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Targeted delivery of Heat shock protein 70 by sortase-mediated ligation with a synthetic mannose receptor-binding ligand

Heat shock protein 70 (Hsp70) is a chaperone protein that is expressed in response to cellular stress. This chapter presents a sortase-mediated ligation strategy to target exogenous recombinant Hsp70 to dendritic cells, via the mannose receptor (MR). For this, a sortagging nucleophile was synthesized, containing a BODIPY dye (BDP) for fluorescent visualization and a MR-targeting mannose cluster (MC). Using SrtA_staph, recombinant Hsp70-LPETGG was transformed into Hsp70-BDP-MC. Successful uptake of this construct by dendritic cells was demonstrated to be dependent on the mannose receptor, showing the ability of the mannose cluster to ensure delivery of a large protein into the lysosomal pathway. Possible applications of targeted Hsp70 lie in the therapeutic intervention of lysosomal storage disorders, where exogenous Hsp70 could lead to decreased breakdown or enhanced activity of lysosomal enzymes.
Chapter 4

4.1 Introduction

The family of heat shock proteins (Hsp) functions as molecular chaperones that assist in the quality control and folding of newly synthesized proteins. Some of the family members are constitutively expressed such as Hsc70 (heat shock cognate protein), whereas others such as the 70 kDa protein Hsp70 (also known as Hsp72 or HSPA1A) are expressed in response to cellular stress. The protein isoforms are highly conserved across species. For example, E. coli derived Hsp70, called DnaK, shares an approximate 50% sequence homology with the human protein. The structure of Hsp70 contains two major domains, an N-terminal nucleotide binding domain (NBD) of ∼45 kDa that binds ATP and a C-terminal substrate binding domain (SBD) of ∼25 kDa that binds short peptides as well as the nascent chain of newly formed proteins. Both domains are connected by a short linker sequence. The ability of Hsp70 to bind and release polypeptide chains is closely linked to its ATPase activity, and several co-chaperones have been identified that are essential for enhancing the ATPase activity and thus proper functioning of Hsp70. While Hsp70 isoforms can be found in all cellular compartments, the majority of expressed Hsp70/Hsc70 is located in the cytosol. However, especially in tumor cells, Hsp70 is also found in lysosomes, where it enhances lysosome stability by preventing lysosomal membrane permeabilization and subsequent release of cysteine cathepsins into the cytosol.

Lysosomes are acidic vesicles containing a plethora of lytic enzymes such as proteases, glycosidases, nuclease, and lipases, and function as the metabolic center of the cell. Lysosomal storage disorders (LSD) are a group of rare but severe diseases in which one of the key enzymes in lipid metabolism is dysfunctioning, leading to accumulation of cellular constituents in the lysosomes (ie (glyco)lipids in case of lipidoses). As shown schematically in Figure 4.1A, parts of the plasma membrane, containing glycosphingolipids (GSL), are engulfed by a variety of processes and transported through the endolysosomal pathway. Along the pathway, both the pH and the amount of cholesterol in the membrane decrease, whereas the amount of bis(monoacylglycerol)phosphate (BMP, Figure 4.1A,B) increases. Lysosomes are confined by a limiting membrane, which is secured from proteolytical degradation by the presence of a glycocalix. Inside the lysosomes, the remains of the endocytosed plasma membrane (consisting mainly of BMP and GSL) form intralysosomal vesicles and the membranes of these vesicles are the main site of GSL catabolism. Enzymes that are responsible for the breakdown of GSLs are usually water-soluble and present in the lysosomal lumen. BMP, along with sphingolipid activator proteins (Saposins), forms a bridge between the internal membrane containing the substrate and the hydrolytic enzymes in the lumen, enabling the catalytic breakdown of GSLs.

A recent study by Kirkegaard et al. has shown that Hsp70 is able to stabilize lysosomes by binding with its amino-terminal NBD domain to the negatively charged BMP, especially at acidic pH (corresponding to the lysosomal environment, Figure 4.1A). They have shown that this has important implications for at least one of the lysosomal storage disorders, Niemann-Pick disease (NPD). Treatment of NPD fibroblasts with exogenous
recombinant Hsp70 resulted in the stimulation of acid sphingomyelinase, stabilization of the lysosomes and reversal of the associated NPD pathology.\textsuperscript{15,16} It is not known whether the lysosomal stabilization of Hsp70 has a beneficial effect on other LSDs as well. It is not unlikely that this effect exists, either by a general mechanism (stabilization of the lysosomes as a whole) or via direct binding interaction between Hsp70 and a lysosomal enzyme, thereby stabilizing it to prevent proteolytical degradation. To study this, efficient uptake of recombinant Hsp70 into the endolysosomal pathway is a requirement. Targeting of cell surface receptors that are constitutively or ligand-induced internalized, provides a point of entry into this pathway. In a previous study by Hillaert \textit{et al.} and a follow-up study, described in Chapter 2, a synthetic mannose cluster has been used as a targeting ligand for mannose receptors on dendritic cells and macrophages, enabling the delivery of cargo attached to the cluster into the lysosomes.\textsuperscript{17,18} In Gaucher disease, an LSD where $\beta$-glucocerebrosidase is deficient, macrophage-like cells form the main population of affected cells (Gaucher cells) and current enzyme replacement therapies use glycan remodeling to expose mannoses on the enzyme for delivery to the mannose receptor.\textsuperscript{19,20}

The research described in this chapter is aimed at the delivery of Hsp70 to the lysosomes via the mannose receptor, by ligation to the synthetic mannose cluster. For this the enzymatic ligation method termed ‘sortagging’ is used, which employs the bacterial enzyme Sortase A (SrtA).\textsuperscript{21,22} This approach combines organic synthesis, to create the sortagging nucleophile, with molecular biology to obtain recombinant Hsp70 that contains the SrtA recognition sequence. Optimization of the sortase reaction is described, followed by

\textbf{Figure 4.1: Proposed mechanism of Hsp70 targeting.} A) Schematic representation of MR-dependent uptake and trafficking of Hsp70-BDP-MC. Also shown is the formation of intralysosomal vesicles, consisting mainly of glycosphingolipids and BMP. Zoom in shows the working model by Petersen \textit{et al.} for the Hsp70-mediated stabilization of lysosomes by binding to BMP.\textsuperscript{15,16} B) Example structure of BMP, containing oleic acid residues.
an investigation of the ability of the synthetic ligand to function as a targeting device for a macromolecule such as Hsp70 in dendritic cells.

