Communication

The Molecular Chaperone Hsc70 Assists the in Vitro Folding of the N-terminal Nucleotide-binding Domain of the Cystic Fibrosis Transmembrane Conductance Regulator

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The most common disease-causing mutation in the cystic fibrosis transmembrane conductance regulator is a single amino acid deletion (ΔF508) in the N-terminal cytosolic nucleotide-binding domain (NBD1). This mutation has previously been shown to be a temperature-sensitive folding mutation that alters the folding pathway but not the native state stability of the isolated domain (Qu, B.-H., and Thomas, P. J. (1996) J. Biol. Chem. 271, 7261–7264). Here we provide evidence that the molecular chaperone Hsc70 productively interacts with NBD1 to increase the folding yield of the domain and inhibit off-pathway associations leading to the formation of high molecular weight aggregates. Furthermore, we have sublocalized a region within NBD1 where Hsc70 binds. Notably, inhibition of NBD1 aggregation is not dependent upon the presence of Hsc70 in the early stages of folding, indicating that the chaperone may act on a folding intermediate. In the presence of K⁺ and Mg²⁺-ATP, conditions where Hsp70 binds substrate rapidly and can release it, Hsc70 is less effective at inhibiting NBD1 aggregation. Thus, the rate of release of unfolded substrate is an important factor in preventing aggregation and promoting folding of the domain. These results demonstrate that Hsc70 promotes the otherwise inefficient folding of AF-NBD1 and provide insight into the mechanisms by which molecular chaperones assist proteins in folding.

Correct protein folding is an essential biological process. When proteins do not obtain correct native structure, their normal function is either impaired or absent. Not surprisingly, mutations that result in misfolded proteins have been implicated in a growing number of diseases (1). To assure their correct folding in the cellular milieu many proteins interact with molecular chaperones during the process of folding (2).

The Hsp70 class of molecular chaperones is thought to bind early in the folding process to extended conformations of polypeptide chains (3) with a preference for hydrophobic sequences (4) and to maintain the polypeptides in a soluble conformation competent for folding upon release. The cycle of ATP binding and hydrolysis on Hsp70 is coupled to substrate binding and release by conformational changes in the chaperone that represent interdomain communication between the substrate-binding and ATPase domains of Hsp70 (5–7). The ADP-bound form of Hsp70 has a tight affinity for substrate and a slow rate of substrate release (8). K⁺ and Mg²⁺-ATP are required for release of substrate from Hsc70 (9, 10). In effect, substrate turnover occurs in the presence of K⁺ and Mg²⁺-ATP with likely consequences for the mechanism by which Hsp70s promote protein folding. Upon release from the sequestration of the chaperone, polypeptides may either fold to the native conformation or enter the degradation machinery of the cell. Chaperones may play an active role in directing misfolded or mutant proteins to proteolysis. Hsp70, for example, has recently been shown to be required for ubiquitination of some proteins (11), the first step toward degradation by the proteasome (12).

Defective folding and maturation of the most common mutant form of CFTR, deletion of phenylalanine 508 (ΔF508), underlie the pathogenesis of most cases of cystic fibrosis (CF) (13). Deletion of phenylalanine 508 in the 22-kDa N-terminal nucleotide-binding domain (NBD1) alters the folding of the domain in vitro (14, 15). Thus, altered folding is the basis of incorrect glycosylation of CFTR, retention in the endoplasmic reticulum, lack of transit to the plasma membrane, and degradation in a proteasome-dependent manner observed in the cell (16–18). The constitutively expressed Hsp70 isoform, Hsc70, has been shown to interact with immature wild type and ΔF508 CFTR in vitro; however, the mutant protein has an extended interaction with Hsc70 (19). Understanding how Hsc70 influences the partitioning of wild type and ΔF508 CFTR proteins into folding and degradation pathways will provide insight into the mechanisms of intracellular quality control.

In the present study we utilize an established in vitro NBD1 folding system that recapitulates the effects of several CF-associated mutants, including ΔF508 (15, 20). Notably, the ΔF508 mutation produces a kinetic defect in CFTR-NBD1 folding. This in vitro system may be manipulated to influence the rate(s) of folding and the proportion of material destined for either off-pathway aggregation or folding to the native state. Here we use the in vitro folding of CFTR NBD1 to demonstrate that interactions with Hsc70 promote folding.

MATERIALS AND METHODS

Expression and Purification of NBD1—Six-histidine-tagged wild type and ΔF508 mutant CFTR-NBD1 from residues Gly⁶⁴⁴ to Ser⁷⁸⁹ were expressed in Escherichia coli and purified by Ni²⁺-resin affinity chromatography as described previously (15). The concentration of NBD1 solubilized in 6 M GdnHCl was calculated from the absorbance at 280 nm using a molar extinction coefficient of 13,490 M⁻¹ cm⁻¹ as determined in Ref. 15. Once resuspended in 6 M GdnHCl, protein was stored at 4 °C and used within a week.

Hsc70 Purification—Hsc70 was purified from bovine brain as de-

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carboxymethylation of cysteine residues, was obtained from Sigma. Bovine (RCMLA), which is unable to reach the native state due to folding was initiated and the time data collection was begun.

