Chapter IV

- *in which an attempt to reconstitute wtHtt function in vivo is undertaken; wtHtt and caspases are produced as a recombinant proteins but no bioactivity of recombinant wtHtt is observed in several assays*

1 Introduction

Currently, experiments that address the question of apoptosome formation and activation are increasingly carried out as *in vitro* experiments (Beere et al., 2000; Zou et al., 1999). These kinds of experiments have the advantage that they generate a completely defined system with no uncharacterized components. The known components of the apoptosome are produced as recombinant proteins and added together *in vitro* together with effector molecules. Subsequently, caspase measurements are performed or the resulting complexes are resolved by SDS-PAGE and analyzed by Western blot. Thus far, this kind of experiment has proven to be very successful in mimicking the 'real life' events surrounding apoptosome function.

These experiments can be made to mimick the *in vivo* situation by the addition of cellular extracts, or reversely, by the addition of recombinant protein to cellular extracts. Since this kind of experiment introduces a level of uncertainty due to the undefined nature of cellular extracts, they are usually only performed when an unknown cellular component is assumed to take part in apoptosome formation. Thus modified, these assays can be used to identify these unknown molecules.

Since Htt affects the formation of the apoptosome as well as caspase-9 function, the same kind of experiment should be possible if recombinant Htt can be produced in sufficient quantities.

2 Results

2.1 Production of recombinant wtHtt and recombinant caspase-9

Since poly-Q fragments form insoluble precipitates (Perutz, 1999), attempts were made to produce recombinant wtHtt only. Thus, only the question of wtHtt was addressed by this approach.

Because a first attempt at producing recombinant wtHtt as a Glutathione-S-Transferase (GST) fusion protein failed, wtHtt was produced as a S-tag/His_6- fusion protein in *E. coli*. The recombinant protein was eluted from Ni-beads using PBS containing 250mM imidazole. Analysis of the elution fraction by SDS-PAGE followed by Coomassie Brilliant Blue staining did not reveal any predominant band (data not shown). The recombinant protein was only detected by Western blot analysis (Fig. 1A). However, the signal generated by Western blot were strong and showed no degradation of the recombinant protein indicating excellent induction but too low of a yield of wtHtt to be detected by Coomassie staining. Most importantly, wtHtt was produced without being degraded.

Similarly, caspase-3- and caspase-9-His_6- fusion constructs were used to produce recombinant caspase-9 and caspase-9D315/330A (Stennicke and Salvesen, 1999). Caspase-3 and -9 were produced as active enzymes showing two bands corresponding to the zymogen and the processed form, respectively, on a Coomassie stain whereas the caspase-9 cleavage site mutant D315/330A showed only a single band of the appropriate size (data not shown). Because the caspase constructs produced excellent yield, the low efficiency of wtHtt production must be due to an unidentified problem with the Htt protein itself.

2.2 Binding experiments

To further demonstrate that caspase-9 and wtHtt interact with each other, recombinant wtHtt and recombinant caspases were added together and wtHtt was immunoprecipitated. Western blot analysis of the immunocomplexes showed that wtHtt was not able to precipitate caspase-3, as expected (data not shown). However, neither caspase-9 nor caspase-9 DD (D315/330A) bound to wtHtt (Fig. 1B). In addition, no interaction was observed when detergent was added to the reaction (data not shown).

Since the lack of interaction of the recombinant proteins could have been due to some missing third protein(s) the above experiment was performed with slight variation by addition of cytoplasmic extracts from ST14A cells. As before, no interaction was observed. Even when the cytoplasmic extracts were pre-activated by addition of cytochrome c, no binding was observed, even though caspase-9 activity was detected (data not shown).

2.3 Htt phosphorylation by Akt

Htt contains a conserved phosphorylation site for the S/T protein kinase Akt (Fig. 2A). Akt has been implicated to play a role in several anti-apoptotic signaling pathways and the pro-apoptotic Bcl-2 family member BAD has been shown to be directly phosphorylated by Akt which leads to binding of BAD to 14-3-3 and its subsequent inactivation (Datta et al., 1997; del Peso et al., 1997). Thus, a possible explanation for the failed binding of Htt and caspase-9 *in vitro* could lie in the non-existent phosphorylation of recombinant Htt. Therefore, *in vitro* phosphorylation studies were performed.

