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CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 12 (2007) pp 435 - 447
*http://www.cmbl.org.pl*

DOI: 10.2478/s11658-007-0014-1

Received: 04 August 2006
Revised form accepted: 26 January 2007
Published online: 15 March 2007



Short communication

# THE CFTR-DERIVED PEPTIDES AS A MODEL OF SEQUENCE-SPECIFIC PROTEIN AGGREGATION

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**Abstract:** Protein aggregation is a hallmark of a growing group of pathologies known as conformational diseases. Although many native or mutated proteins are able to form aggregates, the exact amino acid sequences involved in the process of aggregation are known only in a few cases. Hence, there is a need for different model systems to expand our knowledge in this area. The so-called *ag* region was previously found to cause the aggregation of the C-terminal fragment of the cystic fibrosis transmembrane conductance regulator (CFTR). To investigate whether this specific amino acid sequence is able to induce protein aggregation irrespective of the amino acid context, we altered its position within the CFTR-derived C-terminal peptide and analyzed the localization of such modified peptides in transfected mammalian cells. Insertion of the *ag* region into a different amino acid background affected not only the overall level of intracellular protein aggregation, but also the morphology and subcellular localization of aggregates, suggesting that sequences other than the *ag* region can substantially influence the peptide's behavior. Also, the introduction of a short dipeptide (His-Arg) motif, a crucial component of the *ag* region, into different locations within the C-terminus of CFTR lead to changes in the aggregation pattern that were less striking, although still statistically significant.

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Abbreviations used: CFTR – cystic fibrosis transmembrane conductance regulator; GFP – green fluorescence protein; HA –hemagglutinin antigen; NBF2 – nucleotide-binding fold 2

Thus, our results indicate that even subtle alterations within the aggregating peptide can affect many different aspects of the aggregation process.

**Key Words:** Protein aggregation, Conformational diseases, CFTR, Site-directed mutagenesis

## INTRODUCTION

The ability of a protein to perform a physiological function depends to a significant extent on its tertiary structure forming correctly during the process of protein folding. Although misfolded proteins should be degraded by the ubiquitin/proteosome system, some stable intermediates are able to avoid the degradation pathway, and can go on to form intra- or extracellular protein aggregates. Such protein aggregation is a characteristic feature of many diseases affecting humans (e.g. Alzheimer's disease and Huntington's disease) and other animals (e.g. bovine spongiform encephalopathy) [1]. These conformational diseases constitute a great challenge to biomedical science, as their molecular pathogenesis is not well understood and no effective cures are available.

Numerous studies have indicated that different polypeptides use common mechanisms to form aggregates. As a consequence, many unrelated proteins can accumulate in similar aggregation-related structures, with extracellular amyloid fibrils and intracellular aggresomes being the best known examples [2, 3]. On the other hand, certain features of the aggregation process seem to be specific to particular proteins or even to particular amino acid sequences. For instance, elongated polyglutamine stretches are not only responsible for the disease-related aggregation of several human proteins, but are apparently also involved in the intracellular sequestration of polyglutamine-containing transcription factors and other crucial cellular proteins, which may additionally contribute to disease-specific clinical symptoms [4-9]. It has also been demonstrated that sequences other than the polyglutamine repeats determine the specific nuclear or cytoplasmic localization of aggregates formed by mutated huntingtin, a protein critical for the pathogenesis of Huntington's disease [10-12].

As the number of similar examples increases, protein aggregation is becoming perceived as a more sequence-specific phenomenon. However, the exact amino acids contributing to this specificity are known in only a few cases [13-16]. This exposes the need for new protein models that would facilitate the exploration of the sequence-specific mechanisms governing the aggregation process.

Cystic fibrosis transmembrane conductance regulator (CFTR), a protein that is defective in cystic fibrosis, is commonly used to study protein aggregation [17-22]. Although the aggregation of CFTR seems not to be causally related to the pathogenesis of cystic fibrosis, it was the first protein for which the formation of aggresomes was described [17]. It was also demonstrated that intracellular aggregation of the CFTR-derived C-terminal peptides depends on the presence of a nine-amino acid sequence, termed the *ag* region [22, 23]. The existence of such a relatively simple peptide model provides a unique opportunity to study

the contribution of different amino acids to the aggregation process. Therefore, we used this model system to investigate to what extent the background of the *ag* region within the CFTR C-terminal sequence can influence the peptide's ability to form intracellular aggregates.

