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Identification of peptides in human Hsp20 and Hsp27 that possess molecular chaperone and anti-apoptotic activities

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Abstract

Previous studies have identified peptides in the 'crystallin-domain' of the small heat-shock protein (sHSP) α-crystallin with chaperone and anti-apoptotic activities. We found that peptides in heat-shock protein Hsp20 (G^{71}HFSVLLDVKHFSPEEIAVK^{91}) and Hsp27 (D^{93}RWRVSLDVNHFAPDELTVK^{113}) with sequence homology to α-crystallin also have robust chaperone and anti-apoptotic activities. Both peptides inhibited hyperthermic and chemically induced aggregation of client proteins. The scrambled peptides of Hsp20 and Hsp27 showed no such effects. The chaperone activities of the peptides were better than those from αA- and αB-crystallin. HeLa cells took up the FITC-conjugated Hsp20 peptide and, when the cells were thermally stressed, the peptide was translocated from the cytoplasm to the nucleus. The two peptides inhibited apoptosis in HeLa cells by blocking cytochrome *c* release from the mitochondria and caspase-3 activation. We found that scrambling the last four amino acids in the two peptides (KAIV in Hsp20 and KTLV in Hsp27) made them unable to enter cells and ineffective against stress-induced apoptosis. Intraperitoneal injection of the peptides prevented sodium-selenite-induced cataract formation in rats by inhibiting protein aggregation and oxidative stress. Our study has identified peptides from Hsp20 and Hsp27 that may have therapeutic benefit in diseases where protein aggregation and apoptosis are contributing factors.

Keywords

apoptosis; cataract; chaperone; peptide

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AUTHOR CONTRIBUTION

Ram Nagaraj supervised the study and designed the experiments together with Rooban Nahomi. Rooban Nahomi performed all the experiments, except some of the chaperone assays which were performed by Michael DiMauro. Mass spectrometric analysis was performed by Benlian Wang.

INTRODUCTION

Small heat-shock proteins (sHsps) are a diverse group of molecular chaperones characterized by a crystallin core domain containing 80–100 amino acid residues. The amino acid sequence within this domain is highly conserved in sHsps. In human tissues, heat-shock protein (Hsp)20 (HspB6) and Hsp27 (HspB1) are major members of the sHsp family [1]. When cells are exposed to stressful stimuli, these sHsps are overexpressed to counter protein aggregation and apoptosis [2,3]. Unlike large heat-shock proteins, such as Hsp90, the chaperone function of sHsps is not dependent on ATP; this property is important to protect cells under stressful conditions where ATP is a limiting factor [4].

The chaperone activity of sHsps appears to be multifaceted; they bind to many proteins in cells under normal as well as stressful conditions. A recent study showed that αA-crystallin binds to more than 200 proteins in the lens epithelial cells under basal conditions [5]. In addition, several studies have shown that the sHsps bind to and stabilize cytoskeleton proteins in the cells experiencing stress [6–10]. They also bind to nuclear p53 in the cells undergoing mitosis [11] and pro-apoptotic molecules, such as Bax [12] and procaspase-3 [13–15], during the inhibition of apoptosis. In the plasma, αB-crystallin has been shown to bind to many cytokines [16]. *In vitro* experiments have shown that the sHsps can bind to and prevent the aggregation of various proteins [7,17–19]. In addition, pin array technology has demonstrated that the sHsps can bind various growth factors [20]. sHsps are phosphorylated in cells under stress and are translocated to the nucleus, where they are found to be in conjugation and speckled with the RNA-sensitive fraction [21]. αB-Crystallin is secreted from the apical side by retinal pigment epithelial cells. Whether this secreted form has any effect on the retinal cells has yet to be established. Moreover, whether these interactions are orchestrated bindings or random interactions needs further verification.

Apoptosis, the programmed death of cells, occurs in response to various external stresses, such as hyperthermia, oxidative stress and inflammatory cytokines. In several diseases, including eye diseases such as cataract formation, macular degeneration, glaucoma and diabetic retinopathy, apoptosis is an integral part of pathogenesis [22]. sHsps inhibit apoptosis by chaperoning destabilized proteins [23], activating antioxidant defence systems [24] and blocking steps in the apoptotic pathways [25]. We showed that the anti-apoptotic function of one of the sHsps, αA-crystallin, is directly related to its chaperone function [15]. In the past few years, two peptides, one derived from αA-crystallin and the other from αB-crystallin, have been shown to exhibit chaperone activity very similar to their parent proteins [26,27]. We, along with the others, have shown that these peptides also possess anti-apoptotic activities and that they specifically block the mitochondrial death pathway by inhibiting cytochrome *c* release from the mitochondria and activating caspase-3 [28,29]. Hinton and colleagues [29] have shown that the αB-crystallin peptide binds to an amino acid transporter on the cell surface and is then internalized. Steinman and his group [16] have also shown that the αB-crystallin peptide can inhibit inflammation, similar to αA-crystallin. Furthermore, Hsp20 and Hsp27 are also anti-apoptotic and block cytochrome *c* release from the mitochondria and inhibit caspase activation [30–32].

The present study was designed to investigate whether Hsp20 and Hsp27, peptides with sequence homology to αB-crystallin (Figure 1), show chaperone and anti-apoptotic activity. The study also aimed to determine whether the four native C-terminal amino acids were required for cell penetration and the inhibition of apoptosis. We also tested whether these peptides have any *in vivo* effects in a rat model of cataract formation.

EXPERIMENTAL

Materials

Citrate synthase (CS), insulin, lysozyme, malate dehydrogenase (MDH), staurosporine (STS), sodium selenite, protease inhibitor cocktail and a monoclonal antibody for FITC were obtained from Sigma. The remaining chemicals were of analytical grade. The Hsp20 (G^{71}HFSVLLDVKHFSPEEIAVK^{91}) and Hsp27 (D^{93}RWRVSLDVNHFAPDELTVK^{113}) peptides with or without FITC–Ahx, the scrambled Hsp20 (SLSFVDEPILHGKVHAKFVE) and Hsp27 (VSHFWRPARDLNVDVEKLDT) peptides and the Hsp20 (GHFSVLLDVKHFSPEEKAIV) and Hsp27 (DRWRVSLDVNHFAPDEKTLV) scrambled-end peptides with or without FITC–Ahx conjugation, all at 95%–99%purity, were supplied by Peptide 2.0.

Chaperone assays

Chaperone assays were carried out as previously described [33]. The ratios of peptide to client proteins were as follows: CS (1:2.5), insulin (1:5), lysozyme (1:1) and MDH (1:1). To study the complex formation between CS and the peptides, they were incubated at a 1:1 ratio at 43°C for 1 h and centrifuged through a 5-kDa cut-off filter. The retentate was washed three times with PBS by filtration and analysed by SDS/PAGE.

Measurement of surface hydrophobicity

The surface hydrophobicity of the peptides was measured using 6-(*p*-toluidinyl) naphthalene-2-sulfonic acid (emission 350–600 nm, excitation 320 nm) as previously described [28].

Cellular entry of the peptide and stress-induced nuclear translocation

HeLa cells (~70% confluent) were incubated for 3 h with 10 μg/ml FITC-conjugated Hsp20 peptide. After the incubation period, the cells were washed twice with sterile PBS. Images were acquired by either fluorescence or confocal microscopy. Cell lysates were prepared using the mammalian protein extraction reagent (M-PER; Thermo Scientific) and fluorescence of the cell lysates (equivalent to 10 μg of protein) was measured in a Spectramax4 spectrofluorometer (HORIBA Scientific) at excitation/emission wavelengths of 490/525 nm. In addition, Western blotting was carried out with 60 μg of each cell lysate using a monoclonal antibody for FITC (1:1000 dilution). To study the effect of hyperthermia on the nuclear translocation of the peptide, cells were incubated with the FITC–Hsp20 peptide as described above and then stressed at 43°C for 1 h. Following this, the cells were incubated at 37 °C for 1 h and washed with PBS and the nuclei were stained with Hoechst (Thermo Scientific). Images were taken with a fluorescence microscope and cells were counted using the MetaMorph software (Molecular devices).