4.2 Results and Discussion

Synthesis. The bacterial enzyme Sortase A (SrtA) catalyzes the transpeptidation between a glycine nucleophile and a polypeptide substrate containing a C-terminal recognition sequence, as shown schematically in Figure 4.2A.23 The triglycine sortagging nucleophile 35 was synthesized by standard solid-phase peptide (SPPS) procedures and cleaved from the resin using acidic conditions. Under these conditions, the Boc-protected lysine was deprotected, whereas the Fmoc group on the N-terminal glycine was still in place. This allowed for selective modification of the ε-amino group of lysine with azido-BODIPY-OSu 36 (Scheme 4.1), resulting in Fmoc-protected 37 in 91% yield. This intermediate was deprotected using DBU in DMF, followed by quenching with HOBt and immediate purification by RP-HPLC. The HPLC fractions were lyophilized resulting in the TFA salt of sortase ligand “GGG-BDP” 38 in 16% yield. Other purification procedures, yielding the free amine, resulted in significant degradation of the BODIPY dye, whereas the TFA salt proved to be stable.

Scheme 4.1: Synthesis of the sortagging nucleophiles GGG-BDP (38) and GGG-BDP-MC (40).

Reagents and conditions: [a] N3-BODIPY-OSu 36, 24 DiPEA, DMF, 91%; [b] mannose cluster 20, CuSO4, sodium ascorbate, DMF/H2O; [c] i) DBU, DMF; ii) RP-HPLC purification, 38: 16%; 40: 24% over 2 steps.
Alternatively, compound 37 was used in a Cu(I)-catalyzed Huisgen cycloaddition with mannose cluster 20. After Fmoc removal with DBU and RP-HPLC purification, the envisaged sortagging ligand "GGG-BDP-MC" 40 was obtained in 24% (two steps).

**Sortase-mediated ligation.** Sortase A from *Staphylococcus aureus* lacking the membrane anchoring domain (SrtA*staph*Δ59, referred to here as "WT") has been shown to catalyze the transpeptidation reaction between a triglycine nucleophile and protein in vitro. In case of C-terminal sortagging, the protein of interest needs to contain the sortase recognition sequence (LPXTGG, where X can be any amino acid) near its C-terminus. Recombinant Hsp70 was designed with an LPETGG sortase sequence, followed by a His6 tag for purification purposes at its C-terminus. As depicted in Figure 4.2A, reaction of "Hsp70-LPETGG" with either one of the sortagging nucleophiles 38 or 40 (Scheme 4.1) would result in the

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\text{Hsp70-LPETGG} \quad \text{His}_6-\text{COOH}
\]

\[
\text{Hsp70-BDP}
\]

\[
\text{Hsp70-BDP-MC}
\]

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**Figure 4.2: Optimization of the sortase-mediated ligation of Hsp70 and a triglycine nucleophile.**

A) Schematic representation of sortase-mediated ligation of Hsp70-LPETGG with GGG-BDP or GGG-BDP-MC using either wild-type SrtA*staph*Δ59 (WT) or P94S/D160N/K196T triple mutant SrtA*staph* (mut). B) 7.5% SDS-PAGE of the reaction between Hsp70-LPETGG (1 µg, 1 eq), SrtA WT or mut (5 eq) and GGG-BDP 38 or GGG-BDP-MC 40 (25 eq), 16 h, 37 °C. C) 7.5% SDS-PAGE of Hsp70-LPETGG (1 µg, 1 eq), SrtA WT or mut (5 eq) and GGG-BDP 38 or GGG-BDP-MC 40 (25 eq) samples that were reacted for 65 h at different temperatures. White bands correspond to the BODIPY fluorescence signal (Cy3), black bands to Coomassie brilliant blue staining (CBB). Arrowheads: unidentified degradation products.
formation of fluorescently labeled Hsp70, with (Hsp70-BDP-MC) or without (Hsp70-BDP) a targeting entity. A recent report by Chen et al. has identified a sortase triple mutant (P94S/ D160N/ K196T, here referred to as "mut") with enhanced affinity for the LPETGG sequence and higher catalytic activity allowing for much more efficient transpeptidation.²⁹