## MATERIALS AND METHODS

### Construction of expression plasmids

The construction of the eukaryotic expression plasmids encoding the CFTR C-terminal fragment (a.a. 1370-1480 of the full-length protein), fused to the green fluorescence protein (GFP) or tagged with the hemagglutinin (HA) epitope, was described elsewhere [23]. The introduction of the deletion encompassing amino acids 1395-1403 (Δ*ag*) of CFTR was also previously described [23].

The Transformer site-directed mutagenesis system (Becton Dickinson) was applied to introduce the *ag* region or the HR motif into different positions within the CFTR C-terminal sequence. The sequences of all the oligonucleotide primers used to create these insertions are available upon request. The nucleotide sequence of the newly created constructs was verified by DNA sequencing.

### Cell culture and transient transfections

The IB3-1 cells (immortalized bronchial epithelial cells derived from a patient with cystic fibrosis [24]) were cultured in LHC-8 medium (BioSource) containing 5% FBS (Gibco). These cells lack functional CFTR [24] and do not express detectable amounts of mutant CFTR, as measured by immunoprecipitation followed by radioactive *in vitro* phosphorylation [25 and our unpublished results]. For routine culture, the cells were grown in polystyrene flasks (Sarstedt) coated with collagen (Cohesion), in 5% $CO_2$-balanced air at 37ºC. For protein aggregation analysis, the cells were grown on collagen-coated glass coverslips in 6-well culture plates (Sarstedt). After reaching 60-80% confluency, the cells were transiently transfected with Lipofectin (Invitrogen), in accordance with the manufacturer's instructions. Twenty-two hours after transfection, the cells were fixed with 4% PFA (Sigma) for 20 minutes. If immunostaining was not necessary (as for the GFP-tagged constructs), the cells were mounted in SlowFade (Molecular Probes) containing 0.05 mg/ml of DAPI (Sigma). For Western blot analysis, the cells were grown in 6-well plates and harvested 22 hours after transfection.

### Western blot analysis

To assess the protein level in the transfected IB3-1 cells, the cells were lysed overnight in 1% NP-40, 150 mM NaCl, 1 mM EDTA, 20 mM Hepes, pH 7.0. The lysates were separated by SDS-PAGE (12%) and electrophoretically transferred to PVDF membranes (Amersham Pharmacia Biotech). The probing with monoclonal anti-GFP antibody (Becton Dickinson) and subsequent detection with the ECL+plus system (Amersham Pharmacia Biotech) were performed according to manufacturers' instructions.

**Immunofluorescent staining**

The HA-tagged peptides were detected using monoclonal (12CA5, Roche) or polyclonal (SG77, Zymed Laboratories Inc.) anti-HA antibodies. The mitochondrial marker Grp75 was detected with the anti-Grp75 antibody (SPA-825, Stressgen). The transfected cells were fixed in 4% PFA for 20 minutes, washed with PBS, and permeabilized with 0.1% Triton X-100 (Sigma) for 3 minutes, and then immunostained. Non-specific binding was blocked by incubation with 2.5% goat serum (Sigma) in PBS for 30 minutes. Immunostaining was carried out in two steps separated by a short PBS wash. The cells were first stained with the primary antibody for 1.5 hours and then with the proper secondary antibody, conjugated to Cy3 or FITC (Sigma), for 30 minutes. The coverslips were then washed with PBS and mounted in SlowFade with DAPI.

**Fluorescence microscopy and statistical analysis**

Cells were counted and images were taken using the Eclipse E400 (Nikon) fluorescence microscope. The CytoVision 2.75 software (Applied Imagining Corp.) was used to process the images.

