HSC70 and HSP90 chaperones perform complementary roles in translocation of the cholera toxin A1 subunit from the endoplasmic reticulum to the cytosol

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Cholera toxin (CT) is an AB₅ toxin that is endocytosed after binding to GM1 gangliosides on the plasma membrane of a host cell (1). Most internalized toxin is routed to the lysosomes for degradation, but 5–10% of the cell-associated toxin travels to the endoplasmic reticulum (ER) where it catalytically dissociates from the rest of the toxin, unfolds, and moves through a membrane-spanning translocon pore to the cytosol. Heat shock protein 90 (HSP90) binds to the N-terminal region of CT A1 and facilitates its ER-to-cytosolic export by refolding the toxin as it emerges at the cytosolic face of the ER membrane. HSP90 also refolds some endogenous cytosolic proteins as part of a foldosome complex containing heat shock cognate 71-kDa protein (HSC70) and the HSC70/HSP90-organizing protein (HOP) linker that anchors HSP90 to HSC70. We accordingly predicted that HSC70 and HOP also function in CTA1 translocation. Inactivation of HSC70 by drug treatment disrupted CTA1 translocation to the cytosol and generated a toxin-resistant phenotype. In contrast, the depletion of HOP did not disrupt CT activity against cultured cells. HSC70 and HSP90 could bind independently to disordered CTA1, even in the absence of HOP. This indicated HSP90 and HSC70 recognize distinct regions of CTA1, which was confirmed by the identification of a YYIYV-binding motif for HSC70 that spans residues 83–88 of the 192-amino acid CTA1 polypeptide. Refolding of disordered CTA1 occurred in the presence of HSP70 alone, indicating that HSC70 and HSP90 can each independently refold CTA1. Our work suggests a novel translocation mechanism in which sequential interactions with HSP90 and HSC70 drive the N- to C-terminal extraction of CTA1 from the ER.

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²The abbreviations used are: CT, cholera toxin; CysA, cyclosporin A; DT, diphtheria toxin; CTA1, cognate 71-kDa protein; HSP40, heat shock protein 40; HSP70, heat shock protein 70; HSP90, heat shock protein 90; HOP, HSC70/HSP90-organizing protein; PES, 2-phenylethynesulfonamide; PBST, phosphate-buffered saline with 0.05% Tween 20; RIU, refractive index unit; SPR, surface plasmon resonance.

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identity, and they perform redundant functions in protein qual-
ity control (37–39).

HSP90 can bind directly to CTA1 in an unusual interaction that does not require co-chaperones (17, 18), but we still pre-
predicted that HSC70 and HOP would also be involved with the ER-to-cytosol export of CTA1. HSC70/HSP70 inactivation dis-
rupted CTA1 passage into the cytosol and generated a toxin-
resistant phenotype. HOP, in contrast, was not required for CT
intoxication. HSC70 could directly bind to either folded or dis-
ordered CTA1, whereas HSP90 only interacts with unfolded con-
formations of CTA1 (18). Like HSP90 (18), HSC70 gener-
ated a gain-of-structure in unfolded CTA1. However, HSC70
recognized an internal YYIYVI sequence in CTA1 as opposed
to the previously reported interaction between HSP90 and the
N-terminal region of CTA1 (40). Our work thus demonstrates
atypical client-chaperone interactions, independent of the
foldosome complex, drive CTA1 export from the ER to the
cytosol.

Results
CT intoxication involves HSC70 and HSP70 but not HOP
RNAi was initially used to examine the functional roles of
HSC70, HSP70, and HOP in CT intoxication. Western blot
analysis confirmed the specific depletion of each targeted pro-
tein in siRNA-transfected HeLa cells (Figs. 1, A and B). After a
2-h exposure to CT, each of these cell populations exhibited a
cAMP response comparable with the mock transfected cells
and cells transfected with the control siRNA (Fig. 1C). The indi-
vidual HSC70, HSP70, and HOP proteins were therefore dis-
pensable for CT activity against cultured cells.