Electrophoresis (16.5% gel) and stained with Coomassie Blue (23). The G17A peptide also stimulates the substrate-binding domain contains the temperature-sensitive folding mutation (lane 3) has a shifted mobility compared with the mobility of free Hsc70 (lane 2). Notably, G17A, a peptide fragment from NBD1 corresponding to residues Gly^{545}-Ala^{561}, was able to compete with RCMLA for binding to Hsc70. Not all peptides compete for Hsc70 binding in this gel shift assay (22). G17A is homologous to a region of p53 known to bind Hsc70 (26) and includes several CF-associated mutations that affect maturation (27, 28) and folding (20). The peptide-Hsc70 complex runs at a size consistent with free Hsc70, and the free peptide does not resolve in the gel. The G17A peptide also stimulates the substrate-dependent ATPase activity of Hsc70 at 50 μM peptide (data not shown). It is important to note that additional Hsc70-binding sites may exist within NBD1. The localization of a binding region for Hsc70 within NBD1 is significant because the domain contains the temperature-sensitive folding mutation ∆F508 as well as other known folding mutations (27).

RESULTS AND DISCUSSION

Binding of Hsc70 to CFTR—Although full-length CFTR has been shown to interact with Hsc70 in a tissue culture system by co-immunoprecipitation experiments (19), the location of the binding site(s) for Hsc70 to CFTR have not been identified. Using a peptide representing a portion of CFTR we localized a Hsc70 binding site(s) for Hsc70 to CFTR have not been identified. Using a peptide representing a portion of CFTR we localized a Hsc70 binding site(s) for Hsc70 to CFTR have not been identified. Using a peptide representing a portion of CFTR we localized a Hsc70 binding site(s) for Hsc70 to CFTR have not been identified. Using a peptide representing a portion of CFTR we localized a Hsc70 binding site(s) for Hsc70 to CFTR have not been identified. Using a peptide representing a portion of CFTR we localized a Hsc70 binding site(s) for Hsc70 to CFTR have not been identified. 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Hsc70-assisted Folding of CFTR-NBD1

At 37 °C to a final concentration of 2 mM. Each investigation of Hsc70 on inhibiting aggregation at various temperatures compared with the aggregation of 2 mM long dashed line. In both cases aggregation was inhibited as in Fig. 2A. The fraction of NBD1 that is soluble under conditions where folding is inefficient, Hsc70 also inhibits the aggregation of wild type NBD1 (data not shown). Not surprisingly, considering that wild type CFTR interacts with Hsp70 in vitro, under conditions where folding is inefficient, Hsc70 also inhibits the aggregation of wild type NBD1 (data not shown).

By inhibiting the aggregation of ΔF-NBD1, Hsc70 increases the amount of soluble protein formed during an overnight folding yield experiment performed at various temperatures as shown in Fig. 2B. The fraction of NBD1 that is soluble under these conditions has previously been shown to be functional by binding nucleotide (15, 20). At increased temperatures the folding yield is lowered as the amount of aggregation increases. In the presence of Hsc70 the amount of ΔF-NBD1 in the soluble phase increases. A large fraction of the total ΔF-NBD1 is soluble, implying that solubility of the domain is not merely a consequence of complex formation with Hsc70.

Aggregation in the Presence of Hsc70 and ATP—All of the previous aggregation experiments were performed in the absence of Mg2+-ATP. In the presence of K+ and Mg2+-ATP, conditions that promote binding and rapid release of substrate (8–10), aggregation is no longer effectively inhibited by the chaperone (Fig. 4). ATP has been shown to have similar effects on the ability of Hsc70 to block the aggregation of citrate synthase (29). It is important to note that although NBD1 also binds ATP, this binding has no effect on the folding yield or

FIG. 3. Folding kinetics of ΔF-NBD1. A, to follow the folding process kinetically, ΔF-NBD1 was diluted into either 6 M GdnHCl or Buffer R at 37 °C to a final concentration of 2 μM. The increase in intrinsic fluorescence was monitored over time with excitation and emission wavelengths of 282 and 324 nm, respectively. The burst phase in the 15 s between initiation of folding and data collection is indicated by the difference in fluorescence between dilution into 6 M GdnHCl and dilution into Buffer R. Each dot represents an individual data point. B, to investigate the effect of Hsc70 on inhibiting aggregation at various phases in the folding process, 1 μM Hsc70 was added to 2 μM ΔF-NBD1 either before initiating folding (short dashed line) or 400 s after initiation (long dashed line). In both cases aggregation was inhibited as compared with the aggregation of 2 μM ΔF-NBD1 with no Hsc70 (solid line). Aggregation was performed, and the results are presented as in Fig. 2A.
aggregation (20). Therefore, the rate of release of an intermediate prone to aggregation may be critical for effective inhibition of aggregation by Hsc70. Premature release of NBD1 may result in an accumulation of the partially structured folding intermediate(s) susceptible to intermolecular interactions and, ultimately, accelerated higher order processes of aggregation or proteolysis. Thus, the kinetics of chaperone binding and protein folding must be in balance. Alteration of that balance by a kinetic folding mutation would result in a reduction in folding efficiency and, perhaps, an increased rate of quality control processes such as ubiquitination and subsequent proteolysis. Whereas other chaperones and cofactors such as Hsp40 regulate the turnover of Hsc70 substrates, they may have an important role to play in this process.

Correcting the folding defect of CFTR has promise as a treatment for the disease because ΔF-CFTR forms a functional channel in cell culture systems under conditions that promote folding, such as lower growth temperature(s) or growth in 10% glycerol (30–32). The results here indicate that careful alteration of the kinetics of Hsc70 interaction with CFTR may allow some of the mutant protein to reach a native conformation.

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