Heat shock-induced repression of proteolysis: poly(A)-binding protein degradation patterns can illusorily suggest its specific loss during heat shock

Valérie Lefrère¹ and Roger F. Duncan^{1,*}

Centre de Biologie du Développement, Bat. IVR3, Université Paul Sabatier, 31062 Toulouse Cedex, France and ¹University of Southern California School of Pharmacy, Department of Molecular Pharmacology/Toxicology and School of Medicine, Department of Microbiology, 1985 Zonal Avenue, Los Angeles, CA 90033, USA

Received January 21, 1994; Accepted February 24, 1994

ABSTRACT

Poly(A)-binding protein (PABP) is highly susceptible to proteolysis during cell lysis of *Drosophila* tissue culture cells unless substantial amounts of proteolysis inhibitors are included in the extraction buffer. This intrinsic proteolytic activity is substantially reduced during heat shock. An artifactual appearance that poly(A)-binding protein is specifically degraded by heat shock can result. Several contradictory descriptions of PABP may also be related to its proteolysis. Repression of proteolysis is likely to reflect a physiologically significant regulatory event, based on recent examinations of HSP70 stability during and after heat shock.

INTRODUCTION

Heat shock serves as a preeminent system for investigating gene expression regulation. Heat shock severely inhibits the translation of virtually all non-heat shock (pre-existing) mRNAs (1). Substantial efforts have been directed towards determining the molecular mechanism, most extensively in the fruit fly, *Drosophila* (1). In *Dictyostelium*, the poly(A)-binding protein (PABP), which is required for mRNA translation and cell viability (2, 3), is reportedly specifically degraded during heat shock (4). PABP degradation would be a sufficient cause for protein synthesis repression. To test for the generality of this mechanism, we have examined PABP's heat stability in *Drosophila* cells. We observe, rather than PABP degradation, the specific, heat-dependent loss of several lower M_r , antigenically-related proteins. However, these lower M_r species are proteolytic 'artifacts', and the alterations in PABP forms observed during heat shock reflects altered intrinsic proteolysis during sample preparation. Proteolytic degradation of intact, 70 kDa PABP may have confused previous analyses of PABP during heat shock and under other circumstances, as we describe below.

MATERIALS AND METHODS

Cell culture and lysate preparation

Drosophila S2 cells ($6-10 \times 10^6$ cells/ml) were propagated in monolayer culture in T150 flasks. Heat stressed cells were incubated at 37°C in a water bath, with stirring, temperature monitored to $\pm 0.1^\circ\text{C}$ with a digital thermometer. To prepare extracts, cells were washed twice, then resuspended in extraction buffer. Three different buffers were compared: (i) a 9.8 M Urea based buffer, originally formulated by O'Farrell (11), (ii) an $\sim 100^\circ\text{C}$, 0.3% SDS buffer, originally formulated by Garrells (12), and (iii) a low salt lysis buffer containing 0.5% Triton X-100 (TXC/LSB; 25 mM HEPES pH 7.2, 65 mM KCl, 0.5 mM MgCl_2). Several protease-inhibitors (0.3 U/ml Aprotinin; 2 mM PMSF; 10 $\mu\text{g/ml}$ leupeptin; 100 $\mu\text{g/ml}$ soy bean trypsin inhibitor; 10 mM benzamidine) were added in various combinations to buffer TXC/LSB. For extraction with buffers (i) and (iii), the cell pellet was disrupted and dissolved by repeated pipetting. The lysate was microcentrifuged 30 sec, and the supernatant removed and used in analysis. For the hot SDS lysis, the pellet was disrupted in the lysis buffer, briefly cooled to $\sim 22^\circ\text{C}$; 1/10 volume of {5 mg/ml DNase, 5mg/ml RNase A, 500 mM Tris pH 7.0, 50 mM MgCl_2 } was added, the viscosity removed by pipetting, 1 mg/ μl urea added and dissolved by vortexing, and the sample frozen or used.

Gel electrophoresis and immunoblotting

Two-dimensional NEPHGE analyses was carried out basically as described by O'Farrell and O'Farrell (10). The first dimension gel contained 3.75% acrylamide mix, $\sim 6\%$ 3.5–10 and 0.6% 7–9 (LKB and Serva, respectively) ampholines, and 2% Triton X-100. Gels were run for 8 h at 400 V. One dimensional gels and the second dimension for 2DGE NEPHGE were run using standard Laemmli conditions. Western blotting was carried out using the ECL Western blotting protocols (as described by Amersham in their kit).

*To whom correspondence should be addressed

RESULTS AND DISCUSSION

Unfractionated extracts prepared from *Drosophila* S2 cells were analyzed by Western blotting (Figure 1), probing with antisera specific to *Drosophila* PABP made in *E. coli* (5). 70 kDa PABP is detected in all lanes. This protein M_r corresponds to the predicted M_r based on the cDNA sequence (6). However, in some lysates made with the TXC/LSB, there are several prominent lower M_r proteins (M_r s ~52 kDa and 48 kDa; Panel A, lanes 3–5) which are not detected in lysates made with the urea- or SDS-based buffers (lanes 1, 2). The lower M_r bands are reproducibly observed in TXC/LSB lysates ($\geq 80\%$ of extractions), and never detected in urea or SDS lysates (3–10 determinations in each buffer system). The appearance of the lower M_r bands can be wholly blocked by certain protease inhibitor cocktails, but not by others (compare lanes 3–5 vs. 7–9; see legend for the specific inhibitors used). We conclude

that PABP is very sensitive to proteolysis during extraction, and that the inclusion of certain protease inhibitors is insufficient to block this cleavage. The protease activity is selective. Immunoblot analysis of other proteins, such as HSP70, evidenced no degradation, using the lysates depicted in lanes 3–5 (data not shown).

