Short Technical Reports

Specific Inhibitors Prevent Proteolytic Degradation of Recombinant Proteins Expressed in High Five™ Cells

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ABSTRACT

The insect cell line BTI-TN-5B1-4 (High Five™) is frequently used to express recombinant proteins in large amounts using the baculovirus expression system. However, extensive proteolytic degradation of recombinant proteins is often encountered. Furthermore, we have observed that recombinant proteins migrate in SDS-PAGE in agreement with poly-ubiquitinated forms of the protein, suggesting a ubiquitin/proteasome degradation pathway. Here, we describe a systematic study unraveling the effect of adding proteasome inhibitors or specific protease inhibitors to the growth medium of High Five insect cells infected with recombinant baculovirus. Furthermore, protease inhibitors were added to the lysis buffer to establish the most efficient way to inhibit proteolytic activity after lysis of baculovirus-infected cells expressing recombinant proteins. We conclude that a combination of adding protease inhibitors to the growth medium and to the lysis buffer minimizes the proteolytic activity in High Five cells. The most efficient protease inhibitors were E-64 in the growth medium together with Leupeptin in the lysis buffer at concentrations higher than with available cocktails of inhibitors. The optimal treatment of High Five cells is different from the optimal treatment of Sf9 cells. For proteins susceptible to ubiquitinylation, a treatment of insect cell cultures with the proteasome inhibitor MG132 (LLL) leads to a considerable reduction of the yield of production of recombinant protein.

INTRODUCTION

Extensive proteolytic degradation of recombinant proteins produced in the insect cell line BTI-TN-5B1-4 (High Five™ cells; Invitrogen, Groningen,

Holland) using the baculovirus expression system is a problem associated with most recombinant proteins (4). The high yield of protein produced in High Five cells was hampered because 20%–50% of the protein was degraded in spite of using cocktails of protease inhibitors (Complete™; Roche Molecular Biochemicals, Hvidovre, Denmark). We therefore wanted to examine the possibility of increasing the fraction

of full-length recombinant protein produced in High Five cells by adding specific protease inhibitors to the growth medium and to the lysis buffer. In addition, we have observed that a ladder of proteins with higher molecular weight than the human tryptophanyl-tRNA synthetase (hWRS) recombinant protein expressed in High Five cells appeared in immunoblot analyses, indicating the existence of recombinant

Figure 1. Immunoblot analysis of recombinant proteins from High Five cells after addition of LLL to the growth medium. High Five or Sf9 cells were infected with p46 or hWRS recombinant baculovirus, as indicated. One hour after infection, LLL was added to the growth medium to the final concentrations indicated. Full-length recombinant proteins are marked by arrows. Degradation products are marked with arrowheads. % deg indicates percentage of total proteolytic degradation products compared to amount of full-length protein. % FL indicates percentage of the amount of full-length protein (* is set to 100%). Pre-stained low molecular weight markers from Sigma were used.

Short Technical Reports

Table 1. Proteasome and Protease Inhibitors

Inhibitor	Description	Stock Solution
LLL	Z-Leu-Leu-Leu-CHO	50 mM in DMSO
ALL	N-Acetyl-Leu-Leu-Met-CHO	50 mM in DMSO
Lactocystin	<i>clasto</i> -Lactocystin β -lactone	5 mM in DMSO
Leupeptin		1 mM in water
E-64	N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatin	20 mg/mL in EtOH:water (1:1)
Chloroquine		10 mM in water
TPCK	tosyl-phenylalanin chloromethyl keton	20 mg/mL in water
Pepstatin		1 mM in water
Pefabloc SC	4-(2-Aminoethyl)-benzenesulfonyl-fluoride, HCl (AEBSF)	10 mM in water
Complete	Cocktail of inhibitors	
P8340	Inhibitors for mammalian cell extracts	
P8849	Inhibitors for poly(Histidine)-tagged proteins	
LLL and Lactocystin were purchased from Affiniti (Exeter, UK), E-64 and Pefobloc SC from Roche Diagnostics (Hvidovre, Denmark), ALL and TPCK from Sigma, and Pepstatin and Leupeptin from Calbiochem (Bie & Berntsen, Rødovre, Denmark).		

Short Technical Reports

proteins attached to poly-ubiquitin. We thus wanted to test the effect of adding specific proteasome inhibitors on proteolytic degradation of recombinant proteins in insect cells. Even though a proteasome has not been described in insect cells, ubiquitinylation of proteins has been observed (11), and baculovirus encode a viral ubiquitin (6), which is not essential for viral replication (12).