Measurement of apoptosis

HeLa cells were cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% FBS. Once the cells were 70%–80% confluent, they were treated with the peptides at a concentration of 10 μg/ml for 3 h. After this, STS was added to the medium to a concentration of 100 nM and cells were incubated for 20 h. Cells without any treatment served as control. To study the effect of peptides on H_2O_2-induced apoptosis, cells were treated as mentioned above and then H_2O_2 was added to the medium to a final concentration of 75 μM. The percentage of apoptosis was measured by staining the cells with either Annexin V–FITC (BD Biosciences) for early apoptosis or the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) reagent (Promega) for late apoptosis, per the manufacturer's protocol.

Western blot for cytochrome *c*

The cells treated, as described above, were lysed using the M-PER reagent containing a protease inhibitor cocktail (1:100 dilution). Western blotting was carried out using 25 μg of cell lysate and probing with the anti-cytochrome *c* antibody (1:1000 dilution, Enzo Life Sciences). After the first probing, the membrane was re-probed with an anti-β-actin antibody (1:1000 dilution, Cell Signaling).

Caspase-3 assay

Caspase-3 activity in the cell lysates was measured using the Ac-DEVD-AFC (*N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin) substrate, as previously described [28].

Selenite cataract

Neonatal Sprague–Dawley rat pups (male or female, 11 days old) were used in animal experiments. The animal experiments were performed in strict adherence to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of Animals in Ophthalmic and Vision Research and in accordance with the institutional guidelines. Cataracts were induced by a single subcutaneous injection of sodium selenite at a concentration of 4 mg/kg of body weight. Peptides were diluted from the stock in sterile water (dissolved by adding 2 μl of 10 M NaOH to 1 ml of stock) to a concentration of 1 μg/5 μl and injected intraperitoneally (i.p.) at the concentration 50 μg/animal 4 h prior to selenite injection. This was followed by five consecutive injections for 5 days (i.p., 300 μg/animal). Animals without any treatment and animals that received sodium selenite served as controls. When pups opened their eyes on approximately day 18, the eyes were dilated using tropicamide and analysed using a slit lamp microscope. Transparency of the lenses was analysed by photographing the isolated lenses placed on a copper grid. Some eyes were enucleated and fixed immediately in 10% buffered formalin solution overnight for haematoxylin and eosin staining.

Measurement of the soluble proteins in the lens

Water-soluble proteins were isolated as previously described [28] and their concentration was measured using the BCA protein-assay kit with BSA as the standard (Thermo Scientific).

Measurement of GSH in the lens

Lenses were homogenized in ice-cold 10%TCA (trichloroacetic acid) and the GSH concentration was determined as previously described [34].

Detection of intraperitoneally injected FITC-conjugated Hsp20 peptide in the rat lens

FITC–Hsp20 peptide (100 μg in 100 μl of sterile PBS) was injected i.p. into 12-day-old Sprague–Dawley rat pups (n = 6) on four consecutive days. The control animals (n = 6) received 100 μl of sterile PBS. Three hours after the last injection, animals were killed and their lenses removed. Lenses were sonicated in 700 μl of PBS containing 8 M urea and centrifuged through 10-kDa cut-off filter. The filtrate was analysed by LC–MS/MS using Orbitrap Elite Hybrid Mass Spectrometer (Thermo Electron) coupled with a nanoAcquity UPLC system (Waters). Spectra were acquired by data-dependent methods with an alternative full scan followed by ten MS/MS scans with collision-induced dissociation of the peptide ions at normalized collision energy of 35%. Raw LC–MS/MS data were submitted to customized database constitute of FITC–Ahx–Hsp20 peptide through Mascot Demon (version 2.2.0, Matrix Science). The mass tolerance was set as 10 ppm for precursor ion and 0.8 Da for product ion.

Statistical analysis

The data are presented as the means ± S.D. from the experimental number indicated in the Figure legends. They were analysed using the StatView software (SAS Institute). Statistical significance between the groups was determined using ANOVA with $P < 0.05$ being considered significant.

RESULTS

Hsp20 and Hsp27 peptides inhibit stress-induced aggregation of client proteins

Four client proteins were used to investigate the chaperone activity and aggregation of the peptides from either heat (CS, lysozyme and MDH) or chemical (insulin) stress. Both peptides significantly inhibited stress-induced aggregation of the client proteins (Figures 2A–2D). The Hsp20 peptide was slightly better than the Hsp27 peptide. To study the specificity of the peptides, we used scrambled peptides of Hsp20 and Hsp27 to test against heat-induced aggregation from CS and MDH. In both the assays, the scrambled peptides failed to show chaperone activity (Figures 2E and 2F). We also tested the effect of peptide concentration on the chaperone activity with the two client proteins, CS and insulin. Both the Hsp20 and the Hsp27 peptides inhibited the thermally and chemically induced aggregation of the client proteins in a concentration-dependent manner. The Hsp20 peptide was slightly better at all concentrations (Figure 3).

Hsp20 and Hsp27 peptides form complexes with client proteins during chaperone activity

To determine whether the peptides bind to client proteins during heat stress, we incubated the peptides with the client protein CS at 43°C for 1 h and filtered the sample using a 5-kDa centrifugal filter to remove the unbound peptides. The filtration retentate was analysed by SDS/PAGE. The bound peptides were dissociated during SDS/PAGE and could be detected in the gel (Figure 4A). Scrambled peptides did not show any complex formation. To show peptide separation in the gel, peptides without CS incubation were analysed by SDS/PAGE (Figure 4B).

Hsp20 and Hsp27 peptides perform better than the α-crystallin peptide in their chaperone activity

When CS was used as a client protein, the Hsp20 and Hsp27 peptides reduced aggregation by 55% and 45% more than the αB-crystallin peptide respectively. However, the two peptides displayed similar chaperone activity compared with the αA-crystallin peptide (Figure 5A). In the thermal aggregation of MDH, the Hsp20 peptide reduced aggregation more than any of the other three peptides (Figure 5B). The difference in the chaperone activity was unrelated to the surface hydrophobicity of the peptides, as it was similar for all four peptides (Figure 5C).

Hsp20 and Hsp27 peptides are taken up by cells and hyperthermic stress promotes their nuclear translocation

To determine whether the peptide could enter the cells without the aid of cell permeable reagents, we conjugated the N-terminus of the Hsp20 peptide with FITC and incubated it with HeLa cells. The confocal images confirm that the peptide was taken up by the cells and uniformly distributed in the cytoplasm after a 3 h incubation period (Figure 6A). We also detected the peptide in the cell lysates through Western blotting by using a monoclonal anti-FITC antibody (Figure 6B). The fluorescence of the cell lysate was measured at 490/525 nm; this was detected only in the cells treated with the FITC-conjugated peptide and not in the untreated cells (Figure 6C). To test the effect of stress on the peptide within the cells, we incubated HeLa cells containing the peptide at 43°C for 1 h. We found significant amounts of nuclear translocation of the peptide after this treatment (Figures 6D and 6E).

Hsp20 and Hsp27 peptides inhibit stress-induced apoptosis

Next, we investigated the ability of the peptides to prevent chemically induced apoptosis in HeLa cells. We incubated the cells with the peptides for 3 h and induced apoptosis by treating the cells with either 100 nM STS or 75 μM H_2O_2. Cells treated with STS and without the peptides showed significant early-stage apoptosis (14% of the cells) and late stage apoptosis (5% of the cells). Cells previously incubated with either the Hsp20 or the Hsp27 peptide showed a reduction in both early-stage (9%–10%) (Figure 7A) and late-stage (2%–3%) (Figure 7B) apoptosis. The scrambled peptides did not show such an effect. The inhibition of apoptosis was accompanied by the inhibition of cytochrome *c* release from the mitochondria and the inhibition of caspase-3 activation (Figures 7C and 7D). Similarly, cells treated with H_2O_2 showed 13% apoptotic cells, which was significantly reduced (7%–8%) by the addition of the peptides (Figure 7E).

