) of 37 kDa (Oram and Holmes, 2006). The three-dimensional structure of DT has been elucidated (Choe et al., 1992; Bennett et al., 1994). It is comprised of three domains: the N-terminal catalytic (C) domain corresponds to FA, whereas the transmembrane or translocation (T) domain as well as the C-terminal receptor-binding (R) domain reside in the FB.

FA is a mono-ADP-ribosyltransferase that catalyses the transfer of ADP-ribosyl group of nicotine amide dinucleotide (NAD^+) to a post-translationally modified histidine (diphthamide) residue on eukaryotic elongation factor 2 (eEF2) (Van Ness et al., 1980), abolishing its activity in protein synthesis (Collier, 1967).

FB (R domain) is required for the binding of DT to its receptor on the target cells. This receptor has been shown to be EGF-like growth factor precursor (Naglich et al., 1992). The uptake of the holotoxin is achieved through receptor-mediated endocytosis and early endosomes (Lemichez et al., 1997; Papini et al., 1988). The acidic milieu in endosomes causes conformational changes and denaturation of the toxin. The transmembrane domain becomes disclosed and mediates the release of FA into the cytoplasm through interactions with the endosomal membrane (Draper and Simon, 1980; Kaul et al., 1996; D'Silva and Lala, 1998). Other findings, however, have shown the involvement of the cytosol (Lemichez et al., 1997) and a cytosolic translocation factor (CTF) complex comprised of heat shock protein 90 (Hsp90) and thioredoxin reductase in this step (Ratts et al., 2003).

The recent report (Bektaş et al., 2009) that FA can interact with actin both *in vitro* and *in vivo* also deserves some consideration. Its biological relevance being unknown, this interaction has been approached with reservation. An explanation seems to be provided by the role of the actin cytoskeleton in protein trafficking (Durrbach et al., 1996; Matarrese and Malorni, 2005; Provance et al., 2008). Thus, the observed interaction with actin may reflect an event that is necessary for distribution of FA in the cell, which is, for its transfer to the site of its action. It may also be required during the process of converting denatured FA into the appropriate, functional conformation. These considerations prompted us to address the issue by investigating the effect of cytochalasin D (CD)-promoted breakdown of actin filaments (Ohmori et al., 1992) on FA activity and distribution in the target cell.

Abbreviations: CD, cytochalasin D; CTF, cytosolic translocation factor; DT, diphtheria toxin; EEA1, early endosome antigen 1; eEF2, eukaryotic elongation factor 2; EGF, epidermal growth factor; FA, diphtheria fragment A; F-actin, filamentous actin; FB, diphtheria fragment B; Hsp90, heat shock protein 90; HUVEC, human umbilical vein endothelial cells.

* Corresponding author. Tel.: +90 212 4142187; fax: +90 212 4142187.
E-mail address: muhbektas@hotmail.com (M. Bektaş).

1357-2725/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2011.05.017

2. Materials and methods

2.1. Materials

All reagent grade biochemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. [Adenosine-^{14}C]NAD$^+$, specific activity 535 Ci/mol, and L-[U-^{14}C]phenylalanine ([^{14}C]Phe), specific activity 469 Ci/mol, were products of PerkinElmer Life Sciences (Waltham, MA, USA). The FA-specific monoclonal antibody 7F2 (mouse) was obtained from Santa Cruz Biotech (Santa Cruz, CA, USA), and the eEF2-specific antibody (rabbit) was obtained from GenWay (San Diego, CA, USA). DT was a gift from the Refik Saydam Institute in Ankara. Rat liver eEF2 and rabbit skeletal muscle F-actin were prepared as previously described (Bektaş et al., 1994).

2.2. ADP-ribosylation assay

Cell extracts or cytosolic fractions were assayed in NAD$^+$-and DT-dependent ADP-ribosylation of eEF2 for determination of either ADP-ribosyltransferase or ADP-ribose acceptor activity. In both cases, if not otherwise indicated, the reactions were performed for 10 min at 20 °C in 25 μl reaction mixtures containing 50 mM Tris–HCl, pH 7.4, 7 mM 2-mercaptoethanol, 5 μM [^{14}C]NAD$^+$, and 10 μg cell extract or cytosolic protein (Bektaş et al., 2005). The reaction mixtures also contained 10 μg (about 100 pmol) eEF2 for the ADP-ribosyltransferase assay and 3 μg (about 46 pmol) DT for the determination of free ADP-ribose acceptor sites. Following incubation, 5 μl aliquots were plated onto GF/A glass fiber filters (Whatman, Maidstone, Kent, UK), which were successively washed in cold CCl_3COOH, ether–ethanol and ether. After drying, the filters were transferred to vials containing 5 ml of 0.4% 2,5-diphenyloxazole in toluene, and CCl_3COOH-precipitated radioactivity was determined with a liquid scintillation counter (Packard TriCarb 2100TR).

2.3. Determination of protein synthetic activity

Polyphenylalanine (polyPhe) synthesis was performed as previously described (Bektaş et al., 2005). The 50 μl reaction mixtures contained 20 pmol ribosomes, 20 μg eEF1 protein, 5 μg polyU, tRNA (*Escherichia coli*) (300 μg/ml) charged during the reaction by use of DE52 (Whatman) fractionated S130 protein (*E. coli*) as a source of AA-tRNA synthetases, 2 μM [^{14}C]Phe, 6 mg/ml creatine kinase, 6 mM creatine phosphate, 1.5 mM GTP, and 1.5 mM ATP. Cell extract or cytosolic fractions (S5 and S120) were used as a source of eEF2. The incubations were performed for 3 min at 37 °C, and CCl_3COOH-precipitated radioactivity was determined as above with the inclusion of a prior (5%) CCl_3COOH treatment for 5 min at 90 °C.

2.4. Treatment of cells with DT

Human umbilical vein endothelial cells (HUVEC; ATCC ECV 304) were propagated in DMEM F-12 supplemented with 10% fetal calf serum FCS (Gibco) at 37 °C in 5% CO_2. They were distributed into a 24-well plate prior to the experiment with 5×10^6 cells per well. The concentrations of DT were adjusted as specified in the figure legends. After the indicated periods of time, cells were harvested by use of a cell scraper (Greiner Bio-One) and washed three times in PBS. They were homogenized in 1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) in the presence of protease inhibitors (Complete Tablets, Roche Applied Science). Cell extracts were first clarified by centrifugation for 5 min at 5000 × *g* and then subjected to ultracentrifugation for 2 h at 120,000 × *g*. The cytosolic fractions (S5 and S120) were then assayed in ADP-ribosylation and polyPhe synthesis. Whenever indicated, an initial centrifugation step (7 min at 700 × *g*) was included in this differential centrifugation procedure. To promote the release of residual FA in early endosomes, cell extracts were subjected to either acid or Triton X-100 treatment. To that effect, cell extracts were acidified (pH 5.5) by the addition of a phosphate buffer and incubated for 30 min, followed by readjustment to pH 7.2. Alternatively, cell extracts were treated with 1% Triton X-100.