HEK293 cells were transfected with GST-Akt constructs and stimulated with insulin for 10min. After harvest, the kinase was purified from the cell lysates with glutathione beads. Recombinant wtHtt was then added together with ^{32}P-γ-dATP to the beads and the labeling reaction was resolved by SDS-PAGE. As shown (Fig. 2B), recombinant Htt was phosphorylated by wild type Akt, but not by a kinase deficient Akt. However, the Akt substrate Histone H2B was phosphorylated much more efficiently than Htt itself.

2.4 The final straw

Recombinant Htt was eluted in a buffer containing 250mM imidazole. When enzymatic activity measurements for caspase-9 activity were performed, this buffer dramatically reduced activity. This effect also explains the relatively weak phosphorylation of Htt by Akt, since the control substrate H2B, which was dissolved in water, was phosphorylated efficiently. Therefore, it became imperative to eliminate these buffer effects.

Several buffers were tested for their ability to reduce caspase-9 catalytic activity and only the low salt buffer A showed neutral results. Thus, recombinant Htt was dialyzed versus buffer A. The dialyzed solution still showed strong Htt immunoreactivity and no degradation of the Htt band. Unfortunately, though, when the Akt phosphorylation experiment was performed using dialyzed recombinant Htt, no phosphorylation was observed (data not shown). This suggests that dialysis produced incorrectly folded Htt that could not be phosphorylated anymore. Unfortuneately, no solution for this problem was found. Thus, the effect of Htt phosphorylation on interaction with caspase-9, with or without additional cellular factors, and

consequently on caspase-9 catalytic activity could not be investigated in a defined *in vitro* system.

3 Discussion

In an effort to understand the exact molecular mechanisms that underlie the observed Htt functions, recombinant wtHtt-N548 is produced in *E. coli*. Even though yield is low (and outright miserable compared to the amounts of purified caspases), strong immunoreactivity is observed, sufficient to perform the pertinent experiments. The reasons for this abysmal production are unclear. Several different *E. coli* strains were tested with even worse results; concurrent purification of recombinant caspases is extremely successful, even though some caspases are produced as active enzymes and undergo auto-proteolysis. One reason that Htt can only be produced in small quantities is its poly-Q stretch. Even small, pure poly-Q repeats are insoluble, and while in a cellular context certain chaperones help protein folding and prevent aggregation, the bacterial chaperone machinery might not be able to perform that task efficiently for Htt. However, the bacterial lysates do not show large quantities of inclusion bodies, which are often found in bacteria producing insoluble recombinant proteins. Neither is substantial degradation of recombinant Htt observed, indicating that Htt is stable in the bacteria and not degraded, eliminating another potential reason for low yield. A last explanation for the poor production of recombinant Htt might lie in problems with transcription/translation, especially of the CAG-stretch, that might not be efficiently carried out by the bacteria.

The problem of aggregation and proper folding is further highlighted by the fact that dialysis against low salt buffer A from high salt bacterial lysis/elution buffer results in the loss of any vestiges of Htt bioactivity (i.e. the ability to get phosphorylated). In this respect, it is surprising that any soluble Htt is produced at all.

WtHtt is produced as a single chain protein with no significant degradation. It precipitates with S-tag- (data not shown) and Ni-beads, as well as with α-Htt antibody, indicating that the integrity of the fusion protein is not compromised. It even retains some biologic activity, as it is phosphorylated by Akt in an *in vitro* kinase assay, albeit at very low efficiency. Whether low phosphorylation efficiency is due to buffer effects or because substantial amounts or recombinant Htt are misfolded, or both, is unclear. Nonetheless, the Akt phosphorylation site in Htt is extremely well conserved and fits the consensus remarkably well. It has long been speculated whether this site is actually utilized and the results obtained here strongly suggest that.