Role of the terminal four amino acids on cellular entry of the peptides

To investigate the role of the last four amino acids in the Hsp20 (IAVK) and the Hsp27 (LTVK) peptides, which we reasoned are necessary for cellular entry based on other cell permeable peptides [35], we scrambled the amino acid sequence of the four amino acids and tested their anti-apoptotic activity. The end-scrambled peptides did not enter the cells (Figures 8A and 8B) and failed to show protective effects against STS-induced apoptosis (Figure 8C).

Peptides inhibit selenite cataracts in rats

Because the peptides inhibited apoptosis from stress-induced protein aggregation, we next tested the peptides for their ability to inhibit protein aggregation that typically occurs during cataract formation [36] in selenite-induced cataracts in rats. Cataracts were induced by a single subcutaneous injection of sodium selenite. The Hsp20 and the Hsp27 peptides were administered i.p. before and after the sodium selenite injection. The sodium-selenite-injected animals showed mature cataracts compared with the controls (Figure 9A). However, administration of the Hsp20 and the Hsp27 peptides inhibited cataract development. Treatment with the scrambled peptides had no beneficial effects. The inhibition of cataract development occurred through the inhibition of protein insolubilization and the inhibition of oxidative stress. Animals treated with selenite alone showed a reduction in the total soluble lens proteins and GSH content compared with untreated controls (Figures 9B and 9C). Both the Hsp20 and the Hsp27 peptides significantly prevented protein insolubilization and GSH formation. Again, the scrambled peptides were completely ineffective.

Intraperitoneally injected Hsp20 peptide translocates to the lens

The chromatographic profiles of PBS-injected control and Hsp20–FITC peptide-injected samples (between 31 min and 45 min) are shown in Figure 10(A). The chromatogram for the peptide-injected sample was different when compared with the one from control, possibly because of the peptide binding to proteins and changing their retention time in HPLC. FITC–GHFSVLLDVKHFSPEEIAVK peptide with mass shift of 502 Da was observed at parent ions of 918.7807(3+) and 689.3379(4+) in the peptide-injected sample, but not in the control sample (Figure 10B). Further, tandem MS analysis revealed that the FITC-tag was at the N-terminus of the peptide, the *m/z* of FITC and Ahx together (mass shift of 502 Da), as well as Ahx alone (mass shift of 113 Da) attached to the fragment ions. The detached FITC molecule was also observed at *m/z* of 390 Da (Figure 10C).

DISCUSSION

The primary purpose of the present study was to determine whether Hsp20 and Hsp27, peptides with sequence homology to αB-crystallin, are molecular chaperones and have anti-apoptotic properties. The secondary purpose was to determine what amino acid motif within the peptide is responsible for their cellular entry. We found that both the Hsp20 and the Hsp27 peptides are molecular chaperones and their ability to inhibit thermally and chemically destabilized proteins is superior to that of the αB-crystallin chaperone peptide.

Many studies have shown a direct link between surface hydrophobicity and chaperone activity in the sHsps [37–39]. It is believed that the hydrophobic patches on these proteins interact with the hydrophobic patches of denaturing proteins during chaperone activity. However, the Hsp20 and the Hsp27 peptides, despite performing better than the αB-crystallin peptide in their chaperone activity, had the same degree of surface hydrophobicity as the αB-crystallin peptide. Such a disconnect between surface hydrophobicity and chaperone activity has been observed previously in sHsps in some studies [40] and, thus, casts a doubt on whether the hydrophobic surfaces of sHsps are entirely responsible for the chaperone activity.

The cell permeability of the peptides required the last four amino acids in their native sequence. The sequences of the two peptides are similar to that of the other cell permeable peptides [35]. Whether the cellular entry of the Hsp20 peptide was mediated through a transporter is unknown; however, it is likely as a recent report [29] showed that the αB-crystallin peptide is transported across the plasma membrane through an amino-acid transporter. Further, determining if the transporter requires the last four amino acids in the native sequence of the peptide for binding also needs to be verified.

The anti-apoptotic property of the peptides appear to be due to their inhibition of the mitochondrial apoptosis pathway, as both peptides inhibited cytochrome *c* release from the mitochondria and activation of procaspase-3. These results are similar to the parent protein molecules from which they are derived, as Hsp27 has been shown to inhibit cytochrome *c* release and activation of caspase-3 [14,41]. Similar properties have been observed for the αB-crystallin peptide [28,29]. Whether the Hsp20 and the Hsp27 peptides also bind to Bax and Bcl-Xs during their inhibition of apoptosis, similar to αB-crystallin [12], still needs to be determined.

The nuclear translocation of the Hsp20 peptide in the thermally-stressed cells is not unexpected. sHsps have been shown to translocate to the nucleus in stressed cells [42] and such translocation has been perceived to be required for inhibiting apoptosis. sHsps, αB-crystallin and Hsp27 are all phosphorylated at discrete serine residues before they are translocated to the nucleus. This was confirmed in studies in which non-phosphorylatable mutant proteins of Hsp27 and αB-crystallin were unable to translocate into the nucleus in the stressed cells [43,44]. Given these findings, it is surprising that the Hsp20 peptide is translocated into the nucleus in the absence of phosphorylated serine residues. It is unlikely that the peptide enters the nucleus on its own, as it lacks the nuclear localization signal that is present in many proteins that translocate into the nucleus from the cytoplasm [45]. It is possible that the peptide binds to a carrier protein(s) during its translocation to the nucleus. The other question is: why is it translocated to the nucleus in stressed cells? It could possibly bind to transcription factors and stabilize them to assist in gene regulation. This could be analogous to αB-crystallin, which has been shown to bind the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2), a regulator of type-2 antioxidant enzyme synthesis in the stressed cells [46].

Previous studies indicate the involvement of proteolytic enzymes and disulfide cross-links in the proteins in selenite cataracts [36,47]. Inhibition of protein insolubilization by the Hsp20

peptide in rat lenses suggested that protein aggregation is inhibited by the peptide. The peptide prevented GSH loss in the lens, which supports the notion that it reduces oxidative stress in the lens. We have previously shown a similar reduction in oxidative stress in rat lenses by i.p. injected α-crystallin peptide [28]. It is remarkable that a peptide injected i.p. produced such effects in the lens. This could only happen if the i.p. injected peptide crossed the blood/aqueous barrier and is taken in by the lens. Our similar observation with the α-crystallin peptide [28] supports this idea. Furthermore, the αA-crystallin peptide has been shown to bind Cu^{2+} at His^{79} and inhibit reactive oxygen species (ROS) formation [48]. The Hsp20 and Hsp27 peptides might possess a similar Cu^{2+} -binding property through their histidine residues and inhibit ROS formation in the lenses of sodium-selenite-treated rats. Whether the observed inhibition of cataract development by the Hsp20 peptide is due to the collective effect of chaperone proteins, inhibition of ROS formation or protein aggregation in the lens needs to be determined.

Taken together, the present study identifies two additional peptides of the sHsps with both chaperone and anti-apoptotic activities. The fact that they can be delivered *in vivo* to block cataract development and apoptosis in cultured cells suggests that they could be used as therapeutic agents in diseases where apoptosis and protein aggregation contribute to disease pathogenesis.

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Abbreviations

CS	citrate synthase
Hsp	heat-shock protein
i.p.	intraperitoneally
MDH	malate dehydrogenase
M-PER	mammalian protein extraction reagent
ROS	reactive oxygen species
sHsp	small heat-shock protein
STS	staurosporine

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Figure 1. Sequence homology in sHsps

The amino acid sequences of αA-crystallin, αB-crystallin, Hsp20 and Hsp27 are shown with the chaperone peptides marked in a specific colour.