Two different recombinant baculoviruses were used infecting both High Five and Sf9 cells to monitor differences between proteins and between cell lines. The recombinant non-secret-

ed proteins tested were p46, a splice variant of the human enzyme 2',5' oligoadenylate synthetase (OAS1) (1), and hWRS (3). The aim of the present study has been to find a general method to avoid extensive degradation of non-secreted recombinant proteins expressed in High Five cells.

MATERIALS AND METHODS

Recombinant *Autographica californica* baculoviruses were constructed using the BaculoGold™ transfection

method (BD Biosciences, Brøndby, Denmark). The hWRS cDNA was cloned in the *EcoRI/SmaI* sites in pVL1392 and the p46 cDNA in the *EcoRI* site in pVL1393. Recombinant baculoviruses were plaque-purified twice and amplified using Sf9 cells grown in BaculoGold protein-free insect medium supplemented with 5% fetal calf serum (Life Technologies, Roskilde, Denmark) and 100 IU/mL penicillin and 100 µg/mL streptomycin (Life Technologies). High Five cells were grown in the same medium without serum. Both Sf9 and High Five cells were maintained as suspension cultures in rollerflasks (Corning, Biotech Line, Lyngby, Denmark) circulating at 12 rpm to prevent cell attachment.

Sf9 or High Five cells were seeded at 1.2×10^6 cells/well in a six-well dish (Nalge Nunc International, Roskilde, Denmark) and left to attach (9). The cells were infected with 150 µL recombinant virus of a high titer (5×10^8 to 5×10^9 pfu/mL) for 1 h. Medium was changed, and the cells were grown for 72 h, lysed in 1% Nonidet® P40 in buffer E [20 mM Tris-HCl, pH 7.5, 10 mM KOAc, 10 mM Mg(OAc)₂, and 1 mM EDTA], and total protein concentrations were measured by the BCA method (Pierce, Bie & Berntsen, Rødovre, Denmark). Proteasome or pro-

Figure 2. Immunoblot analysis of recombinant proteins from High Five cells after the addition of ALL to the growth medium. High Five or Sf9 cells were infected with p46 or hWRS recombinant baculovirus, as indicated. One hour after infection, ALL was added to the growth medium to the final concentrations indicated. Markings are the same as described in the legend to Figure 1.

Figure 3. Immunoblot analysis of recombinant p46 from High Five cells after the addition of protease inhibitors to the lysis buffer. Specific inhibitors added to the lysis buffer are marked. DMSO was added as control. Position of full-length recombinant p46 is marked with an arrow, and the degradation product is marked with an arrowhead. Pre-stained low molecular weight markers from Sigma were used.

Short Technical Reports

tease inhibitors (Table 1) were added to the medium at different time points or added to the lysis buffer. The cell extracts were cleared by centrifugation, and 10- μ g aliquots were applied to 10% SDS-PAGE, followed by electrophoretic transfer to a PolyVinylidene Difluoride membrane (Millipore, Glostrup, Denmark). Immunoblot analyses were carried out using specific primary antibodies, a peptide antibody recognizing p46 (2), and a polyclonal antibody raised against purified hWRS protein (3). Horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Sigma, Vallensbæk Strand, Denmark) was used as secondary antibody, and the enhanced chemiluminescence (ECL[®]) method from Amersham Pharmacia Biotech (Hørsholm, Denmark) was used for visualization of the antigen/antibody reaction. Quantification of the degraded proteins compared to the amount of full-length protein was done

by scanning X-ray films and using the Quantity One[™] program (Bio-Rad Laboratories, Herlev, Denmark).

RESULTS AND DISCUSSION

We started this series of experiments by adding Z-Leu-Leu-Leu-CHO (LLL, also known as MG132) or N-Acetyl-Leu-Leu-Met-CHO (ALL) at various concentrations to the growth medium. LLL is a specific inhibitor of proteasome activity in eukaryotic cells (10) and was used elegantly to show that phosphorylated STAT-1 is degraded by the proteasome (8). In contrast, ALL is a potent inhibitor of cathepsins and calpains but not of proteasomes. All experiments were carried out using High Five and Sf9 cells in parallel.