Hsp70-LPETGG, SrtA-WT and SrtA-mut were expressed in E. coli and purified using nickel-affinity chromatography on an automated AKTA system. Fractions containing the protein of interest were pooled and buffer exchanged to 50 mM Tris, 150 mM NaCl, pH 8.0. All proteins were sufficiently pure after chromatography to be used directly in the transpeptidation reaction. The ability of the novel triglycine compounds ³⁸ and ⁴⁰ to function as a nucleophile in the sortagging reaction with Hsp70-LPETGG was first tested on a small scale. Briefly, Hsp70-LPETGG (1 µg, 1 eq) was reacted with GGG-BDP or GGG-BDP-MC (25 eq) in the presence of SrtA-WT or SrtA-mut for 16 h, 37 °C in SrtA reaction buffer (10 µL 50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5). The proteins were resolved on 7.5% SDS-PAGE and the gel slabs scanned for fluorescence of the BODIPY, before total protein staining with coomassie brilliant blue. As shown in Figure 4.2B, more product was formed in case of the mutant enzyme (lane 5), compared to wild-type (lane 2), when using GGG-BDP ³⁸. For the larger nucleophile GGG-BDP-MC ⁴⁰ it was more difficult to assess the progress of the reaction, because of similar molecular weights of the starting protein Hsp70-LPETGG (lane 8) and the product Hsp70-BDP-MC (lanes 3, 6). When the nucleophile was excluded from the reaction mixture (lanes 4 and 7), no reaction was observed in case of the WT enzyme (lane 4) whereas almost all starting Hsp70-LPETGG was converted to a lower molecular weight protein by the mutant enzyme (lane 7). The lower molecular weight protein most likely corresponds to the hydrolyzed Hsp70-LPET-OH, which is formed by nucleophilic attack of H₂O on the SrtA-Hsp70 intermediate. It was also formed when nucleophile was included in the reaction, as judged by the presence of a lower running band in lane 6. In case of nucleophile ³⁸ the product and by-product have similar molecular weights, so these could not be distinguished.

Figure 4.3 shows the proposed mechanism for sortase-mediated ligation.³⁰ Most of the steps in this mechanism are equilibria and thus reversible. The only irreversible step is the hydrolysis reaction, leading to the unwanted Hsp70-LPET-OH as the end-point of the reaction. For wild-type SrtA_staph, the formation of the acyl-enzyme intermediate is the rate-limiting step, and water is recognized very poorly as the nucleophile.³⁰,³¹ The greater rate of hydrolysis for the mutant enzyme is likely explained by its enhanced affinity for the LPXTTG sequence, thereby accelerating the formation of the acyl-enzyme intermediate. Both loss of selectivity for the nucleophile and slower rates for the transpeptidation reaction might contribute to the increased formation of the hydrolysed product.²⁹ In order to increase the amount of product formation for SrtA-WT and decrease the amount of hydrolysis in favor of product formation for SrtA-mut, reactions were performed at different temperatures for longer periods of time (Figure 4.2C). Prolonged (65 h) reaction times at 37 °C with the wildtype enzyme led to degradation of Hsp70, as seen by multiple bands of lower molecular weight on SDS-PAGE. A similar degradation pattern can be observed in lane 4.
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(Figure 4.2C), where no enzyme was present, indicating that it reflects inherent instability of Hsp70. Remarkably, SrtA-mut was still able to catalyze the transpeptidation reaction at 4 °C, albeit slower than at 16 °C. At 16 °C, complete reaction of Hsp70-LPETGG with GGG-BDP was observed, but both the mixture containing GGG-BDP-MC 40 or no nucleophile again showed a lower running band, corresponding to hydrolysis. Since all residues C-terminally of the threonine of the sortase sequence are removed in the transpeptidation or hydrolysis reaction, only the starting Hsp70-LPETGG contained a His\textsubscript{6}-tag. To be able to purify the envisaged product Hsp70-BDP-MC, removal of unreacted starting protein with nickel-affinity chromatography seemed more promising than removal of untagged Hsp70-LPET-OH by for instance size exclusion chromatography. Therefore, wildtype sortase was chosen as the most convenient enzyme for large scale transpeptidation reactions, even though it was anticipated that the reaction would not go to completion.

![Diagram](attachment:image.png)

Figure 4.3: Proposed mechanism for the SrtA-mediated ligation of a target protein containing the C-terminal LPXTGG recognition sequence and a glycine nucleophile.

Preparative scale sortagging was performed on Hsp70-LPETGG (0.5 mg) with GGG-BDP-MC 40 (25 eq) in the presence of SrtA\textsubscript{staph}-WT (5 eq) for 20 h at 37 °C in sortase reaction buffer. A small amount (0.05%) of Tween-80 was included in the reaction buffer and in buffers of all subsequent steps to prevent aspecific interactions of the amphiphilic sortagging nucleophile with tubes, proteins or beads. After the reaction, all His-tagged proteins (unreacted Hsp70-LPETGG, SrtA and SrtA-Hsp70 intermediate) were removed by nickel-affinity chromatography. From each step of the purification, a small sample was analyzed by SDS-PAGE, followed by fluorescence scanning, Coomassie brilliant blue staining or anti-His western blotting. As shown in Figure 4.4A (left), one Ni-column was not sufficient to remove all His-tagged proteins and therefore the flow-through of the first column was subjected to a second nickel purification step (Figure 4.4A, right), which resulted in a mixture free of any His-tagged proteins as judged by an anti-His western blot (WB). All fluorescent signal, corresponding to the product Hsp70-BDP-MC was found in the flow-through and washing steps, indicating that indeed the His-tag was lost during the reaction and that aspecific interaction of the protein with the beads was negligible under the conditions.
used. Removal of excess nucleophile 40 was attempted using 30 kDa MWCO filters, but this did not succeed, probably due to formation of HMW aggregates or a lack of globularity of the molecule. Therefore, the mixture was subjected to gel filtration to remove the excess of nucleophile. Fractions were analyzed for the presence of fluorescent protein using in-gel fluorescence scanning. As shown in Figure 4.4B, the first fractions contained a \( \sim 75 \) kDa protein, corresponding to the MW of Hsp70-BDP-MC, and later fractions contained an unknown degradation product of about 30 kDa and finally the excess of nucleophile. Fractions containing pure Hsp70-BDP-MC were pooled, concentrated and again analyzed on SDS-PAGE. As can be seen in Figure 4.4C, final sample "20" contained one major band, as seen by fluorescence and coomassie staining, which was positively immunostained by an anti-Hsp70 antibody. In conclusion, Hsp70-BDP-MC was successfully obtained by sortase-mediated ligation between Hsp70-LPETGG and GGG-BDP-MC 40, in 27% overall yield of sortagging and purification procedures.