2.5. Western blotting

$NaDodSO_4$-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Laemmli, 1970). For visualization of the protein bands corresponding to FA and actin, the proteins separated by SDS-PAGE were transferred to nitrocellulose (Millipore) membranes that had been treated with TBS (Tris-buffered saline)–Tween (TBST) and 0.5% BSA for 1 h. The membranes were incubated with an actin-specific antibody (rabbit), an eEF2-specific antibody (rabbit) or FA-specific monoclonal antibody (mouse) 7F2 and then with horseradish peroxidase conjugates of anti-rabbit Ig antibody (goat) and anti-mouse IgG (goat), respectively. After three washes with TBST, the substrate solution containing 3-amino-9-ethyl carbonate (Bio-Rad Laboratories, CA, USA) was added. After developing, the membranes were washed with water.

2.6. Immunofluorescence

FA was detected using the murine monoclonal antibody 7F2 and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig antibody. Early endosomal antigen 1 (EEA1) was detected using the EEA1-specific monoclonal antibody (Abcam, Cambridge, UK).

2.7. Imaging

All images were obtained on an Olympus BX51 Research Microscope equipped with a DP72 camera controlled by Olympus DP2-TWAIN software. HUVEC maintained on glass coverslips at semi-confluence were fixed in 3.7% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 and incubated with 0.1% BSA in PBS for 2 h prior to the application of immunofluorescence supplements. They were subsequently processed for mounting with antifade reagents.

2.8. Isolation of early endosomes

A previously described method (Tjelle et al., 1996) was followed. Briefly, HUVEC (10^7 cells/ml) in suspension were incubated for 10 min in the presence of 750 pM DT under constant stirring and collected by centrifugation for 5 min at 270 × *g*. The cell pellet was resuspended in homogenization buffer (250 mM sucrose, 3 mM imidazole, 0.5 mM K-EDTA, pH 7.3) and lysed with an insulin syringe. The cell extract was centrifuged for 7 min at 750 × *g*. The pellet (P07) contained DT-loaded early endosomes and cell nuclei. Hence, it was used as the starting fraction for isolation of early endosomes. Resuspended in 17% Percoll in homogenization buffer (0.5 ml), it was layered on top of a 0.75 ml 65% sucrose cushion in a Sorvall ultracentrifuge tube. The contents of the tube were then brought up to a final volume of 5 ml with 17% Percoll. The sample was centrifuged in a Sorvall AH 650 rotor for 1 h at 59,000 × *g*. After centrifugation, 0.5 ml fractions were collected, analyzed for FA content by western blotting and assayed for ADP-ribosyltransferase activity.

2.9. Gel filtration analysis

Size distributions were analyzed on a Sephacryl S-100 (Hi-prep 16/60) (GE Healthcare) column equilibrated with homogenization

buffer and calibrated using ribonuclease (M_r 13.7 kDa), carbonic anhydrase (M_r 29 kDa), ovalbumin (M_r 43 kDa), conalbumin (M_r 75 kDa), phosphorylase b (M_r 97 kDa) and β-galactosidase (M_r 116 kDa). The fraction volume was 1 ml. Column fractions were assayed for ADP-ribosyl transferase and ADP-ribose acceptor activity as described above.

2.10. Statistical analysis

Results correspond to the means of at least three independent experiments ± standard deviations (SD) as indicated in the presented data. Differences between data groups were tested using the univariate analysis of variance. Statistical significance was accepted at p values <0.01.

3. Results

3.1. Intracellular distribution of FA—effect of CD

We used an EEA1-specific antibody to explore the morphology and localization of early endosomes in HUVEC incubated with DT and CD. We were able to confirm the recent finding (Antignani and Youle, 2008) that DT induces early endosome fusion and the emergence of enlarged vesicles (Fig. 1A and B). DT also gave rise to a perinuclear localization of early endosomes. CD treatment appeared to further enhance the DT-mediated size changes and the perinuclear accumulation of early endosomes (Fig. 1C and D). We explored the effect of CD on the intracellular distribution of FA. Immunofluorescence microscopy using FA-specific monoclonal antibody 7F2 in control cells revealed a rather uniform concentration of FA(DT)-loaded, enlarged early endosomes around the nucleus with diffusion into the cell periphery where a punctuate pattern labeling was also observed (Fig. 1E). The treatment of cells with CD gave rise to a change in the distribution pattern with perinuclear localisation of the punctuate staining (Fig. 1F). It resulted additionally in the disappearance of FA-specific cytoplasmic haze. Immunofluorescence microscopy carried out in parallel using EEA1-specific antibody revealed a similar image, indicating the early endosomal nature of the punctuate pattern.

3.2. FA activity in the cytosolic fraction—effect of CD

In initial experiments, treatment with CD did not appear to have a great effect on FA-specific ADP-ribosyltransferase activity in the cytosolic fraction (S120), which is routinely used in assays for this activity. These findings were obtained with high DT concentrations (≥1 nM). However, because only a few molecules of DT are required to bring about a complete inhibition of protein synthesis in the cell (Yamaizumi et al., 1978; Falnes and Sandvig, 2000). A 'leakage' in the endosomal cargo traffic would very likely mask a possible inhibitory effect of CD treatment on FA activity. Thus, we wanted to determine whether a CD-mediated inhibition of ADP-ribosyltransferase activity would be apparent in lower concentrations, that is, concentrations that would exist in the intra- and inter-cellular milieu following the administration of lethal doses of the toxin (Gill, 1982). The experiments were repeated with 10^6 cells in the presence of 15 and 150 pM DT. As assayed in an ADP-ribosyltransferase reaction, FA activity appeared to plateau in the cytosolic fraction from control cells after incubation for approximately 3 h in the presence of both DT concentrations (Fig. 2A). CD inhibited FA activity in the cytosolic fraction of cells incubated with 15 pM DT by about 65%. On the other hand, 65% and 75% of the ADP-ribose acceptor sites in the cytosolic fraction appeared to be blocked after incubation for 3 h in the presence of 15 and 150 pM DT, respectively (Fig. 2B). CD reduced the extent of blocked sites to about 15% and 35%, respectively. Thus, the CD effect on FA activity became more apparent at lower toxin concentrations. Similar results were also obtained in polyPhe synthesis, with the extent of blocked ADP-ribose acceptor sites being reflected in eEF2 protein synthetic activity (Fig. 2C).