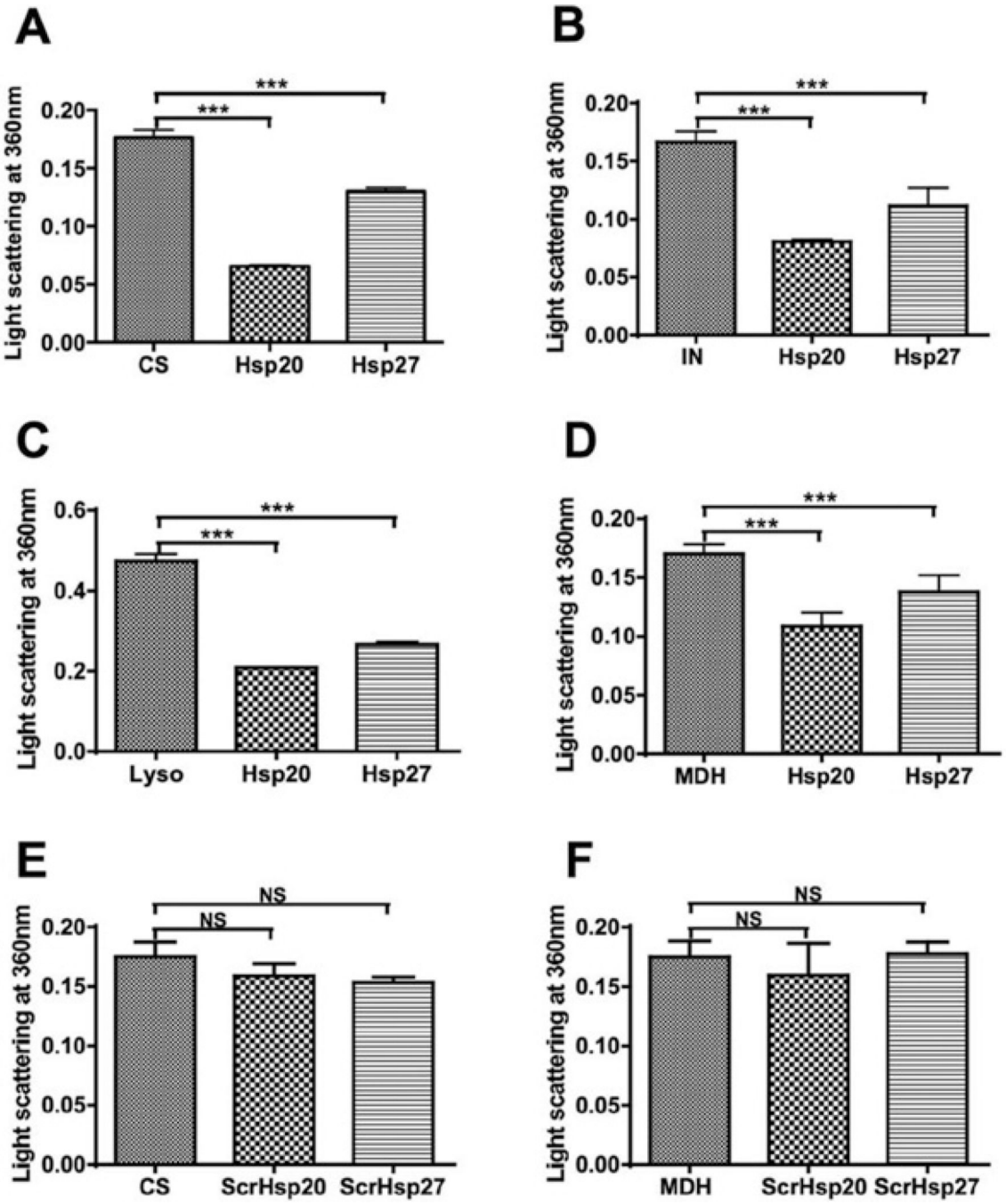

Figure 2. Hsp20 and Hsp27 peptides have chaperone activity

Using four client proteins, we assessed the chaperone activities of the peptides: (**A**) CS, (**B**) insulin (IN), (**C**) lysozyme (Lyso) and (**D**) MDH. The ratios of the client proteins to the peptides are given in the Experimental section. We tested the scrambled (Scr) peptides of Hsp20 and Hsp27 using two of the client proteins (**E** and **F**). The bars represent the means + S.D. of three independent experiments. ***$P < 0.0005$; NS, not significant.

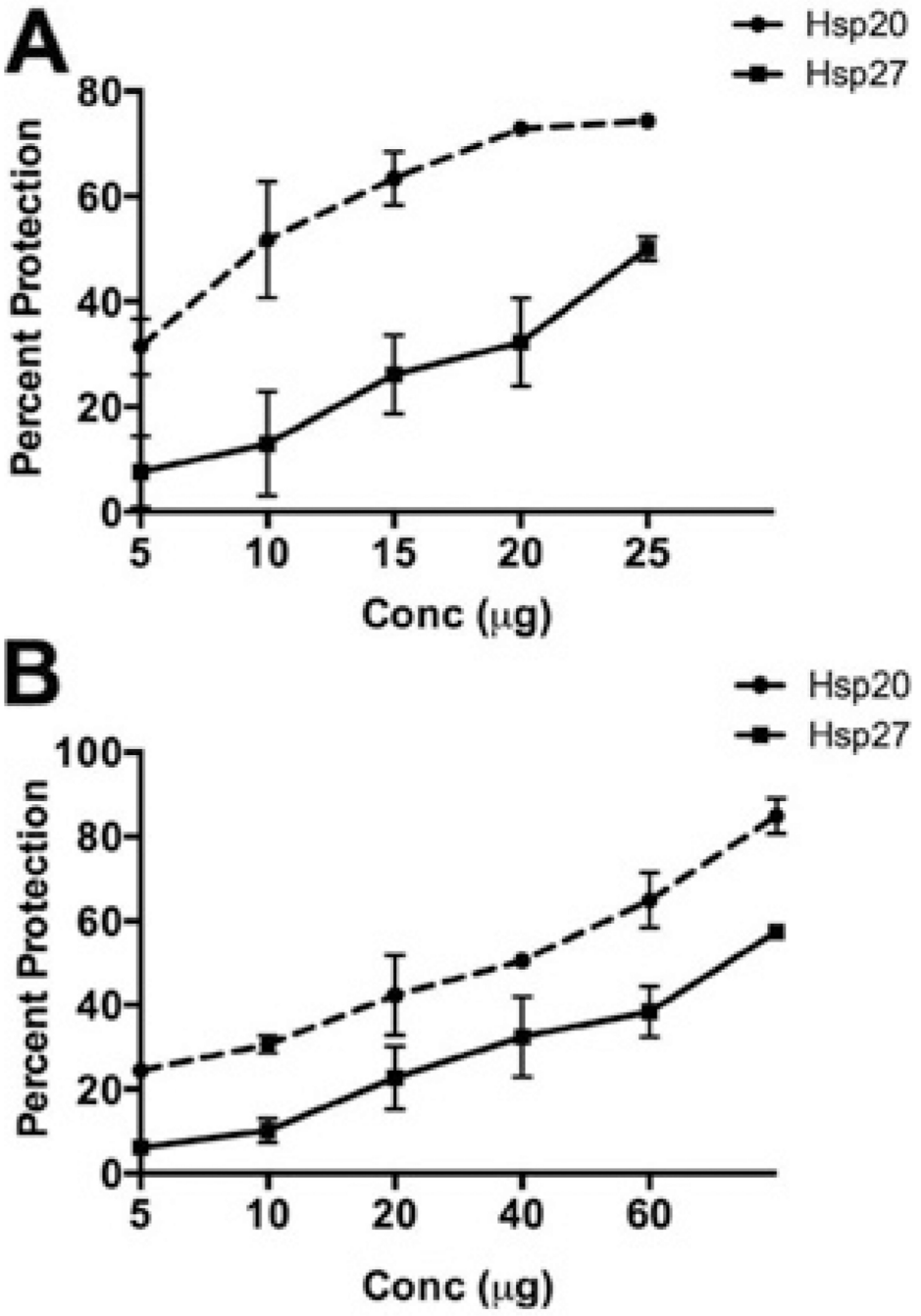

Figure 3. Hsp20 and Hsp27 peptides inhibit hyperthermia and chemically induced protein aggregation in a concentration-dependent manner

Using CS (**A**) and insulin (**B**), we assessed the chaperone activities using increasing concentrations of the peptides. The bars represent the means ± S.D. of three independent experiments.