![Figure 4.4: Large scale sortase-mediated ligation of Hsp70-LPETGG with GGG-BDP-MC and purification of Hsp70-BDP-MC.](image)

**A**) Hsp70-LPETGG (0.5 mg) was treated with SrtA_{staph} WT (5 eq) and GGG-BDP-MC 40 (25 eq) for 20 h at 37 °C. Unreacted Hsp70-LPETGG, Hsp70-SrtA intermediate and SrtA were removed with two subsequent Ni-NTA agarose columns. Shown are 10% SDS-PAGE gels of samples taken from the Ni-purification steps, followed by in-gel fluorescence scanning, Coomassie brilliant blue staining or anti-His Western blotting. **B**) The semi-pure Hsp70-BDP-MC obtained was subjected to gel filtration using a Vision chromatography system to remove excess nucleophile. Shown is a 12% SDS-PAGE in-gel fluorescence scan of fractions eluted from the Vision. Arrowhead: unidentified degradation products. **C**) 10% SDS-PAGE of Hsp70-LPETGG and Hsp70-BDP-MC before and after Vision gel filtration, followed by in-gel fluorescence scanning, Coomassie brilliant blue staining or anti-Hsp70 immunoblotting.

Next, it was investigated whether the synthetic mannose cluster could function as a targeting agent for Hsp70. For this, immature mouse dendritic cells (DCs) were incubated with 10 µg/mL purified Hsp70-BDP-MC for 2 h, 37 °C. Cells were extensively washed, fixed with 4 % formaldehyde, nuclei were stained with Draq5 and imaged using a confocal fluorescence microscope. Bright intracellular vesicles were observed, as shown in Figure 4.5A. Moreover, entry of Hsp70-BDP-MC could be completely blocked by pre-incubation with the yeast polymannoside mannan (3 mg/mL), indicating that the internalization was mediated by mannose-binding lectins such as the mannose receptor (MR). An alternative control experiment would be to make use of Hsp70-BDP, with the fluorophore suited for microscopy, but without the mannose cluster for targeting, to study the efficiency of MR-dependent uptake. Purification of Hsp70-BDP from remaining nucleophile GGG-BDP proved to be cumbersome, and not enough material of good purity could be obtained.

Figure 4.5: MR-dependent uptake of Hsp70-BDP-MC in immature dendritic cells. A) Representative micrographs of immature mouse dendritic cells that were treated for 2 h with purified Hsp70-BDP-MC (10 µg/mL) or pre-incubated for 1 h with mannan (3 mg/mL) followed by 2 h treatment with Hsp70-BDP-MC. Cells were washed with PBS, fixed (4 % formaldehyde in PBS), nuclei were stained with Draq5 (dark grey) and cells imaged for BODIPY fluorescence (white). B) Cells were treated as in A), or with unsortagged Hsp70-LPETGG (10 µg/mL) instead of Hsp-BDP-MC. After incubation, cells were washed with PBS and PBS containing ovalbumin (1 mg/mL) to reduce aspecific binding. Cells were lysed and analyzed by 7.5 % SDS-PAGE, followed by fluorescence scanning/anti-His WB and CBB staining of total protein or anti-S6 (ribosomal protein) WB as loading control. M: molecular weight protein marker, thick black band corresponds to 70 kDa.
to conduct this experiment. In a next experiment, cells were treated using the same conditions, and unsortagged Hsp70-LPETGG was included as a control. Instead of imaging, cells were washed with PBS containing ovalbumin (1 mg/mL, to reduce aspecific binding) and lysed. The lysates were analyzed by 7.5% SDS-PAGE. Gels were scanned for fluorescence, and either stained with Coomassie brilliant blue or immunoblotted against His<sub>6</sub> to show Hsp70-LPETGG (Figure 4.5B). In-gel fluorescence showed the presence of a ∼75 kDa band corresponding to Hsp70-BDP-MC both in samples with and without pre-incubation with mannan. Quantification of the fluorescent signal, normalized against total protein, showed that ∼2.5-fold more Hsp70-BDP-MC was present in lysates of DCs that were untreated compared to those that were treated with mannan. Unexpectedly, immunoblotting showed the presence of Hsp70-LPETGG.

The discrepancy between the SDS-PAGE analysis and the microscopy results, where no signal was detected upon pre-treatment with mannan, can be explained by several factors. Hsp70, being a chaperone, can be expected to interact with a variety of macromolecules present in the medium or on the cell membrane. For example, it has been shown that Hsp70 is able to bind to scavenger receptors such as LOX-1 on dendritic cells. Even though the cells were washed several times before lysis in the presence of a large excess of ovalbumin, it is quite likely that some Hsp70-BDP-MC or Hsp70-LPETGG remained, which was then included in the cell lysate. The fluorescence signal in intracellular vesicles is much easier to detect using confocal microscopy than a diffuse binding pattern on the cell membrane, which might explain the lack of fluorescence in mannan-treated cells. Overall, it can be concluded that the synthetic mannose cluster, although low molecular weight compared to Hsp70, is able to ensure its targeted delivery into dendritic cells in a mannose-binding lectin-dependent fashion.