When High Five or Sf9 cells were infected with p46 or hWRS baculovirus (Figure 1), the amount of recombinant

protein produced was highly decreased when the growth medium included 25 μ M LLL because all lanes contained the same amount of total protein. Comparing p46 produced in High Five cells to the production in Sf9 cells, it can be seen that the degradation pattern is the same, but 50 mM can be added to the medium of the Sf9 cells before the production of p46 is decreased. The same result was obtained with hWRS production in Sf9 cells (data not shown). In conclusion, addition of LLL to the growth medium leads to substantially reduced production of intact recombinant proteins both for p46 and hWRS in both Sf9 and High Five cells.

A different picture was seen when ALL was added to the medium. The yield of full-length recombinant proteins was diminished approximately 30% after adding 125 μ M ALL to High Five cells (Figure 2). However, it is evident that the addition of ALL inhibits most of

Short Technical Reports

the proteolytic degradation of both hWRS and p46. This is in contrast to the production of p46 in Sf9 cells, where no effect was observed with ALL.

In the search for specific protease inhibitors, we added the following to the growth medium of baculovirus infected High Five cells: 50 μ M Chloroquine leading to a pH increase within the cell; 5 μ M *clasto*-Lactocystin β -lactone (Lactocystin), a specific proteasome inhibitor; and 0.1 mg/mL tosyl-phenylalanine chloromethyl keton (TPCK) inhibiting several serine and cysteine proteases. Addition of Chloroquine had no effect on the proteolytic degradation, whereas addition of both Lactocystin and TPCK dramatically decreased protein production (data not shown). Furthermore, no effect could be detected by the addition of 1 mM Pefabloc SC or 10 μ M Pepstatin to the lysis buffer. However, based on these experiments, we cannot exclude the possibility that High Five cells secrete Pepstatin-sensitive proteases to the serum-free medium, as has been described for Sf21 cells (5).

We also examined two protease inhibitor cocktails from Sigma, P8340 and P8849 (1 μ L/mL) in the lysis buffer. These two cocktails differ by their contents of protease inhibitors; thus, P8340 is made for mammalian cell extracts, whereas P8849 is made for recombinant proteins expressed with histidine tags in *E. coli* or *Spodoptera frugiperda*-derived insect cells (Sf9 or Sf21). At the same time TPCK at 0.1 mg/mL in the lysis buffer was tested. For p46 expressed in High Five cells, both Sigma cocktails and TPCK added to the lysis buffer resulted in a partial inhibition of proteolytic degradation (Figure 3). It can be seen that the Sigma P8849 cocktail made for expression of recombinant proteins is the best with approximately 10% degradation of the recombinant protein.

In a final attempt to inhibit the proteolytic activity in High Five cells, we tested a combination of adding protease inhibitors to the medium and to the lysis buffer. We decided to use E-64, a cysteine protease inhibitor that has been shown to be efficient for proteins expressed in Sf21 cells both by addition to the medium and to the lysis buffer. Hom and Volkman (7) have demonstrated that a specific protease encoded

by the baculovirus AcMNPV used for construction of recombinant viruses could be inhibited by the addition of E-64. These authors reported that E-64 could be substituted with Leupeptin, an inhibitor of serine and cysteine proteas-

es. The result of adding ALL or E-64 to the growth medium in combination with addition of Leupeptin to the lysis buffer is shown for hWRS produced in High Five cells and p46 produced in Sf9 and High Five cells (Figure 4).

Figure 4. Immunoblot analysis of recombinant proteins from High Five and Sf9 cells after the addition of protease inhibitors to the growth medium and the lysis buffer. High Five or Sf9 cells were infected with p46 or hWRS recombinant baculovirus, as indicated. ALL (125 μ M) or E-64 (20 μ g/mL) was added to the medium 24 h after infection. DMSO was added as control. The lysis buffer included 100 μ M Leupeptin (+) or no Leupeptin (-). Full-length recombinant proteins are marked by arrows, and degradation products are marked by arrowheads. Pre-stained low molecular weight markers from Sigma were used.

Short Technical Reports

It can be seen for hWRS expressed in High Five cells that adding Leupeptin to the lysis buffer or adding E-64 to the medium give similar effects because a Leupeptin-sensitive degradation product disappears (compare lanes 1, 3, 4, and 5 to lanes 2 and 6 from Figure 4). However, substantial residual degradation of hWRS is still present, leading to the conclusion that recombinant hWRS is susceptible to proteasome degradation, which cannot be inhibited by conventional protease inhibitors.