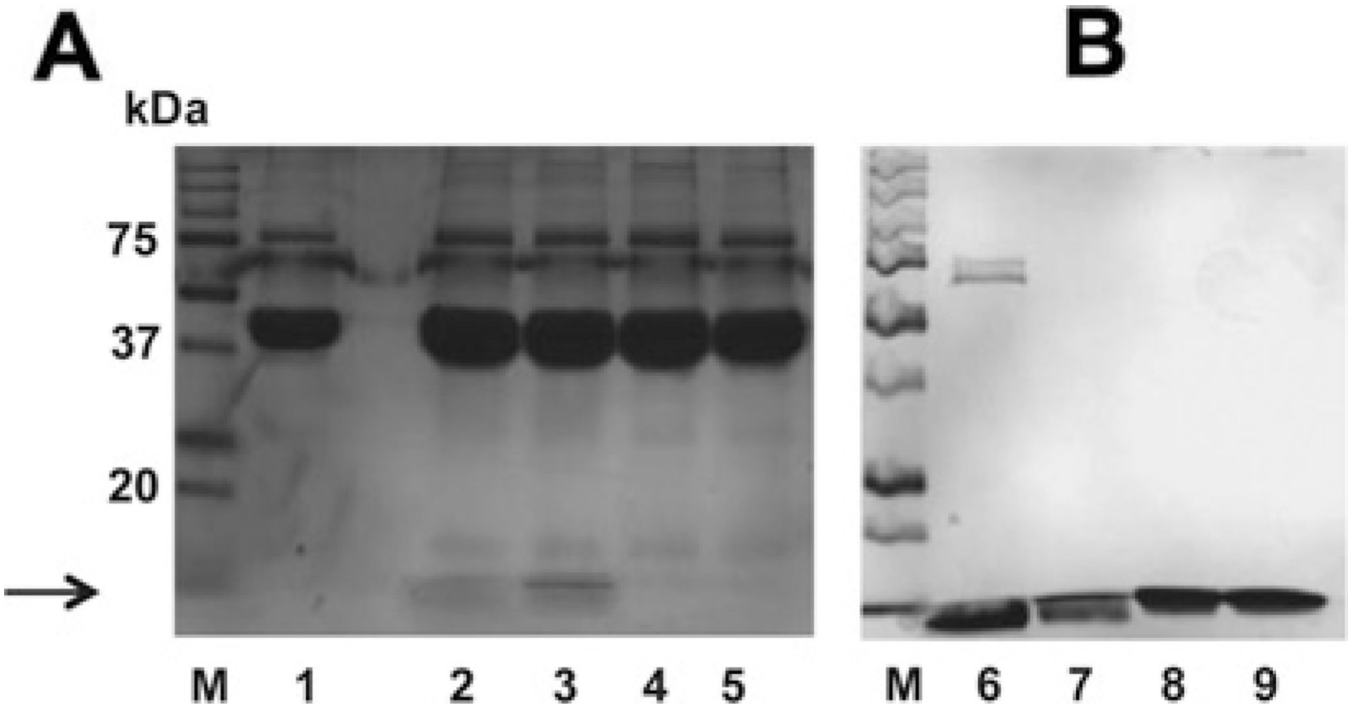

Figure 4. Peptides form complexes with the client protein CS during hyperthermia-induced protein aggregation

CS and the peptides were incubated at a 1:1 ratio at 43°C and analysed by SDS/PAGE (**A**). Corresponding peptides without incubation with CS were analysed by SDS/PAGE to show the peptides in a gel (**B**). M, molecular-mass marker; 1, CS alone; 2, CS + Hsp20; 3, CS + Hsp27; 4, CS + scrambled Hsp20; 5, CS + scrambled Hsp27; 6, Hsp20 alone; 7, Hsp27 alone; 8, scrambled Hsp20 alone; 9, scrambled Hsp27 alone. Arrows indicate the peptide separated from the complex during SDS/PAGE or the peptides.

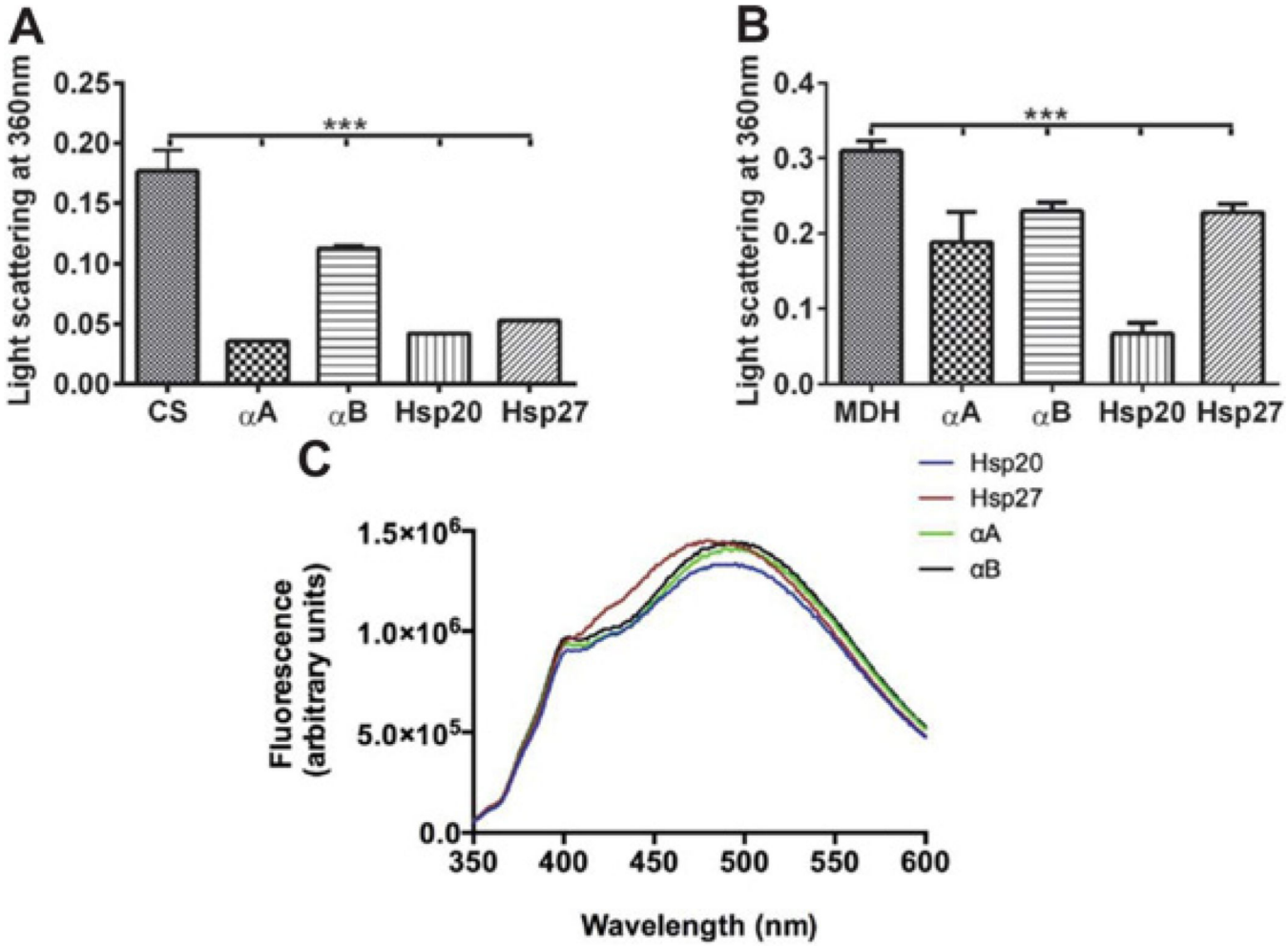

Figure 5. Comparison of the chaperone activity of the Hsp20 and the Hsp27 peptides with α-crystallin

CS (**A**) and MDH (**B**) were used as client proteins in the chaperone assays (**A** and **B**). The ratios of client proteins to the peptides are given in the Experimental section. Surface hydrophobicity was measured using TNS (2,6-toluidinyl-naphthalenesulfonic acid) (**C**). The bars represent the means + S.D. of three independent experiments. ***$P < 0.0005$.