4.3 Conclusion

A sortagging nucleophile containing a BODIPY dye and a synthetic mannose cluster for targeting of the mannose receptor was successfully synthesized and employed in a sortase-mediated ligation reaction with recombinant Hsp70-LPETGG. The ensuing Hsp70-BDP-MC protein could be purified by repeated nickel-affinity and gel filtration chromatography. Treatment of immature dendritic cells with the purified protein resulted in MR-mediated internalization as shown by confocal microscopy and SDS-PAGE analysis. Sortase-mediated modification of a protein with a receptor-targeting, fully synthetic ligand, thus enables selective uptake of the macromolecule into the endolysosomal pathway. The next step would be to determine what the functional consequences are of targeted delivery of Hsp70 to cells that are affected by a lysosomal storage disorder. The current construct, Hsp70-BDP-MC, is ideally suited for delivery to Gaucher cells, and because of the flexible synthetic nature of the sortagging nucleophile, targeting of other receptors is within easy reach.
4.4 Experimental Section

Cloning, expression and purification of Hsp70-LPETGG-His6. Wild-type Hsp70 RNA was isolated from HEK293T cells using RNA-Bee (Bio Connect) and converted to cDNA by RT-PCR. The HSP gene was then PCR amplified using the following primers: 5’-CATATGGCCAAAGCCCGGCGG-3’ (sense) and 5’-GCGGCCGCTGAATTCCTCCTCTCAATGTG-3’ (anti-sense) containing NdeI and NotI restriction sites, respectively. The PCR product (2000 bp) was ligated into the pGEM-T plasmid (Promega). The C-terminal LPETGG-sequence was then introduced by PCR amplification using the following primers: sense primer 5’-CATATGGCCAAAGCCCGGCGG-3’ and anti-sense primer 5’-GCGGCCGTACGCTCGCGGCTCGGGCGCCATCTACCTCCTCAATGTG-3’ containing NdeI and NotI restriction sites, respectively. The PCR product (2000 bp) was again ligated into the pGEM-T plasmid and then cloned into the expression plasmid Pet21A(+) (Novagen) using the NotI and NdeI restriction sites. The resulting construct containing a C-terminal His6-tag after the LPETGG sequence was transformed into competent E. coli BL21(DE3) cells for expression of the recombinant protein. Therefore, two 50 mL cultures in LB-Amp medium were grown overnight at 37 °C, inoculated into 500 mL LB-Amp medium and grown again at 37 °C to an OD600 of ~0.6. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and cultures were grown for 4 h before the cells were pelleted (10,000 g, 4 °C, 2 x 15 min). The pellets were each lysed for 2 h at 4 °C in 20 mL lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 0.1% (v/v) Triton X-100, 100 µM EDTA, 0.5 mg/mL lysozyme, 0.4 µg/mL leupeptin and Roche protease inhibitor cocktail) while rotating. The cells were then sonicated on ice (10x 10 s with 30 s intervals) and centrifuged at 10,000 g for 10 min at 4 °C, after which the supernatant was collected (43 mL). For protein purification, 5 mL 10x binding buffer (500 mM Na2HPO4, 1.5 M NaCl, 100 mM imidazole, pH 8.0) was added to the lysates and the mixture was filtered over a 0.45 µm filter. The protein was then purified on a nickel column (HisPur Ni-NTA chromatography cartridge, Thermo Scientific) using the AKTA Prime Plus System (HisTag purification program with a linear imidazole gradient of 10 → 500 mM in 50 mM Tris, 150 mM NaCl, pH 8.0). Fractions containing the Hsp70-LPETGG-His6 protein were combined, concentrated using 30 kDa MWCO filter tubes (Millipore) and buffer exchanged to 50 mM Tris, 150 mM NaCl, pH 8.0, yielding a total of 6 mg protein (1 mL, 6 mg/mL).

Cloning, expression and purification of SrtAstaph wild-type or P94S/D160N/K196T mutant. SrtAstaph Δ59 (wild-type minus the membrane anchoring domain) in PET28A plasmid was a gift from H. Ploegh (Whitehead institute, MIT, Boston, USA) and was expressed in E. coli as described previously.27 Mutant P94S/D160N/K196T sortase29 was obtained by multi-site mutagenesis using the following primers: CCAGGACCAGCAACATCTGAACATTATAAGAGG (P94S); GACAAGTGATTAGAAAAGAACTGATCG (D160N); GACAGGCTTGGGAAAAACGTTAATCTTTTGTAGG (K196T) and the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) per manufacturers’ instructions. Both recombinant sortases contain a His6-tag for purification purposes. For protein purification 10x binding buffer (500 mM Na2HPO4, 1.5 M NaCl, 100 mM imidazole, pH 8.0) was added to the lysates and the lysate was clarified by filtration over a 0.45 µm filter. The protein was then purified on a nickel column (HisPur Ni-NTA chromatography cartridge, Thermo Scientific) using the AKTA Prime Plus System (HisTag purification program with a linear imidazole gradient of 10 → 500 mM in 50 mM Tris, 150 mM NaCl, pH 8.0). Fractions containing sortase A were combined, concentrated using 10 kDa MWCO filter tubes (Millipore) and buffer exchanged to 50 mM Tris, 150 mM NaCl, pH 8.0. Fractions were pooled, concentrated and glycerol was added to a final concentration of 10% before storage at -80 °C.
Analytical scale sortase-mediated ligation of Hsp70-LPETGG-His$_6$. To find the optimal conditions for sortase-mediated ligation of Hsp70, small scale transpeptidation reactions under varying conditions were performed. A typical reaction contained Hsp70-LPETGG (1 µg, 14 pmol, 1 eq), SrtA$_{staph}$ WT or mutant (5 eq) and triglycine nucleophile 38 or 40 (25 eq) in sortase-reaction buffer (10 µL 50 mM Tris, 150 mM NaCl, 10 mM CaCl$_2$, pH 7.5). Reactions times were varied, together with the temperature. Aspecific interactions between the nucleophile and Hsp70 were examined by excluding SrtA enzyme from the reaction mixture. Hydrolysis was determined in the absence of nucleophile. After reaction, 5x Laemli’s sample buffer (including β-mercaptoethanol) was added and the samples were boiled (100 °C, 5 min) and resolved on 7.5% SDS-PAGE. Gels were scanned on a Typhoon 2000 imager (GE Healthcare) using the Cy3 ($\lambda_{ex}$ 532 nm; $\lambda_{em}$ 580 nm) settings. Total protein loading was determined by staining with Coomassie brilliant blue.