3.3. DT is retained in early endosomes

The inhibition of FA activity after CD treatment could represent the release of denatured FA into the cytosol due to a failure of appropriate folding during translocation through the endosomal membrane (Sandvig and Olsnes, 1981; Draper and Simon, 1980; Kaul et al., 1996; Lemichez et al., 1997; D'Silva and Lala, 1998). Alternatively, as implicated by the findings in immunofluorescence microscopy, it could be a consequence of FA retention in early endosomes. This issue was addressed by assaying the subcellular fractions obtained by differential centrifugation of the extracts of cells exposed to varying DT concentrations for FA(DT) content and ADP-ribosyltransferase activity. As shown in Fig. 3A, over 80% of the FA activity was localized in the P07 fraction following incubation of cells with DT concentrations of 1 and 10 pM. Thus, the nuclear fraction P07 (Roodyn, 1969) appeared to contain the majority DT-loaded early endosomes. When this experiment was repeated with the inclusion of CD-treatment, it was found that CD further increases the FA activity in the P07 fraction in both absolute and relative terms, concomitant with considerable decreases in the already restricted (cytosolic) FA activity in S07 (Fig. 3B). In the presence of 1 or 10 pM DT, FA activity in the S07 fraction accounted for only 6% of the total FA activity. The data indicating the predominant localization of DT-loaded early endosomes in the P07 fraction were further supported by the western blot findings, which revealed a strong accumulation of EEA1 and FA in this fraction of DT-treated cells. CD further enhanced this accumulation at the cost of FA (and EEA1) present in the S07 fraction. The corresponding bands of EEA1 and FA in the S07 fraction, which were already rather weak, became almost undetectable following CD treatment (Fig. 3C). On the other hand, in control samples from cells without DT and/or CD-treatment, the EEA1 band exhibited a nearly equal intensity in both the P07 and S07 samples, indicating a balanced distribution of early endosomes between these two fractions.

3.4. In vitro FA release from early endosomes

An early endosomal fraction obtained by density gradient centrifugation (Tjelle et al., 1996) with P07 as the starting fraction for the isolation procedure was then used in an *in vitro* translocation assay system, similar to the one recently described by Ratts et al. (2003). As shown in Table 1, incubation of early endosomes alone resulted in the release of a slight amount of FA activity in this system. Under conditions presumed to facilitate FA translocation, i.e., low pH and Triton X-100 treatment, there was an increase in released FA activity up to 4.5-fold relative to the background activity (control). Incubation with HUVEC and CD-HUVEC cytosol fractions gave rise to less than 2- and 1.5-fold increases, respectively. Incubation with eEF2 caused nearly a 2.5-fold change alone, and an increase of 3.5-fold when combined with GTP. ADP-ribosylation reduced this increase to around 1.5-fold. F-actin was nearly as efficient as eEF2 in promoting FA release, and eEF2, GTP and F-actin together brought about a 4-fold increase. If eEF2 was replaced with ADP-ribosylated eEF2, there was almost no additional increase in the presence of GTP and F-actin. Finally, Hsp90, a component of CTF (Ratts et al., 2003), also appeared to promote FA release, but it did not enhance the eEF2/F-actin effect.

These results suggest that eEF2, the substrate of ADP-ribosyltransferase activity, may be involved in FA release from