To estimate the percentage of cells with aggregates, at least 150 transfected cells were analyzed in multiple random visual fields on each slide in quadruplicate. To estimate the frequency of occurrence of the different morphological forms of aggregates, at least 50 transfected cells were counted in multiple random visual fields on each slide in quadruplicate. For analysis of aggregates' morphology, the protein depositions in cells expressing the GFP-tagged peptides were classified as aggregates of either compact or loose structure. The aggregates in cells expressing the HA-tagged peptides with the *ag* region in a different position were classified as being: (1) very small and numerous (> 20 per cell); (2) medium-sized and less numerous (5-20 per cell); or (3) very large but scarce (< 5 per cell). Since some cells showed the presence of both small and large protein depositions, they were counted only once as having large aggregates. Such a classification of aggregates was not performed for the Δ*ag* peptides, which rarely aggregated. Additionally, their infrequent aggregates showed no distinct morphology or localization pattern. The aggregates formed by the HA-tagged peptides modified by the introduction of the HR motif were classified as: (1) very small and dispersed throughout the cytoplasm, (2) very small and concentrated in one region within the cytoplasm, (3) medium-sized cytoplasmic, (4) large cytoplasmic, or (5) nuclear. The investigators worked blind, not knowing the identity of constructs used for transfection.

The standard $chi^2$ test was used to estimate the statistical significance of the observed differences in the frequencies of particular localization patterns. Additionally, the Cramer's V coefficient was used as a measure of the strength of the overall association between different positions of the *ag* region/HR motif and the aggregation pattern. For this analysis, we compared four different positions of the *ag* region: the native one and three positions generated by the

mutagenesis. Only three positions, all generated by mutagenesis, were compared in the case of the HR motif. Statistical significance was assumed at $P < 0.05$.

## RESULTS AND DISCUSSION

### The position of the *ag* region affects the peptide's ability to aggregate

To examine first the intrinsic potential of the CFTR-derived *ag* region to promote protein aggregation, we tested whether this short 9-amino acid sequence alone is able to cause aggregation when attached to a soluble non-CFTR protein. The *ag* sequence (C-T-V-I-L-C-E-H-R; a.a. 1395-1403 of CFTR) was fused directly to the C-terminus of GFP, as is commonly practiced when fusing this green fluorescence reporter protein to other polypeptides. By contrast to the non-aggregating GFP protein, the GFP-*ag* fusion formed large cytoplasmic aggregates of irregular shape in 13.4 ± 2.1% of transiently transfected IB3-1 cells ($P < 0.05$). This indicated that the *ag* region is indeed able to induce protein aggregation when present in the non-native amino acid context.

To further explore the ability of the *ag* region to induce protein aggregation, we tested whether the position of this region within the C-terminus of CFTR would affect the aggregation rate of the CFTR-derived peptides. A series of GFP- or HA-tagged constructs was created in which the *ag* region was deleted from the native position and introduced into one of the three new locations within the CFTR C-terminal sequence (Fig. 1A and 1B). The first of these locations (*ag*1) fell within the predicted C-terminal part of the second nucleotide-binding fold (NBF2) [26], a domain also encompassing the native *ag* region and disrupted by the creation of the CFTR C-terminal peptides. In the second position (*ag*2), the *ag* region was placed within the unstructured region between the NBF2 domain and the C-terminus. Finally, when present in the third position (*ag*3), the *ag* region extended the carboxyl terminus of CFTR, thus masking the native C-terminal PDZ-binding motif (D-T-R-L>) [27].