However, no interaction with caspase-9 is observed *in vitro*, even when detergent, cellular extracts or activated cellular extracts were added to facilitate interaction. These results indicate that interaction between Htt and caspase-9 is indirect or needs certain stabilizing factors. Another reason for not observing interaction is that wtHtt needs to be phosphorylated at Ser421 by Akt to be able to bind caspase-9 and perform its anti-apoptotic function (maybe in concert with other cellular factors). This is a very attractive theory, since it directly connects an anti-apoptotic kinase to the regulation of the apoptosome at large. Recently, it was reported that caspase-9 is regulated by Akt-phosphorylation (Cardone et al., 1998). While it is disputed whether caspase-9 is a direct target for Akt, the effects of a phosphorylation event are clearly observed. Thus, Htt might provide the answer to that issue and maybe represents the missing link between Akts anti-apoptotic kinase activity and regulation of caspase-9. Unfortunately, the described experimental approach fails to answer that question due to problems with recombinant Htt.

4 Materials and Methods

4.1 Production of recombinant proteins

The wtHtt fragment was flanked with EcoRI/NotI restriction sites by PCR using KlenTaq-Advantage polymerase (Clontech) and subcloned into pet30a (Novagen) in frame downstream of a His_6-/S-tag. The construct was verified by sequencing (University of Michigan Sequencing Core Facilities) and by *in vitro* transcription/translation using the TNT-Quick kit (Promega). The pet-caspase constructs were described earlier.

Recombinant protein was produced in *E. coli* strain pLysS by induction with 1mM IPTG for two hours (30min for the caspase constructs). Bacteria were lysed in buffer containing 50mM NaH_2PO_4, 300mM NaCl and 10mM imidazole by sonication (six burst of ten seconds) and Talon Ni-beads (Clontech) were added to the supernatant. After one hour incubation, beads were collected and washed five times in lysis buffer adjusted to 20mM imidazole. Beads were eluted in lysis buffer adjusted to 250mM imidazole. Samples of the eluates were separated on SDS-PAGE and either stained with Coomassie brilliant blue (BioRad) or analyzed by Western blot using α-Htt or α-caspase-9 antibody. Eluates containing significant amounts of recombinant proteins were pooled and concentrated using Centricon YM 50,000 or YM 30,000 columns, respectively.

Recombinant Htt was subsequently dialyzed overnight against buffer A with two changes of buffer.

4.2 Binding essays

Recombinant Htt and caspases were added together and diluted tenfold in buffer A to counter any negative effects of the bacterial lysis buffer. In some experiments, dilution was carried out using NP-40 lysis

buffer. To determine, whether cellular proteins facilitate interaction, ST14A S-100 extracts, naive or pre-activated for 30min at 37˚C with cytochrome c, were added to the binding reaction. Immunoprecipitations were then carried out using the indicated antibodies for four hours. Immunocomplexes were separated on SDS-PAGE and analyzed by Western blot.

4.3 In vitro kinase assays

GST-wtAkt and GST-Akt-KD (a kind gift of A.B. Voijtek) were transfected into 293 cells. Cells were harvested 24 hours later and the kinase purified using glutathione-sepharose 4B beads (Pharmacia). After 4 hour incubation, beads were washed four times in kinase buffer (30mM HEPES, pH 7.4, 7mM $MnCl_2$, 5mM $MgCl_2$, 1mM DTT). Kinase assay was performed on beads in kinase buffer with 18μM ATP and trace amounts of ^{32}P-γ-dATP for 30min at 30˚C with agitation. Reactions were separated on SDS-PAGE and the dried gel was autoradiographed.

5 Figures and Legends

Figure 1

(A) S-tag/His_6-wtHtt fusion protein was produced by *E. coli* transformed with the bacterial expression vector. The bacterial lysates were then incubated with Ni-beads and eluted with 250mM imidazole. Aliquots of each fraction were separated on SDS-PAGE and analyzed by Western blot using α-Htt antibody. Good induction of the fusion protein was seen after IPTG treatment for two hours. Binding of the fusion protein to and elution from Ni-beads was not complete. Eluate fractions with α-Htt activity were pooled and concentrated using a Centricon YM 50,000 column.