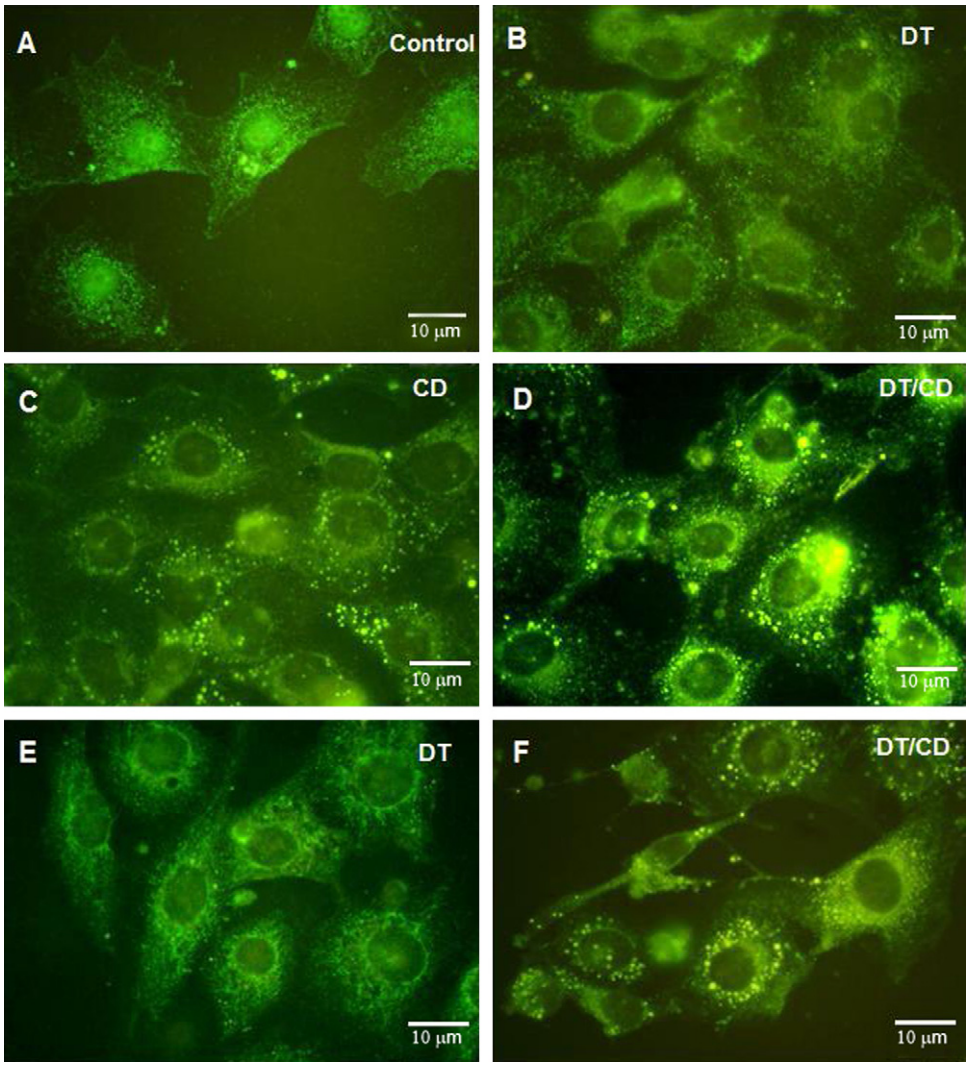

Fig. 1. Morphology and intracellular distribution of FA(DT)-loaded early endosomes. Effect of CD. EEA1-positive early endosomes in the absence (A) and presence of DT (B), CD (C), and DT and CD (D). FA(DT)-positive early endosomes in the absence (E) and presence of CD (F). Experimental conditions are described in Section 2. Whenever present, 750 pM DT and 2 μM CD were used. After incubation, the cells were treated with monoclonal antibodies, followed by staining with FITC-labeled goat anti-mouse antibody. They were examined by fluorescence microscopy, and the diameters of EEA1- and FA(DT)-positive endosomes determined by use of DP2-BSW basic software. Calculated diameter values of early endosomes (means of at least 200 measurements): 0.48 ± 0.25 μm (A); 0.83 ± 0.39 μm (B); 0.87 ± 0.52 μm (C); 1.17 ± 0.82 μm (D); 0.68 ± 0.29 μm (E); 0.89 ± 0.35 μm (F).

DT-loaded early endosomes. They also provide an explanation for the finding that an early endosomal fraction with DT content in a physically segregated and enzymatically inactive state could still exhibit high ADP-ribosyltransferase activity in the presence of eEF2 (as illustrated in Fig. 3). The data further suggest that ADP-ribosylation of eEF2 may give rise to an inhibition of FA release from early endosomes.

In a further set of experiments, we attempted to confirm these findings obtained with isolated early endosomes by using the P07 fraction as a source of DT-loaded early endosomes. The cells were incubated with DT and its non-toxic mutant form, CRM197. The P07 fraction obtained from these cells was then incubated either alone or in the presence of eEF2 in combination with F-actin, GTP and/or NAD^+. After incubation, the P07 fraction was removed by centrifugation at 700 × *g* and the amount of released FA was determined by western blot analysis of the supernatant. Beforehand, the P07 and S07 fractions were examined for their eEF2, actin and FA contents (Fig. 4A). In DT-treated cells, consistent with the previous data, the P07 fraction was relatively rich in FA(DT) and eEF2. In CRM197-treated cells, FA*, enzymatically inactive fragment, appeared to be rather evenly distributed between the P07 and S07 fractions, whereas the eEF2 content in the P07 fraction was lower than that in the S07 fraction, similar to the control sample. As shown in Fig. 4B, eEF2 efficiently promoted FA release from the P07 fraction of DT-treated cells. In line with the data presented in Table 1, this effect was further augmented in the presence of GTP and F-actin, but inhibited by NAD^+. Some FA release was also observed upon incubation of the P07 fraction alone, likely due to the presence of some unmodified eEF2 and/or ADP-ribosylated eEF2, which seemed still to promote FA release to some extent, in this fraction. The weak band in the corresponding sample from CRM197-treated cells could be explained in terms of minor eEF2 amounts present in the P07 fraction. NAD^+, in turn, failed to inhibit the release of FA* from the P07 fraction in CRM197-treated cells. Finally, FA appeared to be released from the P07 fraction as a binary complex of eEF2 and FA (Fig. 4C). Gel filtration analysis of the S07¤ fraction, the supernatant obtained by centrifugation of the P07 fraction after incubation with eEF2, revealed the presence of ADP-ribosylation activity in the fractions corresponding to around M_r 120 kDa, even without the addition of eEF2 or DT(FA). This finding was supported by western blot analysis, which indicated that both eEF2 and FA were present in these fractions. FA released from the P07 frac-

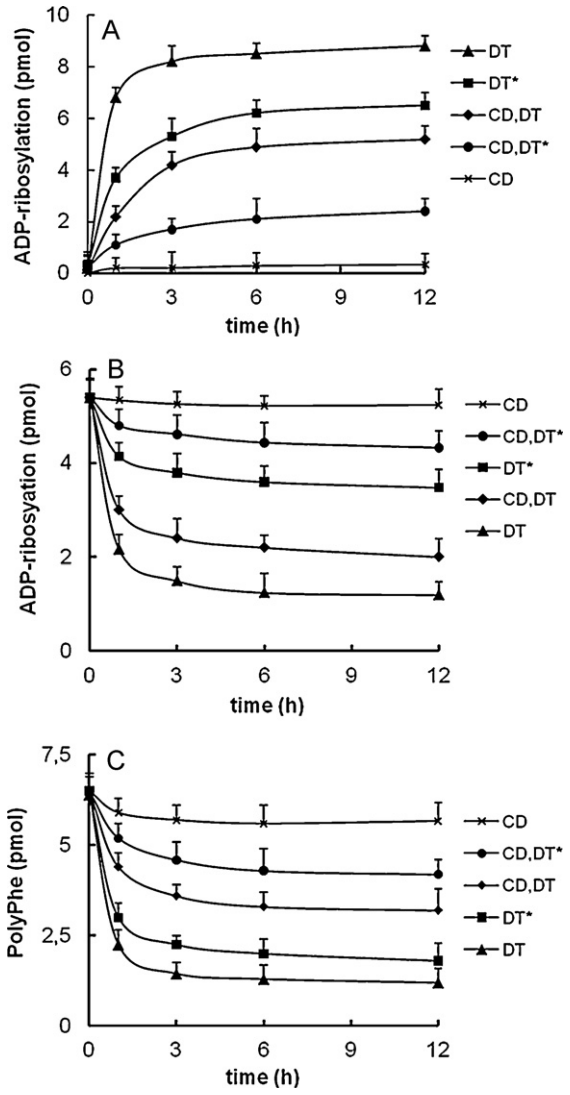

Fig. 2. Effect of CD-treatment on cytosolic FA activity. HUVEC were incubated for the indicated periods of time in the presence of 15 and 150 pM DT. CD concentration, if present, was 2 μM. After incubation, the cells were homogenized and cell extracts were subjected to differential centrifugation, as described in Section 2. The supernatant fraction (S120) obtained after ultracentrifugation for 2 h at 120,000 × *g* was assayed for (A) FA (ADP-ribosyltransferase) activity, (B) free ADP-ribose acceptor sites (and inversely, the extent of *in vivo* ADP-ribosylated eEF2) and (C) protein synthetic activity (eEF2 activity) in polyPhe synthesis. (×–×), CD (2 μM); (▲–▲), DT (150 pM); (■–■), DT* (15 pM); (♦–♦), CD, DT (150 pM); (●–●), CD, DT* (15 pM).