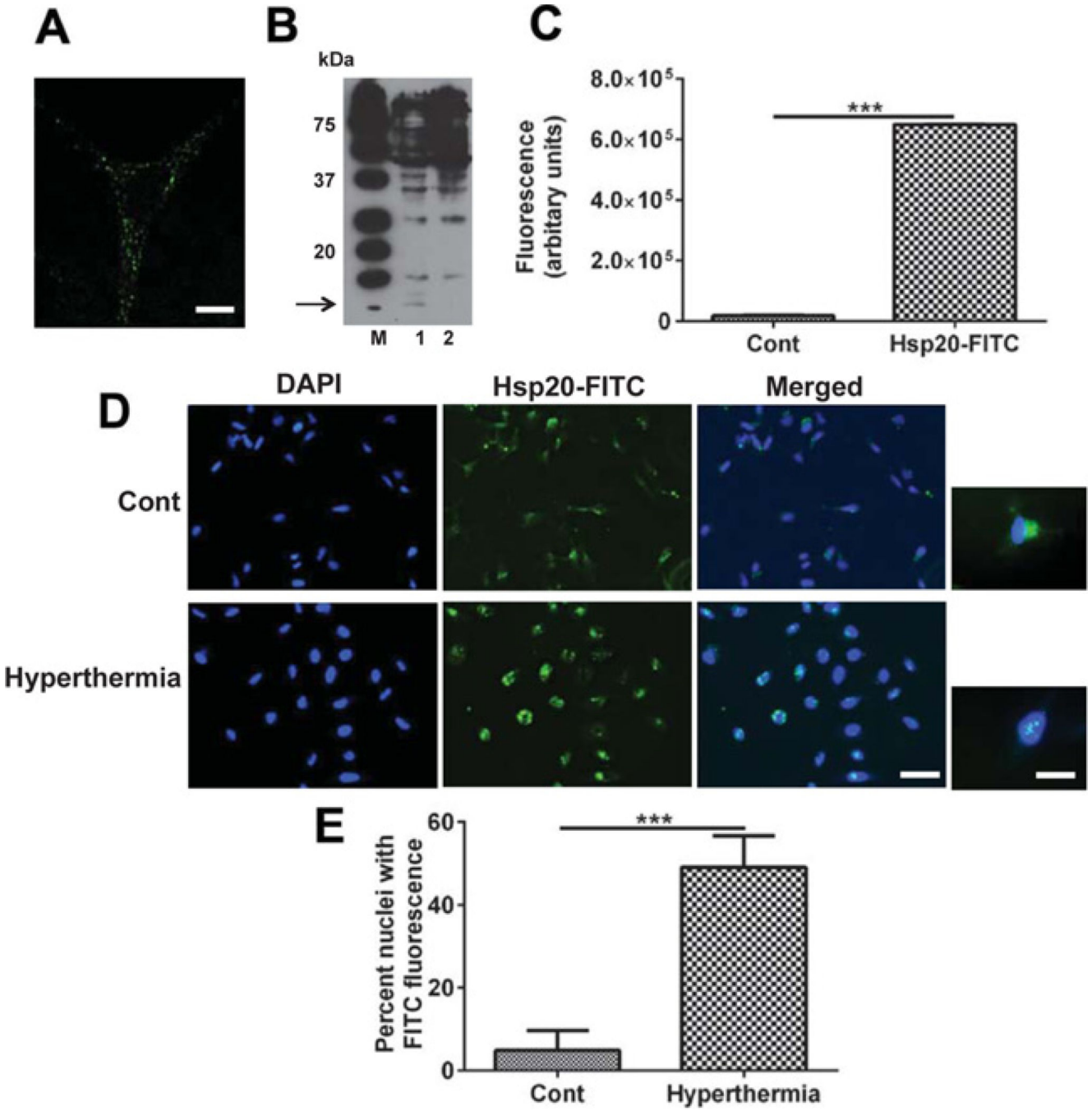

Figure 6. The Hsp20 peptide enters cells and hyperthermia enhances its nuclear translocation

HeLa cells were incubated with the Hsp20–FITC peptide as described in the Experimental section. Nuclear translocation of the peptide was assessed by confocal microscopy (**A**). Western blotting of the cell lysate and fluorimetric measurement of the Hsp20–FITC peptide at 490/525 nm in the cell lysate are shown (**B** and **C**). M, molecular-mass marker; 1, lysate from Hsp20–FITC peptide-incubated cells; 2, lysate from cells without Hsp20–FITC peptide incubation. The arrow indicates the peptide band. Fluorescence microscopic images of the control and stressed cells are shown at ×10 magnification (**D**). Images enlarged to ×63 are

also shown. Percentage of nuclei with the translocated peptide is shown in (**E**). DAPI was used for nuclear staining. Merged images of the DAPI treatment and the FITC–peptide-treated group are also shown. The bars represent the mean + S.D. of three independent experiments and the nuclei staining are from six independent experiments. ***$P < 0.0005$; Scale bar for (**A**) = 10 μm and for (**D**) = 30 μm and for the enlarged ×63 figure = 10 μm.