HSC70 and HSP70 could perform redundant functions in CT
intoxication. To examine this possibility, we first attempted a
cointransfection with HSC70 and HSP70 siRNA. This strategy
apparently generated a stress condition that resulted in
enhanced HSP70 expression, to the point of overcoming the
RNAi effect (Fig. 1B). Toxin-challenged cells were therefore
exposed to 2-phenylethynesulfonamide (PES), an inhibitor of
both HSP70 and HSC70 (38, 41), as an alternative approach.
Cells treated with PES exhibited substantially lower levels of
toxin-induced cAMP compared with the untreated control
cells (Fig. 1C). PES did not affect the basal level of cAMP from
cells, but cells treated with PES and the adenylate cyclase agonist forskolin exhibited a 2.0-fold (0.5 range, n = 2)
increase in cAMP production when compared with cells incu-
bated with forskolin alone. Our intoxication data did not com-
pensate for this stimulatory effect, which highlights the potent
inhibition of CT activity by PES. From these collective observa-
tions, HSC70 and HSP70 appear to play redundant roles in the
CT intoxication process.

HSC70/HSP70 is required for CTA1 translocation to the cytosol
Like HeLa cells, PES-treated CHO cells exhibited a 3.0-fold
(0.25 range, n = 2) increase in forskolin-driven cAMP produc-
tion, which was not corrected for in the corresponding intoxi-
cation assays. Despite this stimulatory effect, PES completely
protected CHO cells from the cytopathic activity of CT
(Fig. 2A).

Loss of HSP90 function has been shown to protect cultured
cells from CT by inhibiting the ER-to-cytosol export of CTA1
(17). We predicted the loss of HSC70/HSP70 activity would
also reduce the efficiency of CTA1 translocation to the cytosol.
To test this prediction, CHO cells were transfected with the
ssCTA1/pCDA3.1 expression plasmid that targets CTA1 for
co-translational insertion into the ER via an N-terminal signal
sequence. Proteolytic processing removes the signal sequence
from CTA1 in the ER lumen, and the toxin is then dislocated
back into the cytosol. Rapid removal of the CTA1 signal
sequence in the ER results in exclusive detection of the pro-
cessed, mature form of CTA1 (42, 43). This strategy bypasses
the trafficking events required for CT delivery to the ER and

Figure 1. Inactivation of both HSC70 and HSP70, but not HOP, blocks the cytopathic effect of CT. HeLa cells were mock-transfected or transfected with
siRNA targeting HOP, HSC70, HSP70, or both HSC70 and HSP70. A and B, cell extracts were probed by Western blot analysis with antibodies against (A) HOP or
(B) HSC70 and HSP70, as indicated. In all panels, blotting against actin was used as a loading control. One of 4 representative experiments is shown for each
panel. C, cells were exposed to 100 ng/ml of CT for 2 h before intracellular cAMP levels were determined. Results from siRNA-transfected cells or PES-treated
cells were expressed as percentages of the values obtained from mock-transfected or mock-treated cells, respectively. Data from at least 4 independent
experiments per condition are presented as box-and-whisker plots. The asterisk indicates a statistically significant difference from the control (p < 0.0001,
Student’s t test).
treatment thus reduced CTA1 translocation to the cytosol by 40%.

**Cyclophilin A is not required for CT intoxication**

The A chains of some ADP-ribosylating toxins move from the endosomes to the cytosol in a process involving HSP90, HSP70, and cyclophilin A (44). CTA1 translocation from the ER involves HSP90 and HSC70/HSP70, and cyclophilins can act as co-chaperones in the foldosome complex (45). We therefore examined the possible role of cyclophilin A in the CT intoxication process. Cells treated with the cyclophilin inhibitor cyclosporin A (CysA) (46) were challenged with either DT or CT. DT is an ADP-ribosylating toxin that inhibits protein synthesis, and its A chain moves from the endosomes to the cytosol in an HSP90-dependent manner (21, 22, 47). Consistent with previous reports (22, 48), we found CysA-treated cells were resistant to the cytotoxic action of DT (Fig. 3A). In contrast, treatment with CysA did not protect cells from CT (Fig. 3B). The endosome-to-cytosol translocation of DT A chain and the ER-to-cytosol translocation of CTA1 thus share some common host factors but are distinct molecular events.