Human IB3-1 cells were transiently transfected with plasmids expressing the above constructs and analyzed for the presence of protein aggregates. All the constructs were expressed at very similar levels, as could be judged from the similar transfection rates and comparable results of Western blot analysis. In agreement with previously published data [22], the removal of the *ag* region (Δ*ag*) significantly reduced the aggregation rate of the CFTR-derived peptides (Fig. 1C). Importantly, the subsequent insertion of the *ag* region into a new position always resulted in a substantial increase in the peptide's aggregation rate, relative to the Δ*ag* constructs of the corresponding series (GFP- or HA-tagged). This suggested that this short amino acid sequence may indeed have some intrinsic potential to induce aggregation. However, the insertion of the *ag* region did not always lead to the full restoration of the peptide's original ability to aggregate. Complete or nearly complete recovery was obtained when the *ag* region was introduced into the *ag*1 or *ag*3 positions. By contrast, the

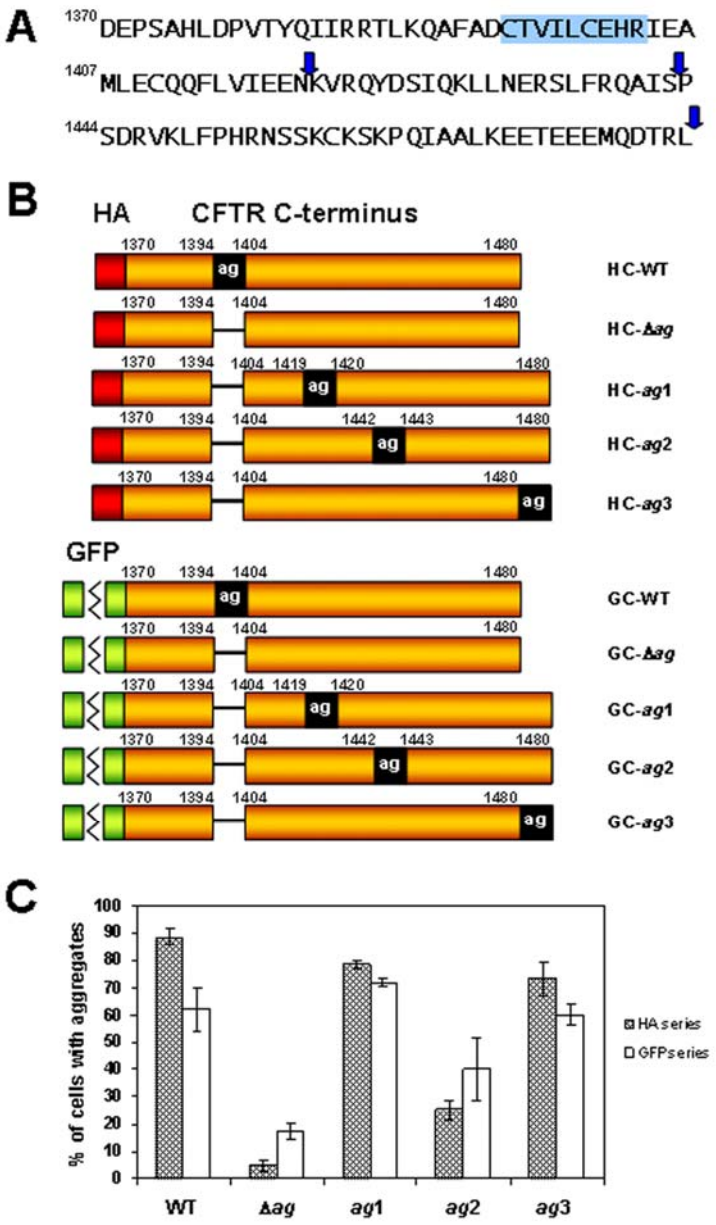


Fig. 1. The effect of the position of the *ag* region on the aggregation rate of CFTR-derived C-terminal peptides. A – The amino acid sequence of the C-terminal CFTR fragment (a.a. 1370-1480 of the full-length CFTR) with the *ag* region highlighted in blue and the additional *a*g insertion sites indicated by arrows. The sequence includes the variant methionine at position 1475. B – CFTR C-terminal constructs, tagged with HA or GFP, and differing in the position of the *ag* region. C – The percentage of aggregate-containing cells among the transfected cells expressing the modified CFTR-derived peptides.

aggregation level of the *ag*2 peptides of both series was significantly lower than in the case of other constructs containing the *ag* region. This indicated that the amino acid background may substantially influence the ability of this region to induce protein aggregation.