(B) The indicated recombinant proteins were diluted 1:10 in buffer A and immunoprecipitations were performed using Htt and caspase-9 antibodies as shown below the figure. After separation on SDS-PAGE, Western blot analysis was performed using α-Htt and α-caspase-9 antibodies, respectively. Cross-reactivity of either antibody with the non-specific protein was tested and not observed. No significant interaction of wtHtt with caspase-9 was observed under these conditions.

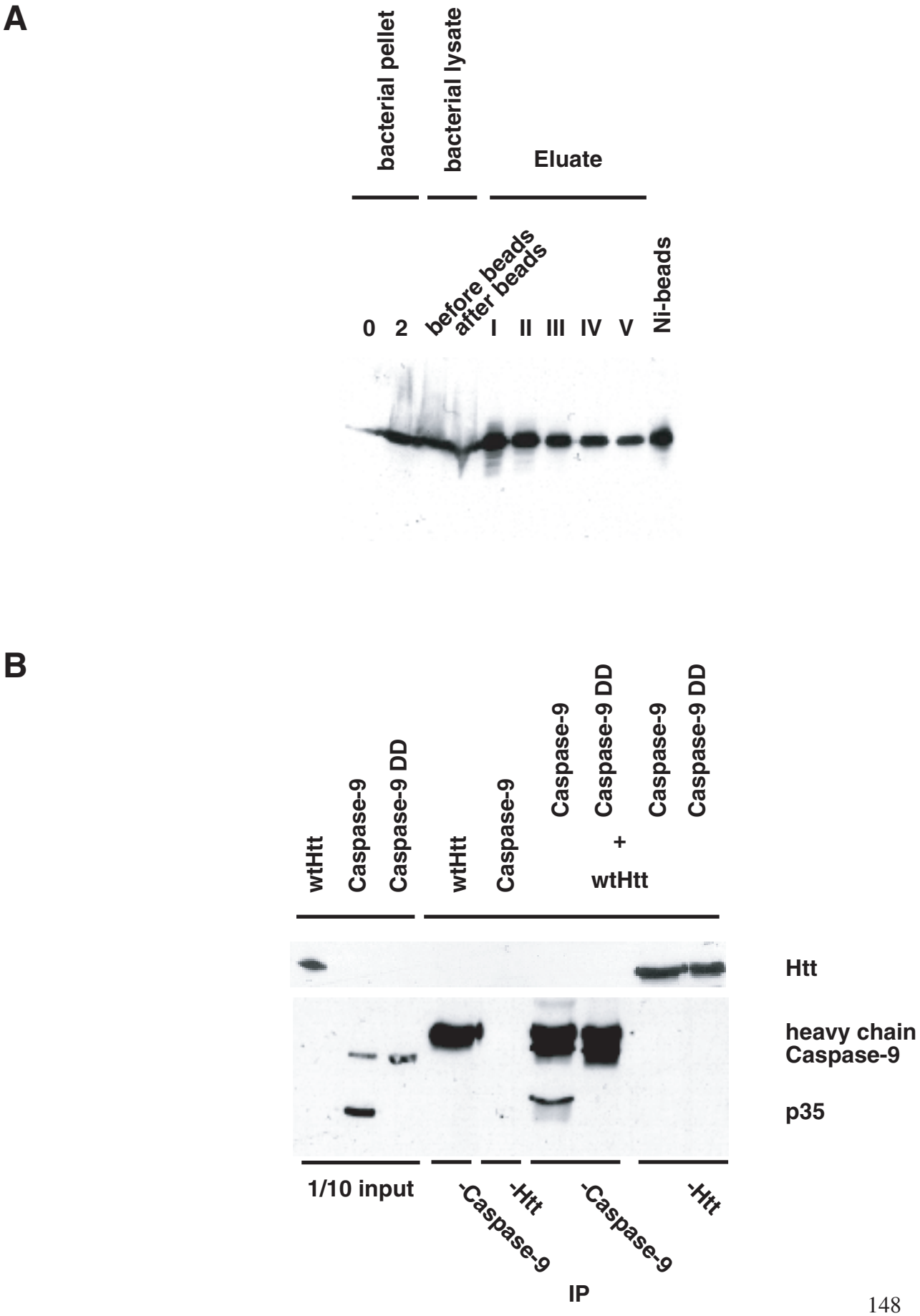
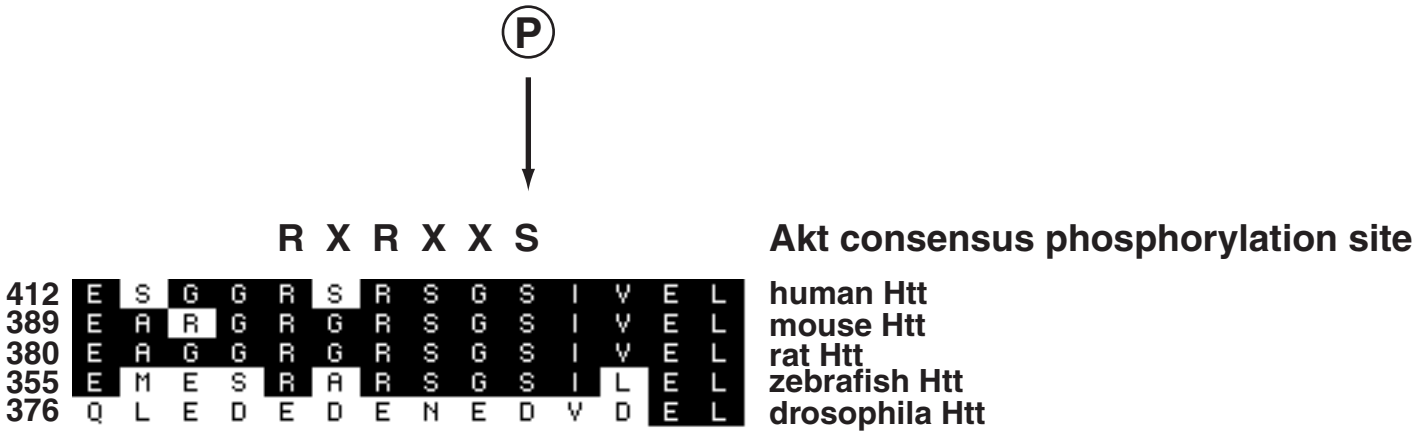