**HSC70 binds directly to CTA1**

HSP90 exhibits a direct, high-affinity interaction with disordered CTA1 that facilitates the translocation event (17). A direct interaction between HSC70 and CTA1 could likewise contribute to toxin translocation. To determine whether HSC70 binds directly to CTA1, we perfused HSC70 over a CTA1-coated surface plasmon resonance (SPR) sensor at 15 or 37 °C (Fig. 4A). Previous studies have shown by CD, tryptophan fluorescence, and FTIR spectroscopy that CTA1 maintains a folded conformation at low temperature but transitions to a partially unfolded state at the physiological temperature of 37 °C (7, 49). HSC70 could bind to both the folded conformation of CTA1 present at 15 °C and the disordered conformation of CTA1 present at 37 °C. Binding did not require HSP40, which is often necessary to deliver client proteins to HSC70 (30–32). Likewise, the binding of HSP90 to CTA1 does not depend upon previous association with HSP40, HSC70, or HOP (17). Both HSC70 and HSP90 form tight complexes with CTA1, as little dissociation is seen after removal of the chaperone from the perfusion buffer (Fig. 4A) (17, 18). We previously calculated a 7 nM $K_D$ affinity between HSP90 and CTA1 at 37 °C (17). HSC70 exhibited a similar 5 nM $K_D$ affinity for CTA1 at 37 °C (Fig. 4B). However, host-toxin interactions are not identical for the two chaperones: HSC70 will bind to either folded or disordered CTA1, whereas HSP90 only recognizes disordered CTA1 (18).

**HSC70 and HSP90 recognize distinct regions of CTA1**

Additional SPR experiments were performed with various combinations of CTA1, HSC70, and HSP90. In Fig. 5A, HSP90 was perfused over a CTA1-coated SPR sensor at 37 °C. HSP90 did not appreciably dissociate from CTA1 after its removal from the perfusion buffer, which was consistent with our published reports (17, 18). Addition of HSC70 to the sensor-bound CTA1/HSP90 complex produced a further increase in the refractive index unit (RIU), which did not appreciably drop
after removal of HSC70 from the perfusion buffer. This demonstrated HSC70 and HSP90 can both bind to disordered CTA1 at the same time. In a related experiment, CTA1 was perfused over an HSC70-coated SPR sensor at 37 °C (Fig. 5B). CTA1 did not appreciably dissociate from HSC70 after its removal from the perfusion buffer. Addition of HSP90 to the sensor-bound HSC70/CTA1 complex produced a further increase in the RIU, which did not decrease after removal of HSP90 from the perfusion buffer. HSP90 could thus bind to an existing complex of CTA1 and HSC70, just as HSC70 could
Atypical chaperone interactions direct CTA1 exit from the ER