Preparative scale sortase-mediated ligation of Hsp70-LPETGG-His$_6$ and subsequent purification of Hsp70-BDP-MC. Hsp70-LPETGG-His$_6$ (7.1 nmol, 0.50 mg, 83 µL 6 mg/mL in Tris buffer pH 8.0) was treated with SrtA$_{staph}$ Δ59 (36 nmol, 0.60 mg, 0.46 mL 1.3 mg/mL in Tris buffer pH 8.0, 5 eq) and GGG-BDP-MC 40 (178 nmol, 9 µL 20 mM in DMSO, 25 eq) in the presence of sortase reaction buffer (65 µL 10x buffer: 500 mM Tris, 1.5 M NaCl, 100 mM CaCl$_2$, pH 7.5) and 0.05% Tween-80 (33 µL 1% in 50 mM Tris pH 8.0). The reaction mixture (0.65 mL) was incubated 20 h at 37 °C while rotating. After reaction, both unreacted Hsp70-LPETGG-His$_6$ and Sortase A could be removed by Ni-affinity purification. To avoid extensive sticking of the product Hsp70-BDP-MC to the nickel column, Tween-80 was included in all buffers. Hence, the reaction mixture was diluted with 75 µL 10x binding buffer (500 mM Na$_2$HPO$_4$, 1.5 M NaCl, 100 mM imidazole, pH 8.0) and 800 µL 1x binding buffer (50 mM Na$_2$HPO$_4$, 150 mM NaCl, 10 mM imidazole, pH 8.0) containing 0.05% Tween-80, before being applied on top of a Ni-NTA agarose (Qiagen) column (0.5 mL column volume; prewashed 3x with binding buffer and 2x with binding buffer + 0.05% Tween-80). The flow-through was collected and the column was washed with binding buffer + 0.05% Tween-80 (1.5 mL). The combined flow-through and washes were concentrated using 30 kDa MWCO filter tubes (Millipore) and buffer-exchanged 3x with 50 mM Tris, 150 mM NaCl and 0.05% Tween-80, pH 7.5 (10x sample volume). The Ni-affinity purification procedure was repeated to further purify the protein, giving a final volume of 250 µL. Finally, the excess of low molecular weight nucleophile was removed by applying the sample to a Vision (BioCad, Applied Biosciences) gel filtration column, using a buffer containing 50 mM Tris and 150 mM NaCl, pH 8.0. The elution fractions containing the desired protein were combined and concentrated using 30 kDa MWCO filter epps (Millipore) to give a final volume of 200 µL containing 135 µg Hsp70-BDP-MC (1.9 nmol, 27%). Each step of the above procedure was monitored by SDS-PAGE analysis of samples containing an estimated 0.5 µg of Hsp70, followed by fluorescence scanning on a Typhoon 2000 imager (GE Healthcare). The gels were then either stained with Coomassie brilliant blue for total protein, or the protein was transferred onto a nitrocellulose membrane using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (1 h, 12V). The membrane was then blocked (1 h, 2% milk in TBST), and incubated (o/n, 4 °C) with primary antibody (1:500 mouse monoclonal α-His IgG(R&D systems, MAB050) or 1:500 mouse α-Hsp70/72 (Enzo life sciences, mAb C92F3A-5)), followed by washing (3x 20 min TBST) and incubation with secondary antibody (1:15000 goat α-mouse IRdye 800CW in 2% milk TBST, 1 h, rt, in the dark). After another wash cycle (3x 20 min TBST + 20 min TBS) the membrane was scanned on an Odyssey scanner (Licor).

Cell culture of primary cells. Immature dendritic cells were obtained from the bone marrow of C75BL/6 mice and were a gift from the Biopharmaceutical Department (Leiden University). The use of animals was approved by the ethics committee of Leiden University. Mice were sedated, bone
marrow of tibiae and femurs was flushed out and washed with PBS. Cells were grown in dendritic cell selection medium (IMDM containing granulocyte-macrophage colony stimulating factor (GM-CSF) 2:1 vol/vol) containing 8% FCS, penicillin/streptomycin (100 units/mL), glutamax (2 mM) and betamercaptoethanol (20 µM). Cells were selected for 10 days (37 °C; 5% CO$_2$) and subcultured every 2-3 days before use in the assays.

**Uptake of Hsp70-BDP-MC - Confocal fluorescence microscopy.** Experiments were conducted on a Leica TCS SPE confocal microscope, using dsRed filter settings (λ$_{ex}$ 532 nm) for BODIPY fluorescence and Cy5 filter settings (λ$_{ex}$ 635 nm) to detect Draq5 nuclear stain (Fisher Scientific). Cells (30-75 x 10$^4$ cells/well, 250-400 µL medium) were seeded onto sterile Labtek II 4- or 8-chamber borosilicate coverglass systems (Fisher Emergo). Dendritic cells were allowed to attach for 2 h before pre-incubation with mannan (3 mg/mL) (1 h, 37 °C, 5% CO$_2$) and subsequent incubation with Hsp70-BDP-MC (10 µg/mL, 2 h). Cells were then thoroughly washed (PBS), fixed (4% formaldehyde in PBS), washed again with PBS, nuclei stained with Draq5 and imaged. All experiments were performed at least in triplicate.