For p46 in High Five cells, the best proteolytic inhibition is obtained after adding either ALL to the medium or E-64 in the medium together with Leupeptin in the lysis buffer. However, addition of ALL to the growth medium clearly decreases the amount of intact protein produced by approximately 30%. Addition of Leupeptin to the lysis buffer prohibits the residual proteolytic activity observed after addition of E-64 to the growth medium. This result is in contrast to p46 expressed in Sf9 cells, where ALL in the medium does not

have any effect, while Leupeptin in the lysis buffer or E-64 in the medium prohibit most proteolytic degradation (compare lanes 6 and 10 with lane 7 from Figure 4). These results obtained with proteins expressed in Sf9 cells are in agreement with previously published data using Sf21 cells (7).

We have demonstrated that adding protease inhibitor cocktails to the lysis buffer after expressing non-secreted recombinant proteins in High Five cells is not adequate to avoid extensive proteolytic degradation of the recombinant protein. Furthermore, we have shown that the proteolytic activities in High Five and Sf9 cell lines are different. For expression in High Five cells, a combination of adding specific protease inhibitors to the medium and to the lysis buffer is necessary. In conclusion, we suggest adding 20 µg/mL E-64 to the growth medium 24 h after infection together with 100 µM Leupeptin in the lysis buffer to avoid extensive degradation of non-secreted recombinant proteins. For recombinant proteins susceptible to proteasome degradation identified by ubiquitinated forms in immunoblots, one might consider using a baculovirus without the viral ubiquitin gene (12) because the addition of proteasome inhibitors dramatically reduces recombinant protein expression controlled by the polyhedrin promoter. How the P10 or other baculovirus promoters respond to the addition of LLL has not been investigated. It is evident that each recombinant protein expressed in High Five cells should be treated individually to avoid extensive proteolytic degradation.

REFERENCES

1. Benech, P., Y. Mory, M. Revel, and J. Chebath. 1985. Structure of two forms of the interferon-induced (2'-5') oligo A synthetase of human cells based on cDNAs and gene sequences. *EMBO J.* 4:2249-2256.
2. Chebath, J., P. Benech, A.G. Hovanessian, J. Galabru, and M. Revel. 1987. Four different forms of interferon-induced 2',5'-oligo(A) synthetase identified by immunoblotting in human cells. *J. Biol. Chem.* 262:3852-3857.
3. Fleckner, J., H.H. Rasmussen, and J. Justesen. 1991. Human interferon gamma potently induces the synthesis of a 55-kDa protein (gamma 2) highly homologous to rabbit peptide chain release factor and bovine tryptophanyl-tRNA synthetase. *Proc. Natl. Acad. Sci. USA* 88:11520-11524.
4. Frolova, L.Y., J.L. Simonsen, T.I. Merkulova, D.Y. Litvinov, P.M. Martensen, V.O. Rechinsky, J.H. Camonis, L.L. Kisselev, and J. Justesen. 1998. Functional expression of eukaryotic polypeptide chain release factors 1 and 3 by means of baculovirus/insect cells and complex formation between the factors. *Eur. J. Biochem.* 256:36-44.
5. Grosch, H.-W. and A. Hasilik. 1998. Protection of proteolysis-prone recombinant proteins in baculovirus expression systems. *BioTechniques* 24:930-934.
6. Guarino, L.A. 1990. Identification of a viral gene encoding ubiquitin-like protein. *Proc. Natl. Acad. Sci. USA* 87:409-413.
7. Hom, L.G. and L.E. Volkman. 1998. Preventing proteolytic artifacts in the baculovirus expression system. *BioTechniques* 25:18-20.
8. Kim, T.K. and T. Maniatis. 1996. Regulation of interferon-γ-activated STAT1 by the ubiquitin-proteasome pathway. *Science* 273:1717-1719.
9. King, L.A. and R.D. Possee. 1992. *The Baculovirus Expression System: A Laboratory Guide*. Chapman & Hall, London.
10. Lee, D.H. and A.L. Goldberg. 1998. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol.* 8:397-403.
11. Löw, P., F.J. Doherty, E. Fellinger, M. Sass, R.J. Mayer, and L. László. 1995. Related organelles of the endosome-lysosome system contain a different repertoire of ubiquitinated proteins in Sf9 insect cells. *FEBS Lett.* 368:125-131.
12. Reilly, L.M. and L.A. Guarino. 1996. The viral ubiquitin gene of *Autographica californica* nuclear polyhedrosis virus is not essential for virus replication. *Virology* 218:243-247.

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