Table 1

<table>
<thead>
<tr>
<th>CTA1 residues</th>
<th>Peptide sequence</th>
<th>HSC70 interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–16</td>
<td>NDDKLYRADSRPPDEI</td>
<td>–</td>
</tr>
<tr>
<td>11–26</td>
<td>RPPEIKEQSGGLMPRG</td>
<td>–</td>
</tr>
<tr>
<td>21–36</td>
<td>GLMPRGQSEYFDRTGQ</td>
<td>–</td>
</tr>
<tr>
<td>31–46</td>
<td>FDRGTQMNINLYDHR</td>
<td>–</td>
</tr>
<tr>
<td>41–56</td>
<td>LYHARTGQTGFVRHID</td>
<td>–</td>
</tr>
<tr>
<td>51–66</td>
<td>GFVYHDDGQVTSYSIL</td>
<td>–</td>
</tr>
<tr>
<td>63–78</td>
<td>SISLBSAHLGYQTLIS</td>
<td>–</td>
</tr>
<tr>
<td>73–88</td>
<td>GTQTLHGSTYJIYVI</td>
<td>+</td>
</tr>
<tr>
<td>83–98</td>
<td>YYIYVIATAPNMVFN</td>
<td>+</td>
</tr>
<tr>
<td>93–108</td>
<td>NMNFNVNDVLGAYSFH</td>
<td>+</td>
</tr>
<tr>
<td>103–118</td>
<td>AYPHPDEEQVSAALG</td>
<td>–</td>
</tr>
<tr>
<td>113–128</td>
<td>VSALGGIPSYQGWY</td>
<td>–</td>
</tr>
<tr>
<td>123–138</td>
<td>QIYGWYRHFVGVLDEQ</td>
<td>–</td>
</tr>
<tr>
<td>133–148</td>
<td>GYLDEQHLRNGYRDR</td>
<td>–</td>
</tr>
<tr>
<td>143–158</td>
<td>RGFDYFYSNLDIAPA</td>
<td>–</td>
</tr>
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<td>153–168</td>
<td>LDIAAAPAGGYGLAGFP</td>
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</tr>
<tr>
<td>163–177</td>
<td>GLAGFPPEHRAWREE</td>
<td>–</td>
</tr>
<tr>
<td>173–188</td>
<td>AWREEPWHHAPPGC</td>
<td>–</td>
</tr>
<tr>
<td>179–192</td>
<td>WHHHAPPGCNAPRF</td>
<td>–</td>
</tr>
<tr>
<td>73–82</td>
<td>GTQTLHGST</td>
<td>–</td>
</tr>
<tr>
<td>73–88m</td>
<td>GTQTLHGSTYILIYVL</td>
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</tr>
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</table>

bind to an existing complex of CTA1 and HSP90. Any CTA1 captured on the HSC70-coated slide would, by necessity, have its HSC70-binding site occupied before the addition of HSP90 to the perfusion buffer. Consistent with previous reports (50), HSP90 only exhibited minimal direct binding to HSC70 (Fig. 5C). HSC70 and HSP90 therefore recognize distinct regions of CTA1 and do not compete for a single binding site.

HSC70 recognizes an internal YYIYVI sequence in CTA1

To identify the CTA1-binding site(s) for HSC70, we generated a series of peptides that contained overlapping amino acid sequences from the entire length of the CTA1 subunit. As summarized in Table 1, HSC70 bound two consecutive peptides spanning CTA1 amino acid residues 73–98. These peptides contained a shared YYIYVI sequence that likely represented the core HSC70-binding site. The YYIYVI sequence alone could not be generated as a soluble hexapeptide, but we found a truncated GQTILSGHST peptide that lacked the YYIYVI motif and a mutant GQTILSGHSTYYLYVL peptide with leucine for isoleucine substitutions in the YYIYVI motif could not bind to HSC70 (Table 1). These results indicated the YYIYVI motif was recognized by HSC70.

A competition assay further established the specificity of HSC70 binding to the YYIYVI sequence (Fig. 6). For this experiment, CTA1 was appended to an SPR sensor slide and exposed to perfusion buffer containing either HSC70 alone or HSC70 in combination with a CTA1-related peptide. HSC70 could bind to CTA1 in the absence of peptide and in the presence of the mutant GQTILSGHSTYYLYVL peptide. However, HSC70 could not bind to CTA1 in the presence of the GQTILSGHSTYYLYVI peptide. These observations confirmed HSC70 specifically recognizes the YYIYVI sequence in CTA1.

HSC70 refolds disordered CTA1

HSP90 couples CTA1 refolding with CTA1 extraction from the ER (18). To determine whether HSC70 could also refold CTA1, HSC70 was combined with uniformly $^{13}$C-labeled CTA1 for structural measurements involving isotope-edited FTIR spectroscopy. $^{13}$C labeling does not affect protein structure but causes a 45–50 cm$^{-1}$ spectral downshift, which allows its amide I spectrum to be resolved from the spectrum of an unlabeled protein in the same sample (49, 51). Measurements of $^{13}$C-labeled CTA1 alone at 10 and 37°C were taken as reference points for the folded and disordered conformations of CTA1, respectively. The folded conformation of CTA1 present at 10°C contained 46% α-helical and 38% β-sheet content (Fig. 7A, Table 2). Due to its thermal instability (7), CTA1 lost substantial secondary structure when it was warmed to 37°C: compared with the 10°C measurement, at 37°C there was a 15% drop in α-helix content with a corresponding increase in irregular (i.e. disordered) structure from 15 to 32% (Fig. 7B, Table 2). Addition of HSC70 to the disordered, 37°C conformation of CTA1 produced a gain-of-structure resulting in a toxin conformation with 38% α-helical, 37% β-sheet, and 17% irregular structure (Fig. 7C, Table 2). These observations demonstrated an interaction between HSC70 and disordered CTA1 leads to the refolding of CTA1.

