Ubiquitin gene expression in *Dictyostelium* is induced by heat and cold shock, cadmium, and inhibitors of protein synthesis

A. MÜLLER-TAUBENBERGER, J. HAGMANN, A. NOEGEL and G. GERISCH*

Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, Federal Republic of Germany

*Author for correspondence*

Summary

Ubiquitin is a highly conserved, multifunctional protein, which is implicated in the heat-shock response of eukaryotes. The differential expression of the multiple ubiquitin genes in *Dictyostelium discoideum* was investigated under various stress conditions. Growing *D. discoideum* cells express four major ubiquitin transcripts of sizes varying from 0.6 to 1.9 kb. Upon heat shock three additional ubiquitin mRNAs of 0.9, 1.2 and 1.4 kb accumulate within 30 min. The same three transcripts are expressed in response to cold shock or cadmium treatment. Inhibition of protein synthesis by cycloheximide leads to a particularly strong accumulation of the larger ubiquitin transcripts, which code for polyubiquitins. Possible mechanisms regulating the expression of ubiquitin transcripts upon heat shock and other stresses are discussed.

Key words: ubiquitin, heat shock, inhibitors of protein synthesis, cadmium effects, Dictyostelium.

Introduction

Ubiquitin is a 76 amino acid protein, which is highly conserved in all eukaryotes examined. Based on sequencing either of the protein or of cDNA clones, the amino acid sequence of ubiquitin proved to be identical in various insects (Gavilanes et al. 1982; Arribas et al. 1986) and vertebrates (Schlesinger & Goldstein, 1975; Dworkin-Rastl et al. 1984; Wiborg et al. 1985; Bond & Schlesinger, 1985). Compared to the human sequence there are only two amino acid substitutions in *Dictyostelium* (Giorda & Ennis, 1987; Müller-Taubenberger et al. 1988) and three in yeast (Ozkaynak et al. 1984) and higher plant sequences (Vierstra et al. 1986; Gausing & Barkardottir, 1986). In most of the ubiquitin genes multiple coding units are aligned in tandem giving rise to polyubiquitin precursors that need to be proteolytically cleaved in order to yield the functional ubiquitin monomers. In man (Lund et al. 1985), *Dictyostelium* (Westphal et al. 1986) and *Saccharomyces* (Ozkaynak et al. 1987) certain ubiquitin genes have been shown to encode fusion proteins in which monoubiquitin is extended at its carboxy terminus by sequences that are rich in basic amino acids. These sequences contain several cysteine residues in an arrangement that is reminiscent of the putative metal-binding sites of nucleic acid binding proteins. These results suggest that the family of ubiquitin genes encodes at least two classes of precursor proteins, which differ in their destination and function within the cells.

The ATP-dependent non-lysosomal proteolysis that is mediated by ubiquitin (Ciechanover et al. 1984; Hershko & Ciechanover, 1986) is most likely implicated in the heat-shock response (Finley et al. 1984; Parag et al. 1987). In chicken embryo fibroblasts (Bond & Schlesinger, 1985) and yeast (Finley et al. 1987) ubiquitin itself is a heat-shock-induced protein. This has not been observed in *Drosophila* (Arribas et al. 1986) or *Dictyostelium* (Giorda & Ennis, 1987). Other functions of ubiquitin are suggested by its location in the nucleus and on the cell surface. Ubiquitin is covalently linked to histones H2A and H2B (Goldknopf et al. 1975), preferentially in regions of actively transcribed chromatin (Matsui et al. 1979). The lymphocyte homing receptor (Siegelman et al. 1986) and the platelet-derived growth factor (Yarden et al. 1986) are cell-surface proteins reportedly ubiquitinylated.