The overall association between the four different positions of the *ag* region and the peptide's ability to aggregate was evident for both series of constructs ($P < 0.001$). However, this association was markedly stronger for the HA-tagged ($V = 0.52$) than for the GFP-tagged ($V = 0.23$) peptides. Numerous studies have reported that GFP may influence the aggregation process when fused to other proteins [9, 15, 22, 28]. Thus, it seems likely that the presence of GFP enhanced the *ag*-independent mechanism of protein aggregation, which in turn weakened the observed relationship between the position of the *ag* region and the peptide's aggregation rate.

**Aggregates formed by modified peptides show different morphologies**

Previous studies have shown that fusing a reporter protein to an aggregating peptide may affect the subcellular distribution and morphology of aggregates [22]. Therefore, we tested whether changing the position of the *ag* region within the CFTR-derived sequence would lead to similar alterations in the aggregation pattern.

The analysis of the morphology of intracellular aggregates in cells expressing the modified HA-tagged peptides revealed that only the HC-*ag*2 peptide predominantly accumulated in small and numerous (> 20 per cell) aggregates resembling those formed by the wild type construct (HC-WT) (Fig. 2A). This similarity included not only the size and the overall distribution of the aggregates within the cell, but also their previously reported association with the mitochondria (data not shown) [22]. On the other hand, two other modified peptides, HC-*ag*1 and HC-*ag*3, showed significantly different aggregation patterns. The HC-*ag*1 peptide often accumulated in large perinuclear aggregates that were much less frequent in cells expressing the wild-type peptide ($38.2 \pm 3.3$ and $7.5 \pm 1.0\%$, respectively, $p < 0.001$). An even more unique aggregation pattern was associated with the HC-*ag*3 construct, accumulating almost exclusively ($97.5 \pm 2.5\%$ of all cells with aggregates) in aggregates of medium size and uniform spherical shape (Fig. 2A). These cytoplasmic aggregates were usually less numerous (10-20 per cell) than the previously described small aggregates, and unlike them, showed no association with the mitochondria (data not shown). Both the above-described predominant forms of aggregates formed by the HC-*ag*1 and HC-*ag*3 peptides were frequently accompanied by small nuclear deposits (Fig. 2A).

By contrast to the HA-tagged peptides, different peptides fused to GFP displayed a similar aggregation pattern (Figs 2B and 2C). Most of the cells transfected with particular GFP-tagged constructs showed the presence of large protein inclusions of spherical or irregular shape, localized in the cytoplasm