Discussion

Unfolded CTA1 passes through an ER translocon pore to reach the cytosol and its G protein target. Translocation does not require p97 (15, 16) but instead involves an HSP90-dependent ratchet mechanism that couples CTA1 refolding with CTA1 extraction from the ER: co-translational refolding ensures unidirectional toxin export, as the refolded toxin could not slide back into the translocation pore and could therefore only move out of the ER (18). In this work, we provide evidence that HSC70/HSP70 also plays a role in CTA1 extraction to the cytosol.

HSC70 and HSP90 perform complementary but distinct functions in CTA1 translocation. Both HSC70 and HSP90 are required for toxin export to the cytosol and productive intoxication. Both chaperones also independently refold CTA1. However, there are also differences in these toxin-chaperone interactions.
interactions. HSC70 recognizes both folded and disordered CTA1, whereas HSP90 only interacts with disordered CTA1 (18). HSC70 and HSP90 thus appear to recognize distinct motifs in CTA1 and, as such, do not compete for a single binding site in the toxin. We indeed found that HSC70 recognizes a YYIYVI motif spanning residues 83–88 of the 192-amino acid CTA1 polypeptide, whereas HSP90 binds to the N-terminal region of CTA1 (40).

Several chaperones in the ER lumen interact with CTA1 either before or after its release from the CT holotoxin (49, 52–55). Chaperone binding to the free, disordered CTA1 subunit prevents toxin aggregation. It also directs the toxin to the Hrd1 translocon pore for export to the cytosol. HSP90 and HSC70 then recognize CTA1 at the cytosolic face of the ER membrane and continue the translocation event. A continuum of chaperone interactions thus guides CTA1 from the ER lumen to the cytosol.

Figure 7. HSC70 induces a gain-of-structure in disordered CTA1. The FTIR spectrum of uniformly $^{13}$C-labeled CTA1 was recorded in the absence or presence of HSC70. In the curve fitting panels of the left column, the dashed line represents the sum of all deconvoluted components from the measured spectrum (overlapping gray line). Deconvoluted components are presented in dark blue (α-helix), light blue (β-sheet), red (irregular), gray (other/turns), and black (side chains and, in C, the unlabeled protein). The right column presents the respective second derivatives. A, $^{13}$C-labeled CTA1 structure at 10 °C. B, $^{13}$C-labeled CTA1 structure after 30 min at 37 °C. C, $^{13}$C-labeled CTA1 structure in the presence of unlabeled HSC70 at 37 °C. CTA1 was heated to 37 °C for 30 min before the addition of HSC70.
Table 2

<table>
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<th>Condition</th>
<th>α-Helix</th>
<th>% of CTA1 structure</th>
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<tbody>
<tr>
<td>10 °C</td>
<td>46 ± 1</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>37 °C</td>
<td>31 ± 1</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>37 °C + HSC70</td>
<td>38 ± 0</td>
<td>37 ± 2</td>
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Deconvolution of amide I bands from the FTIR spectroscopy data of Fig. 7 was used to calculate the percentages of CTA1 structure under the stated conditions. The averages ± ranges from two curve fittings are shown.