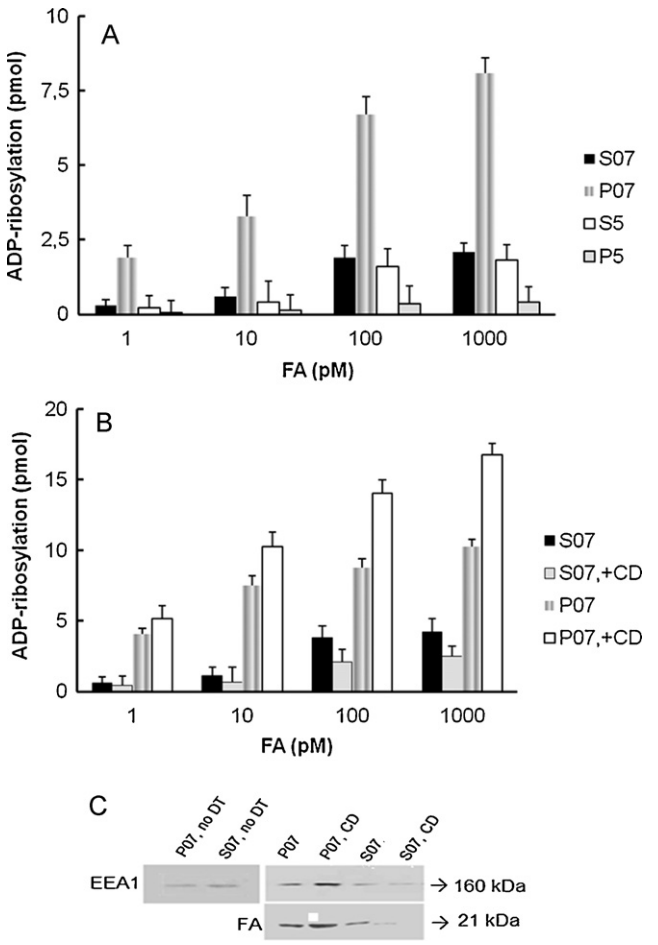

Fig. 3. (A) Distribution of FA(DT) in cytosolic and early endosomal compartments. Incubation of HUVEC was performed for 10 min in the presence of the indicated concentrations of DT. Cells were then homogenized and cell extracts centrifuged for 7 min at 700 × *g*. The supernatant fraction (S07) was centrifuged again for 10 min at 5000 × *g*. Both pellets (P07 and P5) were resuspended in PBS and assayed along with the supernatant fractions (S07 and S5) for ADP-ribosyltransferase activity for 5 min. (B) Effect of CD on the distribution of FA(DT) into cytosolic and early endosomal compartments. Experimental conditions were the same as (A), with the inclusion of a CD incubation and an extension of the reaction time to 20 min as indicated. For the sake of simplicity, only data pertaining to the P07 and S07 fractions are included. (C) Distributions of EEA1 and FA between the P07 and S07 fractions. Cells were incubated for 30 min in the presence of DT (750 pM), and EEA1 and FA were visualized by western blotting as described in Section 2. Control (left) panel denotes EEA1 bands from cells incubated in the absence of DT.

tion was also found in unbound form corresponding to around M_r 20 kDa.

3.5. F-actin and eEF2 binding to early endosomes

The results from our *in vitro* translocation assay indicated that eEF2 and F-actin can promote FA release from early endosomes to varying degrees. Accordingly, one might expect that these agents first bind to DT-loaded early endosomes *in vitro*. With this consideration in mind, we explored whether an interaction between DT-loaded early endosomes and eEF2 and/or F-actin occurs *in vitro*. Thus, following incubation with these agents, early endosomes were isolated by centrifugation and subjected to analysis by SDS-PAGE and western blotting. As shown in Fig. 5A, both eEF2 and F-actin appeared to bind by themselves to DT-loaded early endosomes. Moreover, as revealed by an increase in the intensity of the respective bands, this binding became stronger if these two agents were incubated together with early endosomes. Hence, our data suggest that an interaction between these two proteins occurs during this binding. On the other hand, eEF2 and F-actin could bind only slightly to early endosomes with cargo other than DT (Fig. 5B). This result could explain why weak, but still discernable, eEF2- and actin-specific immunoreactive bands were present in early endosomal samples loaded with transferrin.

4. Discussion

The precise mechanisms of FA translocation across endosomal membranes and the steps that determine its cytosolic refolding and distribution are well documented (Collier, 2001). Unfolding of the toxin is known to occur in the acidic milieu of early endosomes (Blewitt et al., 1985; Wiedlocha et al., 1992; Falnes et al., 1994), leading to the exposure of the hydrophobic T-domain with an increased tendency to bind to membrane lipids (Sandvig and Olsnes, 1981; Montecucco et al., 1985). The membrane insertion of the T-domain gives rise to the formation of a cation-selective chan-