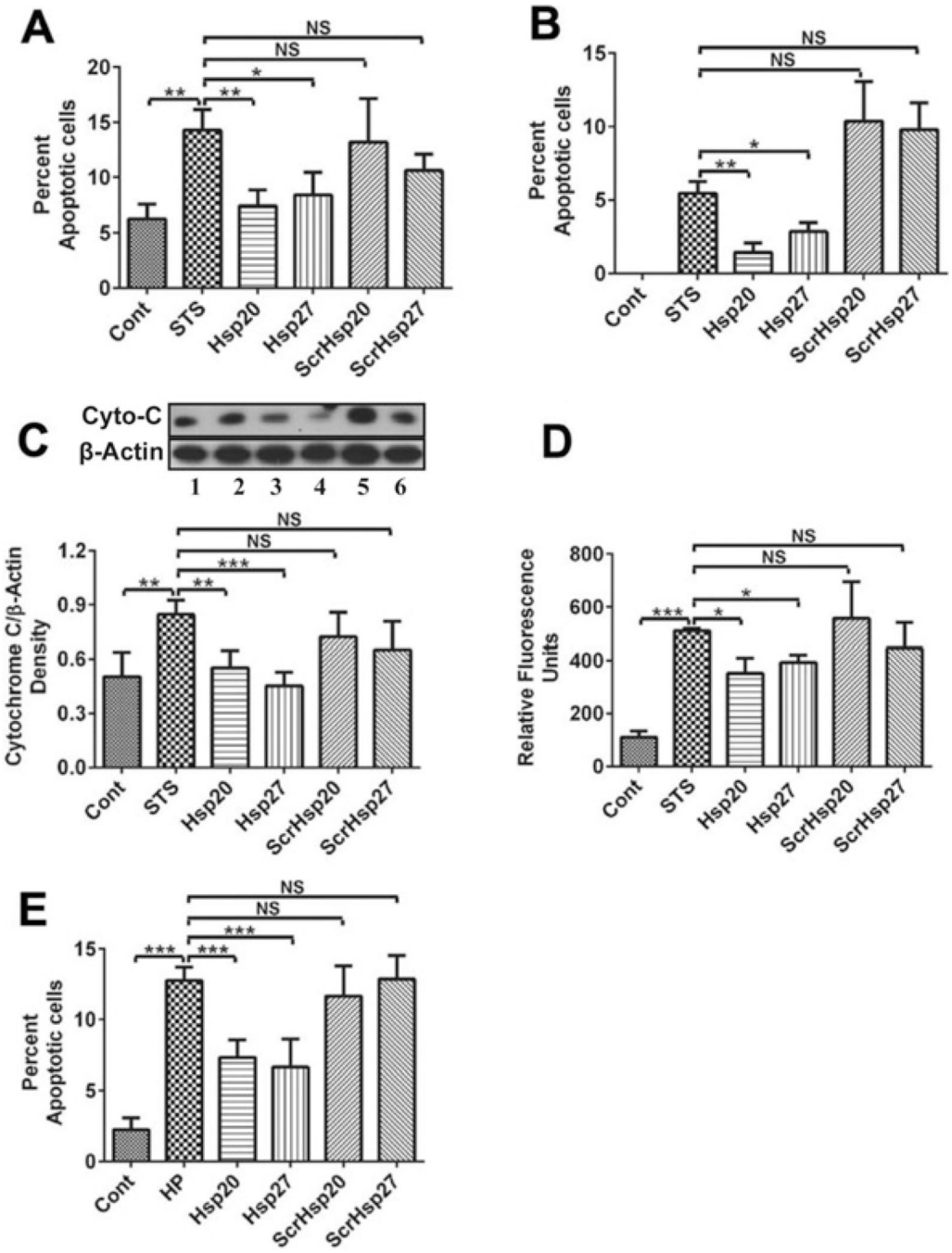

Figure 7. Hsp20 and Hsp27 peptides inhibit chemically induced apoptosis in HeLa cells

HeLa cells were treated with the peptides and apoptosis was induced by treating the cells with either STS or H_2O_2, as described in the Experimental section. Apoptotic cells were counted after Annexin V–FITC staining (**A**) or TUNEL staining (**B**). The peptides inhibited STS-induced cytochrome *c* release from the mitochondria. These data are shown in Western blotting results (bar graph obtained from densitometry). Western blot shown is for representative sample from each group. 1, control; 2, STS; 3, Hsp20; 4, Hsp27; 5, ScrHsp20; 6, ScrHsp27 (**C**). The peptides inhibited caspase-3 activity (**D**). The peptides also blocked

H_2O_2-induced apoptosis in HeLa cells (**E**). β-Actin was used as the loading control. The bars represent the means + S.D. of three independent experiments. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005; NS, not significant.

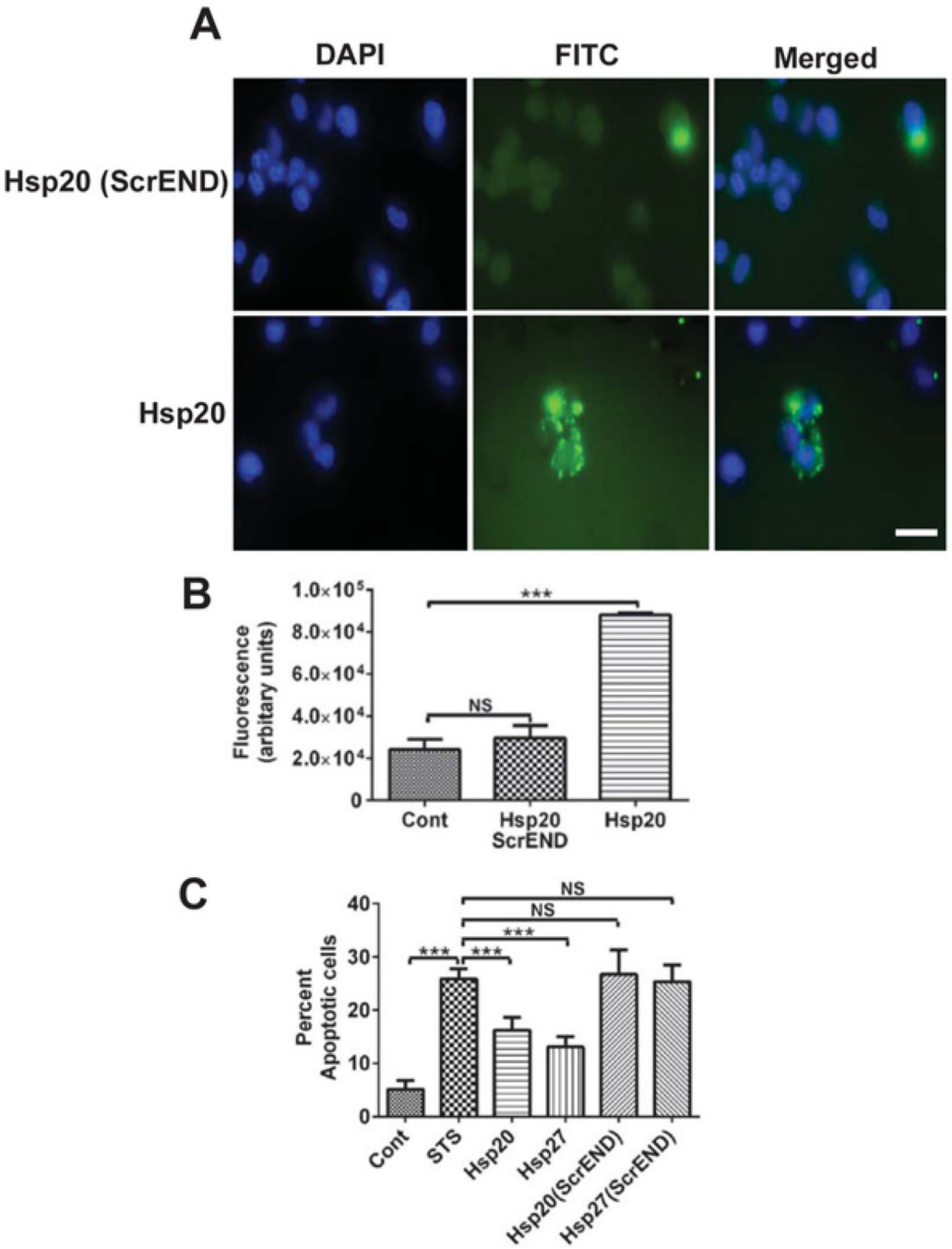

Figure 8. The native sequence of the terminal four amino acids is required for the cellular entry of the peptides

HeLa cells were incubated with the scrambled-end Hsp20 peptide as described in the Experimental section. Cellular entry of the peptide was assessed by fluorescence microscopy (**A**) and fluorimetric measurement of the cell lysate at 490/525 nm (**B**). HeLa cells were treated with the end-scrambled or native Hsp20 and Hsp27 peptides and treated with STS as detailed in the Experimental section. The apoptotic cells were counted after Annexin V–

FITC staining. The bars represent the means + S.D. of three independent experiments. ***$P < 0.0005$; NS, not significant; Scale bar = 10 μm.