Our collective data support a model of CTA1 translocation that involves (i) extraction of CTA1 from the ER in an N-terminal to C-terminal direction; (ii) initial contact of HSP90 with the N terminus of CTA1 as the toxin emerges from the translocon pore; and (iii) subsequent interaction between HSC70 and an internal region of CTA1. The ability of HSC70 to bind both folded and disordered conformations of CTA1 may facilitate its interaction with CTA1 after HSP90 has initiated the refolding process that is coupled with toxin extraction from the ER. Although HSC70 alone can refold CTA1 in vitro, the internal location of its binding site in CTA1 suggests HSP90 must initiate the coupled refolding/translocation process at the N terminus of CTA1 before HSC70 can contribute to CTA1 refolding in vivo. The combined action of HSP90 and HSC70 thereby delivers CTA1 to the cytosol in a folded conformation. Demonstration of a functional role for HSC70 in CTA1 translocation and identification of the HSC70-binding site in CTA1 has thus provided additional molecular detail regarding the process of toxin translocation from the ER to the cytosol.

The 5 nM affinity between HSC70 and CTA1 is unusual but not unprecedented for protein-protein interactions involving HSP70/HSC70 (56, 57). This tight binding suggests the two proteins remain in a complex after toxin delivery to the cytosol. The free CTA1 subunit is in a disordered state at physiological temperature (7), so continued association with HSC70 could allow CTA1 to maintain a folded, functional conformation for the ADP-ribosylation of Gα. HSP90 appears to play a similar role in facilitating CTA1 activity at 37 °C (18). Future work using recombinant CTA1 with a mutant YYLYVL motif that cannot bind HSC70 will examine the possible additional role of HSC70 in promoting the cytosolic activity of CTA1. Bacterial pathogenesis often involves the manipulation and modification of host factors by toxins and other virulence factors. The interplay between CTA1 and HSC70/HSP90 represents another example of this phenomenon.

HSP90 is thought to recognize a feature of ADP-ribosylating toxins that allows it to facilitate the translocation of CTA1 and the A chains from several AB toxins that enter the cytosol from acidified endosomes (40, 44). HSC70 may also recognize a general feature of ADP-ribosylating toxins, as this chaperone works with HSP90 to deliver CTA1 and several endosome-translocating toxins to the cytosol (22, 58, 59). Peptidyl-prolyl cis/trans isomerases such as cyclophilin A also function in the endosome-to-cytosol export of ADP-ribosylating toxins (44), yet we found cyclophilin A was not required for the cytopathic activity of CT. A general HSP90/HSC70-driven translocation mechanism in which toxin folding is coupled with toxin extraction to the cytosol may therefore be active at both the ER and endosome membrane, but the contributing co-chaperones apparently differ for the two translocation sites and possibly for different toxins. Moreover, the HSP90-driven mechanism appears to be specific for ADP-ribosylating toxins: HSP90 and HSC70 are not required for the ER-to-cytosol export of ricin A chain (an RNA N-glycosidase) (60); HSP90 is not required for the endosome-to-cytosol export of anthrax toxin lethal factor (an A chain metalloprotease) (48); and HSC70 is not required for the endosome-to-cytosol export of botulinum toxin light chain (another A chain metalloprotease) (61).

HSP90 and HSC70 are core components of the foldosome, a multimeric cytosolic machine active in protein renaturation. Typical foldosome activity begins with substrate binding to HSP40, transfer to HSP70, and further complex formation with HOP and HSP90. Our studies have recorded unique toxin-chaperone interactions in which HSP90 and HSC70 can each bind independently to CTA1 in the absence of HSP40 or HOP. The work presented here documents a role for HSC70/HSP70 in the ER-to-cytosol export of a soluble ERAD substrate and establishes a noncanonical pattern of chaperone-mediated protein folding that drives toxin extraction from the ER.