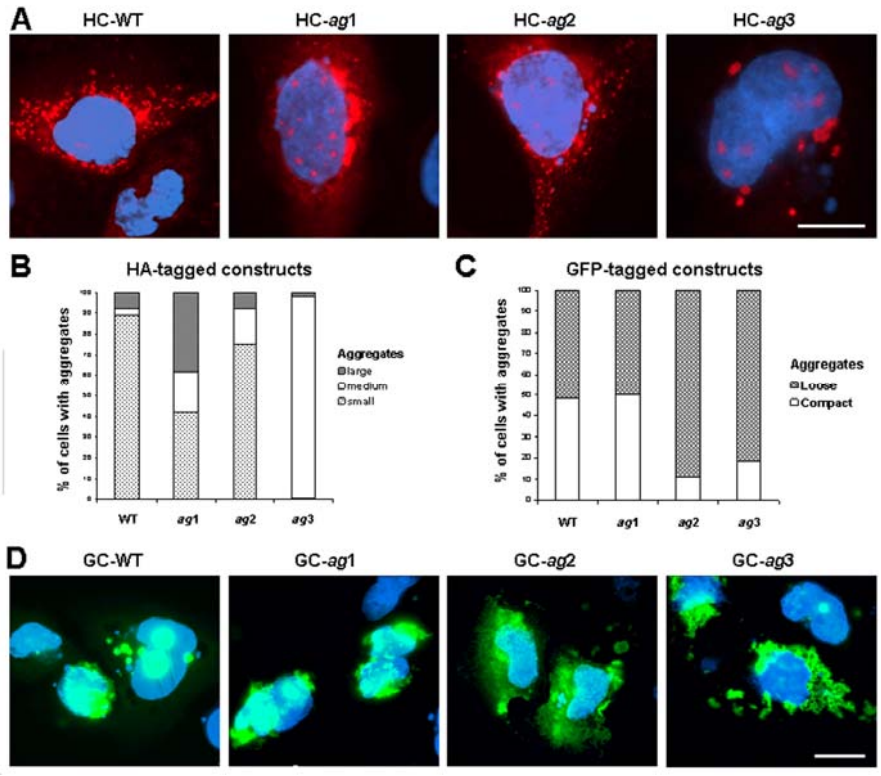


Fig. 2. The morphology and localization of aggregates formed by modified CFTR-derived peptides. A – The subcellular distribution of peptides tagged with the HA epitope (red) in transiently transfected IB3-1 cells. The cells were counterstained with DAPI (blue). Bars, 10 μm. B and C – Frequencies of different types of aggregates in cells transfected with HA-tagged (B) or GFP-tagged (C) constructs. D – The subcellular distribution of peptides fused to GFP (green) in transiently transfected IB3-1 cells. The cells were counterstained with DAPI (blue). Bars, 10 μm.

(mostly in the perinuclear region) or occasionally inside the nucleus. This aggregation pattern was previously found to be associated with the presence of GFP, suggesting that the contribution of GFP determines the aggregates' morphology, superseding any additional *ag*-related position effect. However, there were some subtle differences regarding the relative compactness of aggregates formed by particular constructs (Figs 2C and 2D). While about half of the aggregate-containing cells expressing the GC-WT or GC-*ag*1 peptides (48.7 ± 1.9 and 49.8 ± 4.9%, respectively) exhibited the presence of densely structured aggregates, such dense inclusions were significantly less frequent in cells expressing other GFP-tagged constructs. Instead, most aggregates formed by the GC-*ag*2 or GC-*ag*3 peptides (88.8 ± 5.0% and 81.7 ± 3.0% of the aggregate-containing cells, respectively) showed relatively loose and partially

dispersed structure (Fig. 2D), suggesting that despite the predominant impact of the GFP protein, the position of the *ag* region within the CFTR-derived sequence may also contribute to the morphology of the aggregates.
Summarizing the above observations, the morphology of aggregates formed by the *ag*1-3 peptides of both series showed significant associations with the position of the *ag* region within the CFTR-derived sequence ($P < 0.001$). This overall relationship was stronger for the HA ($V = 0.64$) than for the GFP series ($V = 0.35$), suggesting again that the GFP fusion may additionally influence the aggregation process.

**Insertion of the HR motif affects the aggregation pattern**
The histidine-arginine (HR) motif within the *ag* region was previously shown to be critical for the aggregation of the CFTR C-terminus [22]. To investigate whether this dipeptide motif alone could increase the aggregation rate when present in a different amino acid background, we altered its position within the CFTR C-terminus. Similarly to the strategy used for the whole *ag* region, the HR motif was inserted into three different positions, replacing amino acids 1419 and 1420 (HR1), 1442 and 1443 (HR2) or 1479 and 1480 (HR3) of the HA- or GFP-tagged peptide devoid of the *ag* region.