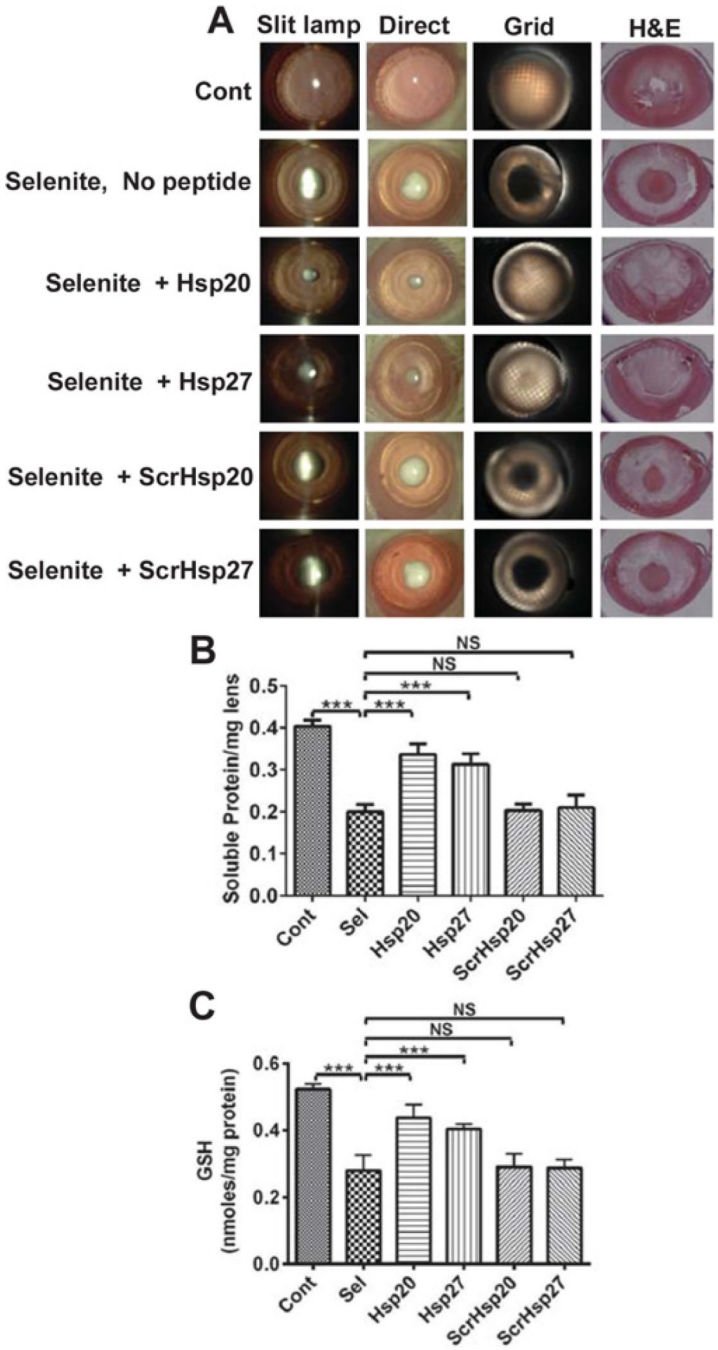

Figure 9. The Hsp20 peptide inhibits cataract development in rats by blocking protein insolubilization and inhibiting oxidative stress

Cataract formation was induced in rat pups by treatment with sodium selenite. The Hsp20 peptide was administered at 10 μg/animal i.p. 1 day prior to and 4 days after sodium selenite injection. Cataract development was assessed by slit lamp microscopy and direct imaging (**A**). Isolated lenses were kept on a copper grid and microscopic images were taken to assess their transparency. Haematoxylin and eosin staining staining was performed to visualize protein aggregation in the nuclear region of the lens. The data shown are from one representative lens per group. The level of soluble proteins and GSH in the peptide-treated

and control rat lenses are shown (**B** and **C**). The bars represent the means + S.D. of three independent experiments. ***$P < 0.0005$; NS, not significant.

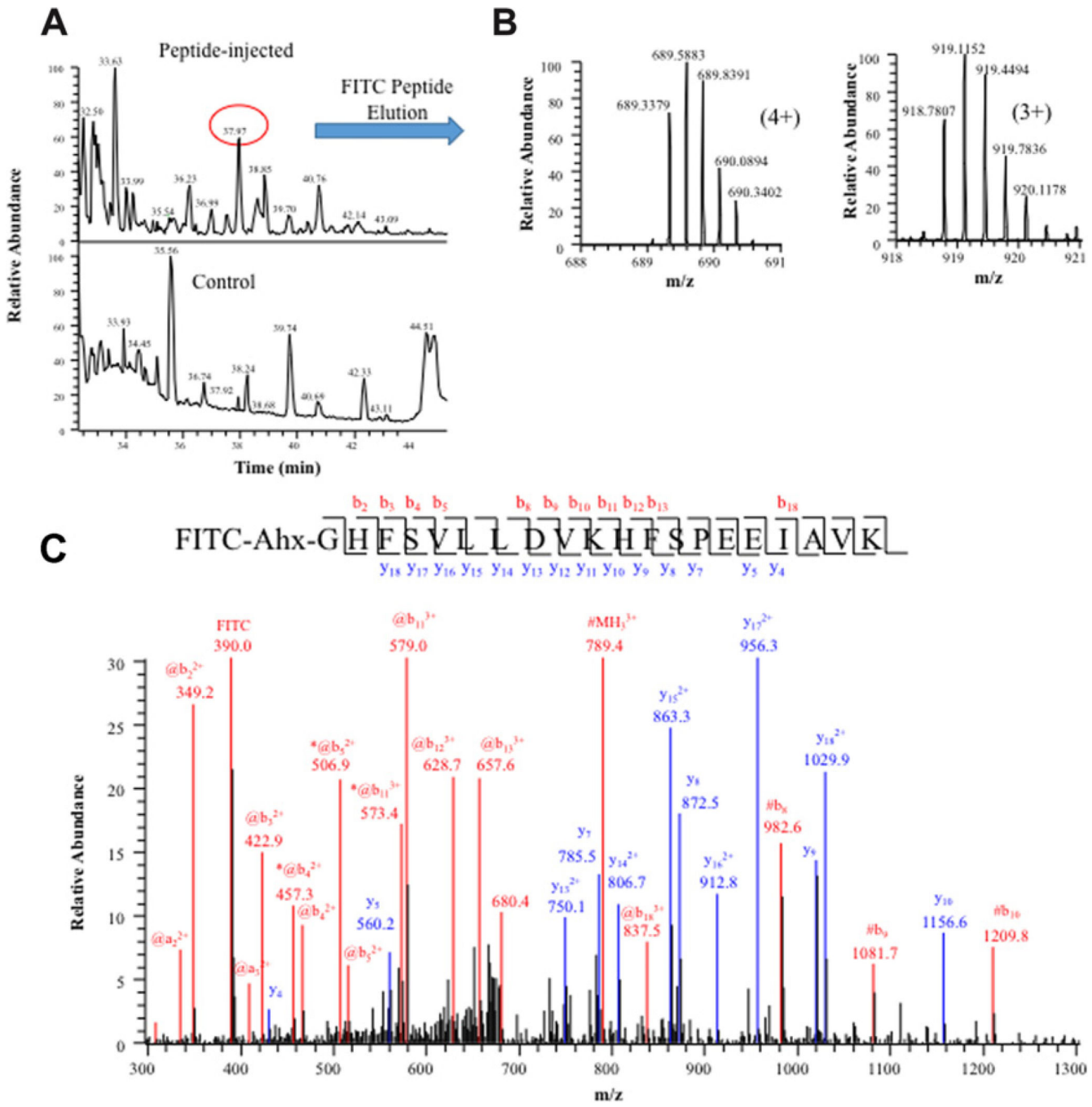

Figure 10. Mass spectrometric detection of i.p. injected FITC–Ahx–Hsp20 peptide in rat lens

Chromatographic profile (between 31 min and 45 min) of the control and peptide-injected lens extracts (**A**). The full mass spectrum of FITC–Ahx–GHFSVLLDVKHFSPEEIAVK shows triply charged parent ions and quadruply charged parent ions at *m/z* 918.7807(3+) and 689.3379(4+) in the peptide-injected sample (**B**), but not in the control sample (not shown). Tandem mass spectrum of FITC–Ahx–GHFSVLLDVKHFSPEEIAVK with parent ion of 689.3379(4+) **(C)**. A mass shift of 502 Da was observed at the parent ion, as well as b-series ions from b2 to b18, marked as @b or *@b (water loss), but not the y-series ions from y4 to

y18, which suggested that FITC–Ahx was at the N-terminus of the peptide. Detached FITC with *m/z* of 390 Da was observed, whereas the b-series ion marked as #b indicated the loss of FITC with only Ahx remaining on the fragment ion (mass shift of 113 Da).