Experimental procedures

Materials

CysA and PES were purchased from Sigma. DT and CT were purchased from List Biological Laboratories (Campbell, CA). His-tagged CTA1 and 13C-labeled CTA1 were purified in the lab as previously described (49, 62). HSC70 was purchased from Enzo Life Sciences (Farmington, NY) or Abcam (Cambridge, United Kingdom), and HSP90 was purchased from Biovision (Milpitas, CA). Antibodies against HSP90 were purchased from Calbiochem (Darmstadt, Germany). Tissue culture reagents, Lipofectamine, and Lipofectamine 2000 were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Tablets of PBS with 0.05% Tween 20 (PBST) were purchased from Medicago (Research Triangle Park, NC). [35S]Methionine was purchased from PerkinElmer Life Sciences (Waltham, MA). HSP70 siRNA (sc-29352), STI1/HOP siRNA (sc-106905), HSC70 siRNA (sc-29349), and Control siRNA-A (sc-37007) were purchased from Santa Cruz Biotechnology (Dallas, TX). Peptides containing amino acid sequences from CTA1 were purchased from Peptide 2.0 (Chantilly, VA).

siRNA transfections

HeLa cells grown to 60–80% confluence in 24-well-plates were transfected with the indicated siRNAs (1 μM final concentrations) using Lipofectamine 2000 and Opti-MEM according to the manufacturer’s instructions. After an overnight incubation, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% FBS. Western blot analysis and intoxication assays were performed 48 h post-transfection.

Western blot analysis

For siRNA experiments, cell extracts were resolved by SDS-PAGE with 15% polyacrylamide gels and probed using the following antibodies: 1:5,000 rabbit anti-HOP (STI1) (Abcam);
1:5,000 rat anti-HSC70 (Abcam); 1:5,000 mouse anti-HSP70 (StressMarq Biosciences, Victoria, BC, Canada); 1:10,000 rabbit anti-β-actin (Abcam); and 1:10,000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), 1:10,000 HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), or 1:10,000 HRP-conjugated rabbit anti-rat IgG (Abcam). Incubations with primary antibodies were overnight at 4 °C, whereas incubations with secondary antibodies were 30 min at room temperature.

**Intoxication assays**

HeLa or CHO cells grown to ~80% confluence in 24-wellplates were transfected with varying concentrations of DT or CT in serum-free medium. Where indicated, cells were also exposed to 20 µM PES for 1–2 h or 10 µM CysA for 30 min before toxin addition. Drugs were also present during the toxin incubations.

After a 2-h incubation with CT, the cells were washed and lysed at 4 °C with acidic ethanol (1 M HCl in ethanol at a 1:100 dilution). Cell extracts were dried, resuspended in assay buffer, and processed for cAMP content using a commercial competition assay according to the manufacturer’s instructions (GE Healthcare). The basal levels of cAMP from unintoxicated cells were background subtracted from all experimental values, which were then expressed as percentages of the cAMP value obtained from cells exposed to 100 ng/ml of CT in the absence of drug or siRNA treatment (i.e., the maximal control cAMP response). Triplicate wells were used for each condition.

After a 4-h incubation with DT, cells were bathed in methionine-free medium supplemented with 10 µCi/ml of [35S]methionine for 30 min. Radiolabeled cells were washed with PBS and placed in PBS containing 10% trichloroacetic acid (TCA) for 30 min at 4 °C. After a second 10-min 4 °C wash with PBS containing 10% TCA, the cells were solubilized in 0.2N NaOH for 30 min at 4 °C. After a second 10-min 4 °C wash with PBS containing 10% trichloroacetic acid (TCA) and thionine for 30 min. Radiolabeled cells were washed with PBS (ssCTA1) using Lipofectamine as the transfection reagent (17).

**CTA1 transfection/translocation assay**

CHO cells grown to 80% confluence in 6-well plates were transfected with the pcDNA3.1/ssCTA1 plasmid encoding a CTA1 construct with the N-terminal CTA signal sequence (ssCTA1) using Lipofectamine as the transfection reagent (17). At 24 h post-transfection, the cells were placed in methionine-free medium for 1 h, incubated in the same medium supplemented with 150 µCi/ml of [35S]methionine for 1 h, and then lifted from the plate with PBS/EDTA. The collected cells were exposed to 0.04% digitonin in HCN buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM CaCl2, 10 mM N-ethylmaleimide, and protease inhibitor mixture) for 10 min at 4 °C before a 10-min centrifugal spin at 16,000 × g generated separate cytosol (supernatant) and organelle (pellet) fractions. The cytosol-containing supernatant was placed in a 1-ml final volume of PBS containing 1% Triton X-100. The pellet was reconstituted in 1 ml of PBS with 1% Triton X-100. An anti-CT antibody (Sigma) coupled to protein A-conjugated Sepharose beads was then added to both fractions for an overnight incubation at 4 °C. Immunoprecipitated samples were resolved by SDS-PAGE using 15% polyacrylamide gels and visualized with PhosphorImager analysis. To determine the extent of CTA1 export from the ER to the cytosol, background-subtracted band intensities were quantified for the ER pellet and cytosolic supernatant fractions. Export efficiency was calculated by dividing the intensity of the supernatant band by the sum intensities of the pellet and supernatant bands. When indicated, 20 µM PES was present during the methionine starvation and radiolabeling steps.