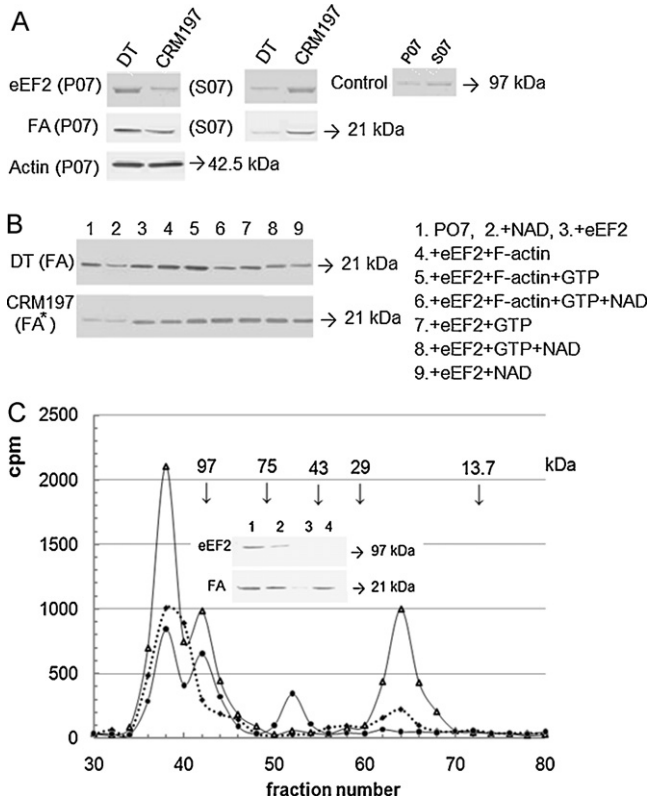

Fig. 4. eEF2-promoted FA release from early endosomes in the P07 fraction. (A) Determination of eEF2, actin, and FA or its mutant counterpart (FA*) from CRM197 in the P07 and S07 fractions by western blot analysis. (B) eEF2-promoted FA and FA* release from early endosomes in the P07 fraction: stimulation by F-actin and GTP, and a differential effect of NAD^+ on FA versus FA* release. Cells were incubated for 30 min with 750 pM DT or CRM197, and the P07 and S07 fractions were obtained by homogenization followed by centrifugation at 700 × *g*. The P07 fraction was then washed by repeated cycles of resuspension in PBS and centrifugation at the same speed. The P07 and S07 fractions were then subjected to western blot analysis (A). Additionally, P07 was incubated either alone or in the presence of eEF2 (2 μM) in combination with the other agents, as indicated (B). After incubation, the P07 fraction was removed by centrifugation and the supernatant (denoted as S07¤, to distinguish it from the original S07 obtained after the initial centrifugation of cell extract) was analyzed for FA or FA*. (C) FA is released from early endosomes as a binary complex with eEF2. Following incubation with eEF2, the P07 fraction was removed by centrifugation and the S07¤ was subjected to gel filtration on a Sephacryl S-100 column. Column fractions were assayed in ADP-ribosylation alone (♦–♦) or in the presence of either eEF2 (2 μM) (▲–▲) or DT (2 μM) (●–●). The inset shows the results from western blot analysis: lanes 1–4 correspond to the peak fractions 38, 42, 52 and 65, respectively.

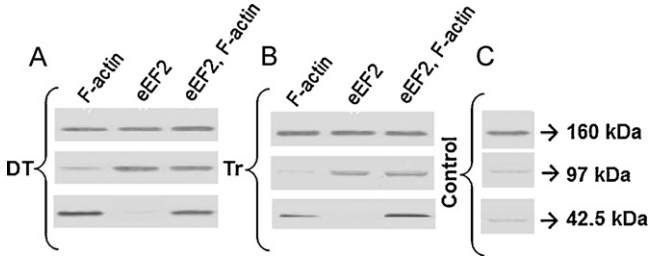

Fig. 5. *In vitro* binding of eEF2 and F-actin to early endosomes. The early endosome fraction obtained by density gradient centrifugation was centrifuged for 1 h at 15,000 × *g*. The pellet was resuspended in PBS and centrifuged again in the same way. This step was repeated once more, and the final early endosomal pellet resuspended in PBS was used in the *in vitro* binding assays. Early endosomes were incubated for 20 min in the presence of 50 pmol eEF2 and/or 50 pmol F-actin and then collected by centrifugation for 20 min at 15,000 × *g*. The pellets were once again resuspended in sample buffer for gel electrophoresis, and aliquots from the suspension (2 mg protein) were subjected to SDS-PAGE and analyzed for eEF2 and actin by western blot. (A) Binding to early endosomes from cells treated with DT (750 pM) for 30 min. (B) Binding to early endosomes from cells incubated with transferrin (Tr, 2 μM) for 60 min. (C) Control samples with no additions.

Table 1
In vitro FA release from early endosomes: effects of different agents. Early endosomal fraction was obtained by density gradient centrifugation of the P07 fraction from HUVEC incubated for 20 min in the presence of 750 pM DT. It was preincubated for 10 min at 37 °C alone or in the presence of the components, as indicated. Early endosomes were then removed by centrifugation for 20 min at 15,000 × *g*. After the addition of a saturating amount of eEF2 (20 pmol) and [^{14}C]NAD^+, the samples were assayed for 10 min for FA activity. The samples preincubated in the presence of 20 pmol eEF2 received no additional eEF2, but those incubated in the presence of ADP-ribosylated eEF2 did, such that in all reaction mixtures the final amount of the unmodified factor was 20 pmol. The concentrations of F-actin and Hsp90 were each 2 μM, and those of ATP (added together with F-actin) and GTP were 0.5 mM. Whenever added, ADP-ribosylated eEF2 was used following gel filtration. The eEF2 content of HUVEC cytosol was 1 pmol. Values of the samples with ATP and GTP alone were close to the control level.

Components added/treatments	ADP-ribosylation (pmol/20 pmol eEF2)
Early endosomes alone (control)	2.8 ± 0.6
+Triton X-100	12.7 ± 0.7
+Low pH	10.4 ± 0.4
+HUVEC cytosol (S15)	4.7 ± 0.3
+HUVEC-CD (cytosol) (S15)	3.6 ± 0.3
+F-actin	5.9 ± 0.2
+G-actin	4.6 ± 0.4
+eEF2	6.8 ± 0.5
+eEF2, +GTP	9.4 ± 0.5
+eEF2, +F-actin	9.9 ± 0.6
+eEF2, +GTP, +F-actin	10.6 ± 0.5
+eEF2, +G-actin	6.7 ± 0.4
+ADPR-eEF2	4.8 ± 0.3
+ADPR-eEF2, +G-actin	5.4 ± 0.4
+ADP-eEF2, +GTP, +F-actin	5.8 ± 0.4
+ADPR-eEF2, +GTP, +F-actin	5.9 ± 0.5
+Hsp90	5.4 ± 0.4
+Hsp90, +eEF2, +F-actin	9.2 ± 0.6

nel involving the TH8 and TH9 helices (Papini et al., 1988; Sandvig and Olsnes, 1988). The relationship of this channel to the translocation of FA requires further elucidation. Along with the findings indicating that channel formation may not be strictly essential (Lanzrein et al., 1996), other studies suggest that the T-domain may provide necessary machinery for and play a direct role as a chaperone in the translocation process (Ren et al., 1999; Oh et al., 1999). Another study shows that, in addition to the T-domain, translocation requires cytosolic factors and ATP (Lemichez et al., 1997). In line with this last finding, Hsp90 has been shown to act as a chaperon in translocation and refolding, and thioredoxin reductase is a necessary component in the reduction of the DT interchain disulfide bond (Ratts et al., 2003).