A
bacterial pellet
bacterial lysate
Eluate
0
2
before beads
after beads
I
II
III
IV
V
Ni-beads
B
wtHtt
Caspase-9
Caspase-9 DD
wtHtt
Caspase-9
Caspase-9
Caspase-9 DD
+
wtHtt
Caspase-9
Caspase-9 DD
Htt
heavy chain
Caspase-9
p35
1/10 input
-Caspase-9
-Htt
-Caspase-9
-Htt
IP

Figure 2

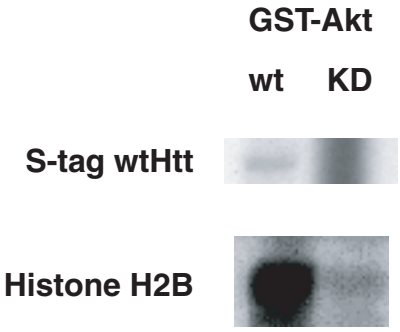
(A) Four of five Htt proteins identified by public database search show a conserved phosphorylation site for the protein kinase Akt (consensus: RXRXXS). The drosophila homologue lacks this site. This is not surprising since this protein is a poor homologue that misses the poly-Q stretch.

(B) GST-Akt fusion proteins were expressed in HEK293 cells and purified using glutathione-beads. The Akt constructs used were either the wildtype or a kinase deficient K-D substitution at the active site of the enzyme. Freshly prepared Akt was then used in *in vitro* kinase assays with recombinant wtHtt and Histone H2B as a control substrate. Weak phosphorylation was observed with recombinant Htt. High background smear, but no specific bands were observed with Akt-KD.

A


P
R X R X X S
Akt consensus phosphorylation site
412 E S G G R S R S G S I V E L human Htt
389 E A R G R G R S G S I V E L mouse Htt
380 E A G G R G R S G S I V E L rat Htt
355 E M E S R A R S G S I L E L zebrafish Htt
376 Q L E D E D E N E D V D E L drosophila Htt

B


GST-Akt
wt KD
S-tag wtHtt
Histone H2B

6 References

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