**Uptake of Hsp70-BDP-MC - SDS-PAGE analysis.** Immature mouse dendritic cells (200,000 cells/well) were seeded on a 24-wells plate (300 µL medium) and cultured for 2 h (37 °C; 5% CO$_2$) before start of the experiment. For competition experiments, cells were pre-treated with mannan (3 mg/mL) for 1 h. After subsequent incubation with Hsp70-BDP-MC (10 µg/mL, 250 µL) or Hsp70-LPETGG-His$_6$ (10 µg/mL, 250 µL) for 2 h, cells were washed (2x 0.5 mL PBS, 1x 0.2 mL PBS + 1 mg/mL ovalbumin (Sigma)) and lysed (35 µL Invitrogen complete cell extraction buffer). Proteins were resolved on 7.5% SDS-PAGE, followed by fluorescence scanning and either Coomassie brilliant blue staining or anti-His WB as described above. Rabbit α-S6 ribosomal protein (1:4000) primary antibody was included as a loading control for total protein. After washing, the membrane was incubated with secondary antibodies (1:15000 goat α-mouse IRdye 800CW and 1:15000 goat α-rabbit IRdye 680CW in 2% milk TBST, 1 h, rt, in the dark). Fluorescence intensities were quantified using ImageJ, and normalized against total protein to correct for differences in protein levels.

**Synthesis**

**General.** All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3 Å molecular sieves. Dichloromethane was distilled over CaH$_2$ prior to use. Solvents used for column chromatography were of technical grade and distilled before use. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Flash chromatography was performed on silica gel (Screening Devices BV, 0.04-0.063 mm, 60 Å). Reactions were routinely monitored by TLC analysis on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254/366 nm) where applicable and spraying with a solution of (NH$_4$)$_6$Mo$_7$O$_{24} \cdot 4$ H$_2$O (25 g/l) and (NH$_4$)$_4$Ce(SO$_4$)$_4 \cdot 2$ H$_2$O (10 g/l) in 10% sulfuric acid in water followed by charring at ∼150 °C. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV-400 (400 MHz) or Bruker AV-500 (500 MHz). Chemical shifts are given in ppm (δ) relative to the residual solvent peak or TMS (0 ppm) as internal standard. Coupling constants are given in Hz. Peak assignments are based on 2D $^1$H-COSY and $^{13}$C-HSQC NMR experiments. LC-MS measurements were conducted on a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer (ESI+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a standard C18 (Gemini, 4.6 mmD x 50 mmL, 5µ particle size, Phenomenex) analytical column and buffers A: H$_2$O, B: ACN, C: 0.1% aq.TFA. High resolution mass spectra were recorded on a LTQ Orbitrap (Thermo Finnigan) mass spectrometer equipped with an electrospray ion source in positive
mode (source voltage 3.5 kV, sheath gas flow 10 mL min⁻¹, capillary temperature 250 °C) with resolution R=60000 at m/z 400 (mass range m/z=150-2000) and dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). For reversed-phase HPLC purification of the final compounds an automated HPLC system equipped with a C18 semiprep column (Gemini C18, 250x10 mm, 5µ particle size, Phenomenex) was used.

**Synthesis of LysAhxGly3Fmoc (35).**

The peptide was synthesized using standard solid-phase peptide synthesis protocols. Starting from Rink amide resin HL (100-200 mesh, 0.78 mmol/g) the following steps were followed:

- Removal of the Fmoc-group with 20% piperidine in NMP for 20 min.
- NMP (3x), DCM (3x), NMP (1x) wash.
- Coupling of the appropriate amino acid (2 eq) with HBTU (2 eq) as the coupling agent, DiPEA (4 eq) as the base, in NMP as the solvent for 5-16 h.
- NMP (3x), DCM (3x), NMP (1x) wash.

Each coupling was monitored by LC/MS analysis and Kaiser test for completion. After the final coupling step, all acid-labile protecting groups were removed and the peptide cleaved from the resin by treatment with TFA/H₂O/TIS (95:2.5:2.5; v/v/v) for 1 h. The peptide was crystallized from ice-cold Et₂O and collected by centrifugation as a white solid (40 mg, 0.061 mmol, 85%). Rf = 0.1 (1:1 DCM:MeOH + TEA). ¹H NMR (500 MHz, CDCl₃/MeOD): δ 7.78 (d, J = 7.5 Hz, 2H, 2 x CH₆), 7.65 (d, J = 7.4 Hz, 2H, 2 x CH₆), 7.40 (t, J = 7.5 Hz, 2H, 2 x CH₆), 7.31 (t, J = 7.5 Hz, 2H, 2 x CH₆), 4.39 (d, J = 7.0 Hz, 2H, 2 x CH₂), 4.23 (t, J = 6.8 Hz, 1H, CH), 3.90 (s, 2H, CH₂), 3.84 (s, 4H, 2 x CH₂), 3.21 - 3.13 (m, 2H, CH₂), 2.92 - 2.87 (m, 2H, CH₂), 2.24 (t, J = 7.3 Hz, 2H, CH₂), 1.88 - 1.80 (m, 1H, CH₂-Ha), 1.69 - 1.59 (m, 5H, 2 x CH₂, CH₂-Hb), 1.54 - 1.48 (m, 2H, CH₂), 1.47 - 1.38 (m, 2H, CH₂), 1.33 - 1.28 (m, 2H, CH₂). ¹³C NMR (126 MHz, CDCl₃/MeOD): δ 174.86, 174.29, 171.45, 170.23, 169.49, 157.51, 143.27, 140.77, 127.13, 126.45, 124.42, 119.28, 66.65, 52.03, 46.54, 43.55, 42.29, 41.85, 38.78, 38.52, 35.04, 30.71, 28.02, 25.49, 24.56, 21.95. LC/MS analysis (linear gradient 10 → 90% ACN) tR: 4.92 min, ESI-MS (m/z): [M + H]+: 652.5. ESI-HRMS (m/z): calcd. for [C₁₃H₂₄N₅O₇]+ H+: 652.34532; obsd. 652.34536.