As previously mentioned, this investigation was initiated to explore whether the recently reported interaction between FA and actin (Bektaş et al., 2009) had any biological relevance. With this objective in mind, we studied the effect of actin filament breakdown on FA activity and distribution in CD-treated cells. CD was shown to alter the intracellular distribution of FA, enhance the already enlarged size of DT-loaded early endosomes (Antignani and Youle, 2008), and inhibit FA activity in the cytosol. Endosomal segregation of FA caused by blocking its cytosolic entry appeared to account for this inhibition in CD-treated cells. These findings suggest that actin filaments are somewhat involved in the cytosolic release of FA from early endosomes. The results indicate that considerable amounts of internalized DT remain in the endocytic compartment and are in line with previous reports that the release of FA into the cytosol is a limiting step (Lemichez et al., 1997; Hudson and Neville, 1985).

The CD effect was particularly evident in the presence of low DT concentrations, which are sufficient for lethality. The FA

release, which was considerably reduced at these DT concentrations, appeared to be an F-actin-facilitated process. Actin filaments are well known for their involvement in vesicular trafficking (Durrbach et al., 1996; Lanzetti, 2007; Smythe and Ayscough, 2006). As the findings presented under Supplementary data shows, primaquine, a compound that blocks recycling of several receptors without affecting the endosomal pH (Van Weert et al., 2000; Woods et al., 2004) inhibits also FA release. Thus, CD-promoted breakdown of actin cytoskeleton may account for the inhibition of FA release by blocking of vesicular trafficking. A recent report has additionally shown that secretory vesicles are tethered to actin filaments (Abu-Hamdah et al., 2006). This bond is thought to be important for the appropriate transport and localization of secretory vesicles. Breakdown of actin filaments brings about the disruption of this bond (Abu-Hamdah et al., 2006), which, according to the data provided in this report, may be primarily involved in FA translocation across endosomal membranes and subsequent refolding. An explanation that assumes the involvement of actin filaments not only in vesicular trafficking but also in the translocation process finds support from the results presented here and from other reports (Durrbach et al., 1996; Su et al., 2007; Ji et al., 2007), which attest to a role of actin and Hsp90 interactions in the regulation of certain cellular reactions. Thus, actin filaments may provide a structural framework for the cellular components involved in interactions with early endosomes. Hsp90 and thioredoxin reductase appear to be such components (Ratts et al., 2003), and eEF2, the substrate of ADP-ribosyltransferase reaction, may be another candidate in the context of FA release. In the light of the reported interactions between eEF2, FA, and F-actin (Bektaş et al., 2009; Bektaş et al., 1994), such a hypothesis does not seem to be too unlikely, and the results of this report, indicating eEF2- and F-actin-promoted FA release, provide strong support for this view.

The scarcity of FA molecules present in the cytosolic compartment at (low) DT concentrations deserves some consideration. It appears to be the result of a highly efficient negative feedback that allows for the release of only a few FA molecules into the cytosol. These FA molecules are known to be sufficient for producing the cytotoxic effect (Yamaizumi et al., 1978; Falnes and Sandvig, 2000). On the other hand, shortly after the release of the very first FA molecules, a component is thought to emerge in the cytosol that has a universal effect on all DT-loaded early endosomes, blocking further entry of FA molecules. ADP-ribosylated eEF2, as the product of the enzymatic action of FA, may be a candidate for exerting this effect. Indeed, ADP-ribosylation seems to reduce the extent of *in vitro* FA release. The inhibitory effect observed here is partial, but it suggests that unmodified diphthamide residue may still be one of the requirements for FA release. The fact that relatively larger amounts of the enzymatically inactive fragment A (FA*) of CRM197 are found in the cytosolic compartment also seems to support this possibility.

A rather unexpected finding of this investigation has been the stimulatory effect of GTP on eEF2- and F-actin-mediated release of FA. Endosomes are rich in G-protein content, with several members of Rab family, such as Rab5, being involved in (early) endosome-associated activities (Stein et al., 2003). On the other hand, eEF2 itself is a well-known motor and G-protein. The relationship of these G-protein/GTPase features to FA release is unclear. The implications of these findings will be as next investigated in our laboratory by using dominant negative mutants of Rab4, Rab5, and Rab11.

Acknowledgements

This work was supported by the TÜBİTAK (Grant no. 107S032) and in part, by the Research Fund of the İstanbul University (Grant no. 463-04012006).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2011.05.017.