Lysates were also prepared after heat shock (30 min at 37°C). Using the TXC/LSB buffer only very low levels of the lower M_r proteolytic products are detected during heat shock (Panel A, lane 10), consistent with a heat-shock suppression of protease activity (7). Clearly, PABP is not significantly degraded during heat shock of *Drosophila*.

Our primary cell line has produced differing absolute levels of degradation in different experiments, characteristic of artifactual, extraction degradation. In the extreme case, depicted in panel B, the heat-altered protease activity creates the illusion that PABP is specifically degraded during heat (compare lane

Figure 1. PABP proteolysis before and during heat shock in *Drosophila*. Cells were cultured, extracted, and analyzed as described in Materials and Methods. Panel A: immunoblot comparison of PABP forms in cells extracted in several different buffer systems, with and without protease inhibitors. lane 1, urea-based buffer, as described by O'Farrell; lane 2, hot SDS buffer, as described by Garrells; lane 3–10, Triton X-100 low salt buffer containing protease inhibitors as follows: lane 3, none; lane 4, Aprotinin; lane 5, Aprotinin and PMSF; lane 6, Aprotinin, PMSF, and leupeptin; lane 7, Aprotinin, leupeptin, and soy bean trypsin inhibitor; lane 8, Aprotinin, benzamidine, and soy bean trypsin inhibitor; lane 9, Aprotinin and benzamidine. Lane 10: 30 min, 37°C extracted in TXC/LSB without protease inhibitors. Panel B: second immunoblot comparison of PABP forms in cells with high intrinsic protease activity. lane 1, 100°C SDS buffer, for comparison; lanes 2–8, TXC/LSB extracts with aprotinin; lane 2, pre-heating (normal temperature); lane 3, 30 min, 37°C; lane 4, 30 min recovery at normal temperature; lane 5, 60 min recovery; lane 6, 2 h recovery; lane 7, 3 h recovery; lane 8, 4 h recovery. Panels C and D: two-dimensional NEPHGE/immunoblotting analyses. Panel C: cells lysed into TXC/LSB plus Aprotinin, benzamidine, and soy bean trypsin inhibitor. Panel D: cells lysed into TXC/LSB without protease inhibitors.

2 (non-heated) vs. 3 (heated)). This pattern appears quite similar to that reported for *Dictyostelium*, and a similar heat-sensitive protease may account for the *Dictyostelium* result. Furthermore, the *Dictyostelium* 'PABP' bands are indicated to be 28–30 kDa (4), whereas yeast, sea urchin, fly, and mammalian PABP is 60–70 kDa (6, 8–10). The failure to detect a heat-insensitive higher M_r PABP in *Dictyostelium* may reflect virtually complete cleavage of PABP during extraction. The *Drosophila* PABP degradative activity progressively returns to nearly the normal-temperature state during recovery from heat shock (Panel B, compare lane 2 (pre-heat) to lane 8 (4 h recovery)).

PABP has been purified from a variety of organisms, including yeast and sea urchins. In both cases, two (or more) M_r forms were detected (as well as multiple isoelectric pI variants) (9). However, mammalian PABP, as identified in two-dimensional gel patterns, is a single, highly basic protein spot (10). Two-dimensional Western blots of NEPHGE-resolved *Drosophila* lysates detect a single PABP isoform at 70 kDa, pI ~9.5 when protease inhibitors are included (Panel C), corresponding to the predicted M_r , pI of PABP and verifying the specificity of the antisera. In the absence of protease inhibitors the 70 kDa spot is decreased, plus several additional highly-basic spots at 52 kDa and 48 kDa appear (Panel D). These likely correspond to the cleavage products observed in the one-dimensional analysis. We suggest that the lower M_r PABPs reported by others may likewise have been generated by this fickle cleavage activity.

ACKNOWLEDGEMENTS

We wish to thank Dr. A. Vincent for encouragement and critical reading of the manuscript. This research was supported by grant GM42644 to RFD.

REFERENCES

1. Lindquist, S. and Petersen, R. (1990) *Enzyme* **44**, 147–166.
2. Sachs, A. B. and Davis, R. W. (1989) *Cell* **58**, 857–867.
3. Sachs, A. B., Davis, R. W., and Kornberg, R. D. (1987) *Mol. Cell. Biol.* **7**, 3268–3276.
4. Manrow, R. E. and Jacobson, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1858–1862.
5. Lefrère, V. (1991) Ph. D. thesis, Université Paul Sabatier, Toulouse, France.
6. Lefrère, V., Vincent, A., and Amalric, F. (1993) *Gene* **126**, 295–296.
7. Luscher, B. and Eisenman, R. N. (1988). *Mol. Cell. Biol.* **8**, 2504–2512.
8. Sachs, A. B., Bond, M. W., and Kornberg, R. D. (1986) *Cell* **45**, 827–835.
9. Drawbridge, J., Grainger, J. L., and Winkler, M. W. (1990) *Mol. Cell. Biol.* **10**, 3994–4006.
10. Standart, N., Vincent, A., and Scherrer, K. (1981) *FEBS Letters* **135**, 56–60.
11. O'Farrell, P. and O'Farrell, P. Z. (1977) *Methods Cell Biol.* **16**, 407–420.
12. Garrells, J. I. (1983) *Meth. Enzymol.* **100**, 411–423.