**SPR**

As previously described (63), experiments were performed with a Reichert (Depew, NY) SR7000 SPR refractometer using a flow rate of 41 µl/min for all steps. His-tagged CTA1 was appended to a nickel-nitrilotriacetic acid sensor slide (52), and amide coupling was used to append HSC70 or CTA1-derived peptides to a Reichert SPR sensor slide with a mixed self-assembled monolayer (63). Our SPR experiments used a dual-channel SPR instrument. After activation of both channels with NHS/EDC, ligand was appended to only one channel. Both channels were then blocked with ethanolamine and subjected to a 10-min perfusion with PBST buffer to establish the 0 RIU baseline that corresponded the mass of the sensor-bound protein/peptide. Both channels were subsequently exposed to the analyte(s) of interest (CTA1, HSP90 with 1 mM ATP, and/or HSC70). The second channel without immobilized ligand was used as a reference to account for nonspecific binding to the sensor. Analyte concentration was 1600 ng/ml in PBST unless otherwise noted. A 5-min perfusion of ligand was followed by a 5-min PBST wash. Reichert Labview software was used for data collection. The BioLogic (Campbell, Australia) Scrubber 2 software and WaveMetrics (Lake Oswego, OR) Igor Pro software were used to analyze the data and generate figures. Because the SPR measurements to measure K_d did not reach steady state, affinity calculations were derived from kinetic rather than equilibrium analysis. The Scrubber 2 software generated K_a and K_d values for each of our replicate experiments, which were then used to calculate K_d.

For competition assays, heat-denatured CTA1 was appended to a Biacore (GE Healthcare) Dock CM5 SPR sensor chip by amide coupling. HSC70 was mixed in PBST with a 10-fold molar excess of the indicated peptide for 10 min at room temperature before injection into the Biacore X100 instrument at a flow rate of 10 µl/min. Data were collected with Biacore X100 Control Software version 2.0.1 and analyzed with Scrubber 2 and Igor Pro software.

**Isootope-edited FTIR spectroscopy**

Samples for FTIR spectroscopy (25 µg of 13C-labeled CTA1-His8, and 83 µg of HSC70) were prepared in D2O-based 20 mM sodium borate buffer (pD 7.0) as previously described (49). All measurements were performed on a Jasco 4200 FTIR spectrometer (Easton, MD) with Peltier temperature controller (Pike Technologies, Madison, WI). When indicated, measurements were taken 10 min after chaperone addition. All traces were background-subtracted using a buffer alone control.
Atypical chaperone interactions direct CTA1 exit from the ER

Analysis of secondary structure was performed as described in detail in Ref. 49, using the following wavenumber assignments for $^{13}$C-labeled CTA1 secondary structures: 1568–1596 cm$^{-1}$ for the main $\beta$-sheet component, 1596–1604 for irregular structure, 1605–1628 for $\alpha$-helix, 1629–1635 for the higher-frequency and low extinction coefficient component of antiparallel $\beta$-sheet, and 1635–1648 cm$^{-1}$ for various turns tabulated as “other” structures.


References


Atypical chaperone interactions direct CTA1 exit from the ER


47. Murphy, J. R. (2011) Mechanism of diphtheria toxin catalytic domain delivery to the eukaryotic cell cytosol and the cellular factors that directly participate in the process. Toxins (Basel) 3, 294–308. CrossRef Medline