References

Abu-Hamdah R, Cho WJ, Hörber JKH, Jena BP. Secretory vesicles in live cells are not free-floating but tethered to filamentous structures: a study using photonic force microscopy. Ultramicroscopy 2006;106:670–3.
Antignani A, Youle RJ. Endosome fusion induced by diphtheria toxin translocation domain. Proc Natl Acad Sci USA 2008;105:8020–5.
Bektaş M, Nurten R, Gürel Z, Sayers Z, Bermek E. Interactions of eukaryotic elongation factor 2 with actin: a possible link between protein synthetic machinery and cytoskeleton. FEBS Lett 1994;356:89–93.
Bektaş M, Akçakaya H, Aroymak A, Nurten R, Bermek E. Effect of oxidative stress on in vivo ADP-ribosylation of eukaryotic elongation factor 2. Int J Biochem Cell Biol 2005;37:91–9.
Bektaş M, Varol B, Nurten R, Bermek E. Interaction of diphtheria toxin (fragment A) with actin. Cell Biochem Funct 2009;27:430–9.
Bennett MJ, Choe S, Eisenberg D. Refined structure of dimeric diphtheria toxin at 2.0 A resolution. Protein Sci 1994;3:1444–63.
Blewitt MG, Chung LA, London E. Effect of pH on the conformation of diphtheria toxin and its implications for membrane penetration. Biochemistry 1985;24:5458–64.
Choe S, Bennett MJ, Fujii G, Curmi PM, Kantardjieff KA, Collier RJ, et al. The crystal structure of diphtheria toxin. Nature 1992;357:216–22.
Collier RJ. Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. J Mol Biol 1967;25:83–98.
Collier RJ. Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. Toxicon 2001;39:1793–803.
D'Silva PR, Lala AK. Organisation of diphtheria toxin in membranes. A hydrophobic photolabeling study. J Biol Chem 1998;275:11771–7.
Draper RK, Simon MI. The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. J Cell Biol 1980;87:849–54.
Durrbach A, Louvard D, Coudrier E. Actin filaments facilitate two steps of endocytosis. J Cell Sci 1996;109:457–65.
Falnes PO, Choe S, Madshus IH, Wilson BA, Olsnes S. Inhibition of membrane translocation of diphtheria toxin A-fragment by internal disulfide bridges. J Biol Chem 1994;269:8402–7.
Falnes PO, Sandvig K. Penetration of protein toxins into cells. Curr Opin Cell Biol 2000;12:407–13.
Gill DM. Bacterial toxins: a table of lethal amounts. Microbiol Rev 1982;46:86–94.
Hudson TH, Neville Jr DM. Quantal entry of diphtheria toxin to the cytosol. J Biol Chem 1985;280:2675–80.
Ji Y, Ferracci G, Warley A, Ward M, Leung KY, Samsuddin S, et al. beta-Actin regulates platelet nitric oxide sythase 3 activity through interaction with heat shock protein 90. Proc Natl Acad Sci USA 2007;104:8839–44.
Kaul P, Silverman J, Shen WH, Blanke SR, Huynh PD, Finkelstein A, et al. Roles of Glu 349 and Asp 352 in membrane insertion and translocation by diphtheria toxin. Protein Sci 1996;5:687–97.
Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
Lanzetti L. Actin in membrane trafficking. Curr Opin Cell Biol 2007;19:453–8.
Lanzrein M, Sand O, Olsnes S. GPI-anchored diphtheria toxin receptor allows membrane translocation of the toxin without detectable ion channel activity. EMBO J 1996;15:725–34.
Lemichez E, Bomsel M, Devilliers G, vanderSpek J, Murphy JR, Lukianov EV, et al. Membrane translocation of diphtheria toxin fragment A exploits early to late endosome trafficking machinery. Mol Microbiol 1997;23:445–57.
Matarrese P, Malorni W. Human immunodeficiency virus (HIV)-proteins and cytoskeleton: partners in viral life and host cell death. Cell Death Differ 2005;12:932–41.
Montecucco C, Schiavo G, Tomasi M. pH dependence of the phospholipid interaction of diphtheria toxin fragments. Biochem J 1985;231:123–8.
Naglich JG, Metherall JE, Russell DW, Eidels L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding, EGF-like growth factor precursor. Cell 1992;69:1051–61.
Oh KJ, Senzel L, Collier RJ, Finkelstein A. Translocation of the catalytic domain of diphtheria toxin across planar phospholipid bilayers by its own T domain. Proc Natl Acad Sci USA 1999;96:8467–70.
Ohmori H, Toyama S, Toyama S. Direct proof that the primary site of action of cytochalasin on cell motility processes is actin. J Cell Biol 1992;116:933–41.
Oram DM, Holmes RK. Diphtheria toxin. In: Alouf JE, Popoff MR, editors. The comprehensive sourcebook of bacterial protein toxins. third ed Amsterdam: Academic Press, Elsevier; 2006. p. 245–56.
Papini E, Sandoná D, Rappuoli R, Montecucco C. On the membrane translocation of diphtheria toxin: at low pH the toxin induces ion channels on cells. EMBO J 1988;7:3353–9.
Pappenheimer Jr AM. Diphtheria toxin. Annu Rev Biochem 1984;46:69–94.
Provance Jr DW, Addison EJ, Wood PR, Chen DZ, Silan CM, Mercer JA. Myosin-Vb functions as a dynamic tether for peripheric endocytic compartments during transferin trafficking. BMC Cell Biol 2008;9:44.

Ratts R, Zeng H, Berg EA, Blue C, McComb ME, Costello CE, et al. The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. J Cell Biol 2003;160:1139–50.

Ren J, Kachel K, Kim H, Malenbaum SE, Collier RJ, London E. Interaction of diphtheria toxin T domain with molten globule-like proteins and its implications for translocation. Science 1999;284:955–7.

Roodyn DB. Some methods for the isolation of nuclei from mammalian cells. In: Birnie GD, Fox SM, editors. Subcellular components. London: Butterworths; 1969. p. 15–42.

Sandvig K, Olsnes S. Rapid entry of nicked diphtheria toxin into cells at low pH. Characterisation of the entry process and effects of low pH on the toxin molecule. J Biol Chem 1981;256:9068–76.

Sandvig K, Olsnes S. Diphtheria toxin-induced channels in Vero cells selective for monovalent cations. J Biol Chem 1988;263:12352–9.

Smythe E, Ayscough KR. Actin regulation in endocytosis. J Cell Biol 2006;119:4589–98.

Stein MP, Dong J, Wandinger-Ness A. Rab proteins and endosomal trafficking: potential targets for therapeutic intervention. Adv Drug Deliv Rev 2003;55:1421–37.

Su Y, Kondrikov D, Block ER. Beta-actin: a regulator of NOS-3. Sci STKE 2007;404:pe52.

Tjelle TE, Brech A, Juvet LK, Griffiths G, Berg T. Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. J Cell Sci 1996;109:2905–14.

Van Ness BG, Howard JB, Bodley JW. ADP-ribosylation of elongation factor 2 by diphtheria toxin, NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. J Biol Chem 1980;255:10710–6.

Van Weert AW, Geuze HJ, Groothuis B, Stoorvogel W. Primaquine interferes with membrane recycling from endosomes to the plasma membrane through a direct interaction with endosomes which does not involve neutralisation of endosomal pH nor osmotic swelling of endosomes. Eur J Cell Biol 2000;79:394–9.

Woods AJ, White DP, Caswell PT, Norman JC. PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. EMBO J 2004;23:2531–43.

Wiedlocha A, Madshus IH, Mach H, Middaugh CR, Olsnes S. Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector. EMBO J 1992;11:4835–42.

Yamaizumi M, Mekada E, Uchida T, Okada Y. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell 1978;15:245–50.