**Synthesis of protected sortase nucleophile BODIPY-Gly3Fmoc (37).**

Peptide 35 (65 mg, 0.10 mmol) was dissolved in dry DMF (10 mL) and azido-BODIPY-OSu 36 (56 mg, 0.10 mmol) and DiPEA (36 µL, 0.20 mmol, 2 eq) were added. After 2 h, LC/MS revealed complete consumption of starting materials, toluene was added and the solvents removed in vacuo. Pure product was obtained by silica column chromatography (0 → 100 % MeOH in DCM) followed by crystallization from DMF/Et₂O as a dark-purple solid (100 mg, 91 µmol, 91%). ¹H NMR (400 MHz, DMF-d7): δ 8.47 (t, J = 5.2 Hz, 1H, NH), 8.24 (t, J = 5.6 Hz, 1H, NH), 8.01 - 7.90 (m, 5H, NH, 4 x CH₂), 7.86 (d, J = 8.0 Hz, 1H, NH), 7.82 - 7.73 (m, 5H, CH₂, 3 x CH₂), 7.51 - 7.44 (m, 5H, CH₂, 2H, 2 x CH₂), 7.38 (t, J = 7.4 Hz, 2H, 2 x CH₂), 7.22 (d, J = 4.0 Hz, 1H, CH₂), 7.10 (d, J = 8.8 Hz, 2H, 2 x CH₂), 7.05 (s, 1H, NH), 6.77 (d, J = 4.0 Hz, 1H, CH₂), 4.40 - 4.26 (m, 3H, CH₂, CH), 4.21 (t, J = 6.1 Hz, 2H, CH₂), 3.92
(t, J = 5.2 Hz, 4H, 2 × CH₂), 3.84 (d, J = 5.8 Hz, 2H, CH₂), 3.68 - 3.64 (m, 2H, CH₂), 3.19 - 3.10 (m, 4H, 2 × CH₂), 2.74 (s, 2H, CH₂), 2.56 (s, 3H, CH₃), 2.37 (t, J = 7.5 Hz, 2H, CH₂), 2.31 (s, 3H, CH₃), 2.24 (t, J = 7.4 Hz, 2H, CH₂), 2.11 (p, J = 6.4 Hz, 2H, CH₂), 1.83 - 1.71 (m, 1H, CH₃H⁺), 1.67 - 1.53 (m, 3H, CH₂, CH₃H⁺), 1.53 - 1.26 (m, 8H, 4 × CH₂). ¹³C NMR (101 MHz, DMF-d7): δ 174.50, 172.68, 171.53, 170.49, 169.54, 168.86, 160.09, 159.65, 157.17, 154.35, 144.28, 141.20, 140.93, 135.29, 134.62, 131.69, 130.74, 128.25, 127.80, 127.22, 125.72, 125.50, 124.09, 120.16, 118.00, 114.29, 66.60, 65.04, 52.88, 48.17, 47.11, 44.21, 42.83, 42.44, 38.85, 35.58, 32.03, 29.22, 28.58, 26.40, 25.34, 23.28, 20.15, 12.64, 8.91. LC/MS analysis (linear gradient 10 → 90% ACN) tᵣ: 9.13 min, ESI-MS (m/z): [M + H]⁺: 1101.33. ESI-HRMS (m/z): calcd. for [C₁₆₅H₇₅BF₂N₁₂O₉ + H]⁺ 1101.52878; obsd. 1101.53115.

**Synthesis of sortase nucleophile GGG-BDP (38).**

Peptide 37 (50 mg, 0.046 mmol) was dissolved in DMF (5 mL) and DBU (8.2 µL, 55 µmol, 1.2 eq) was added. After 40 min at room temperature, HOBt (31 mg, 0.23 mmol, 5 eq) was added to quench the reaction and the solvent was removed under reduced pressure. The product was purified by RP-HPLC (A: 0.2 % TFA in H₂O, B: linear gradient 39 → 50 % ACN in 12’) and lyophilized from water to give the corresponding TFA salt as a purple powder (6.4 mg, 7.3 µmol, 16%). LC/MS analysis (linear gradient 0 → 90% ACN) tᵣ: 7.72 min, ESI-MS (m/z): [M + H]⁺: 879.33. ESI-HRMS (m/z): calcd. for [C₄₁H₅₇BF₂N₁₂O₇ + H]⁺ 879.46071; obsd. 879.46174.

**Synthesis of GGG-BDP-MC (40).**

Mannose cluster 20 (11.6 mg, 3.8 µmol) and peptide 37 (4.2 mg, 4.7 µmol, 1.2 eq) were dissolved in DMF (2 mL) and aqueous solutions of sodium ascorbate (76 µL 100 mM, 2 eq) and CuSO₄ (19 µL 100 mM, 0.5 eq) were added. The resulting mixture was heated to 75 °C for 24 h. Toluene was added and the Fmoc-protected peptide 39 was collected by centrifugation and used without further purification. LC/MS analysis (linear gradient 0 → 90% ACN) tᵣ: 6.60 min, ESI-MS (m/z): [M + 3H]³⁺: 1391.00. The peptide was again dissolved in DMF and DBU (1.2 µL, 8 µmol, 2 eq) was added. After 2 h at room temperature, HOBt (5.2 mg, 38 µmol, 10 eq) was added to quench the reaction and the solvent was removed under reduced pressure. The product was purified by RP-HPLC (A: 0.2 % TFA in H₂O, B: linear gradient 20 → 30 % ACN in 12’, tᵣ: 11.5 min) and lyophilized from water to give the corresponding TFA salt as a purple powder (3.5 mg, 0.9 µmol, 24%). LC/MS analysis (linear gradient 10 → 90% ACN) tᵣ: 4.88 min, ESI-MS (m/z): [M + 2H]²⁺: 1974.40. ESI-HRMS (m/z): calcd. for [C₁₇₂H₂₇₁BF₂N₄₄O₅₉ + 2H]²⁺ 1974.99159; obsd. 1974.99467; calcd. for [C₁₇₂H₂₇₁BF₂N₄₄O₅₉ + 3H]³⁺ 1316.99682; obsd. 1316.99846.

**References**