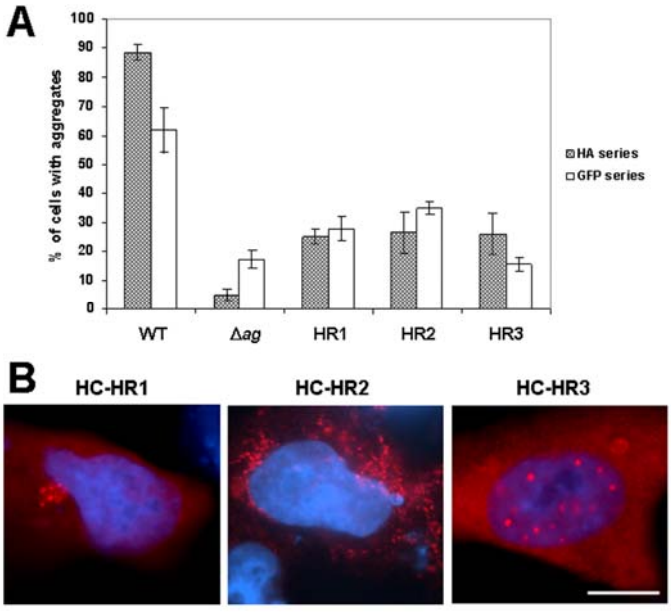


Fig. 3. The influence of the HR motif on the aggregation of CFTR-derived peptides. A – The aggregation rates of different CFTR C-terminal peptides, modified by insertion of the HR motif, in transiently transfected IB3-1 cells. B – The subcellular distribution of the modified HA-tagged peptides (red), differing with respect to the position of the HR motif. Transiently transfected IB3-1 cells were counterstained with DAPI (blue). Bars, 10 μm.

The effect of the HR insertions on the peptides' ability to aggregate in transfected IB3-1 cells was noticeably different from the outcome produced by the *ag* insertions. The overall aggregation level of the HR1, HR2 and HR3 peptides was never as high as in the case of the original peptide with the intact *ag* region (Fig. 3A). However, with the one exception of the GFP-tagged HR3 construct, the aggregation level was significantly higher than in the case of the peptide modified by the deletion of the *ag* region only. Together, these results indicated that the HR motif alone, when extracted from the context of the *ag* region, shows only limited ability to induce protein aggregation following its insertion into a non-native amino acid background.

Although having only a moderate influence on the peptide's overall ability to aggregate, the insertion of the HR motif was still able to significantly alter the subcellular distribution of protein aggregates, at least in the case of peptides that were not fused to GFP (Fig. 3B). For example, in most (53.3 ± 2.3%) aggregate-containing cells expressing the HC-HR1 construct, the modified peptide accumulated in small aggregates concentrated in one region within the cytoplasm (Fig. 3B). By contrast, the HC-HR3 peptides aggregated predominantly (61.3 ± 6.1%) within the nucleus, where they formed small discrete accumulations (Fig. 3B). Only the HC-HR2 construct was able to form the small mitochondria-associated aggregates typical for the wild type peptide (data not shown). These examples clearly indicate that even relatively subtle changes within the amino acid sequence of the aggregating peptide may substantially influence the subcellular localization of the aggregates.

**Concluding remarks**

Our results demonstrate that the *ag* region shows remarkable potential to induce protein aggregation, as can be judged from its ability to increase the aggregation rate following insertion into a new amino acid context. However, the position of this region within the aggregating peptide may influence the aggregation process, suggesting that the ability of the *ag* region to induce aggregation has certain natural limits. This is supported by the fact that when present in the native position within the NBF2 domain of CFTR, the *ag* region does not cause protein aggregation as long as the integrity of the whole domain is preserved. In our studies, the position associated with a decreased aggregation rate (*ag*2, between a.a. 1442 and 1443) corresponds to the presumably unstructured fragment of CFTR, suggesting that the *ag* region causes stronger protein aggregation when present within this relatively highly structured non-native background. Insertion of the *ag* region could introduce a structural element that disrupts a local fold and exposes neighboring aggregation-prone sequences that are normally buried inside the molecule.

It is noteworthy that not only the overall aggregation rate but also the morphology and subcellular distribution of aggregates can be affected by even subtle modifications within the protein's amino acid sequence. Many studies on the intracellular aggregation of huntingtin and other polyglutamine-containing

proteins have indicated that the localization of protein aggregates is critical for their toxicity [29-33]. However, little is known about the sequences in disease-related proteins that modulate the localization of protein aggregates. Identifying these sequences and the mechanisms responsible for targeting protein aggregates to different subcellular compartments should facilitate designing new therapies aimed at directing the aggregating protein to locations associated with lower toxicity.

**Acknowledgments.** This study was supported by a grant from State Committee for Scientific Research (PBZ/KBN/042/P05/06 and 2 P05A 157 29), Poland.

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