*Dictyostelium discoideum* is a eukaryotic microorganism in which the expression of specific genes is linked to discrete steps of development. Upon starvation of growing cells, development proceeds through a pre-aggregation phase to cell aggregation, which is
followed by cell-type specification and terminal differentiation within a multicellular body. Among the cDNA clones that we have used to identify mRNA species that are expressed during early development (Gerisch et al. 1985), there was one that hybridized to multiple mRNA species. This clone proved to encode ubiquitin. Using this clone, a multigene family encoding seven ubiquitin mRNA species with different sizes could be demonstrated (Westphal et al. 1986). Larger mRNA species have been shown to represent polyubiquitin messages (Giorda & Ennis, 1987). All the larger mRNAs were found to accumulate during early development while the small ones declined (Westphal et al. 1986). In the present paper we show that heat shock and other stresses cause accumulation of ubiquitin mRNAs, and that these mRNAs are dramatically overexpressed in cells treated with cycloheximide or other inhibitors of protein synthesis.

Materials and methods

Cell culture and development

Cells of Dictyostelium discoideum strain AX2-214 were grown axenically at 21°C as described (Malchow et al. 1972). To initiate development, cells were harvested at densities of 2-4×10⁶ cells ml⁻¹, washed and cultivated in suspension on a gyration shaker at 150 rev. min⁻¹ in 17 mM-Sørensen phosphate buffer at a cell density of 1×10⁷ per ml.

Heat and cold shock and heavy metal treatment

Temperature shocks and heavy metal ions were applied in the presence of nutrient medium to cell cultures grown up to densities of 1-4×10⁶ cells ml⁻¹. The cells were heat shocked by shifting the temperature from 21°C to 30°C. For recovery the temperature was shifted back to 21°C. The cells were cold shocked by shifting the temperature from 21°C to 4°C and kept in the nutrient medium under shaking conditions at 100 rev. min⁻¹.

Inhibition of protein synthesis

Inhibitors of protein synthesis were added either to growing cells in the presence of nutrient medium as described for heat shock (see Fig. 5), or to cells starved in phosphate buffer as for the initiation of development (see Figs 4, 6). Cycloheximide and anisomycin were purchased from Sigma, emetine from Serva (Heidelberg). The rate of protein synthesis was determined by adding [³⁵S]-L-methionine (Amersham, 370 kBq per 1×10⁶ cells) at 30 min after the addition of inhibitors and to control cells. Incorporation of the ³⁵S-label was stopped after 10 min of incubation by the addition of trichloroacetic acid, final concentration 10%. The pellets were washed twice with trichloroacetic acid and the radioactivity in pellets obtained after zero time of incorporation was subtracted as background from the precipitable radioactivity. [³⁵S]Methionine incorporation into untreated control cells was set as 100%.

RNA isolation, Northern blotting and hybridization

For Northern blots total cellular RNA was purified by phenol-chloroform extraction, separated by gel electrophoresis in 1-2 % (Figs 3, 4, 6) or 1-5 % (Figs 1, 2, 5) agarose in the presence of 6% formaldehyde (Maniatis et al. 1982), and transferred to nitrocellulose (BA 85, Schleicher and Schuell). A 10-μg sample of RNA was applied per lane. The blots were hybridized for 16-18 h with the nick-translated ubiquitin-specific cDNA fragment UBIU (Westphal et al. 1986) at 37°C in 50% formamide, 2×SSC (1×SSC is 0-15 M-NaCl, 15 mM-sodium citrate), 4×Denhardt’s, 1% Sarcosyl, 0-12 M-sodium phosphate buffer, pH 6.8, and 0-1% SDS. RNA sizes were determined using an RNA ladder (Bethesda Research Laboratories) as marker. For comparison, either a cloned 1-5-kb EcoRI cDNA fragment from the coding region of the D. discoideum 120K (10⁻³ M₄) gelation factor, or a 1-2-kb EcoRI fragment from the coding region of α-actinin was used (Witke et al. 1986).

Results

Differential expression of ubiquitin genes during temperature shocks and cadmium treatment

In growing D. discoideum cells four major ubiquitin transcripts of 0-6, 0-7, 1-5 and 1-9 kb and one minor transcript of 1-4 kb were expressed. In cells starved for 5 h ubiquitin transcripts of 0-9 and 1-2 kb appeared, and the amounts of the 1-4-, 1-5- and 1-9-kb transcripts increased (Fig. 1, first and fourth lane). By shifting growing cells from 21°C to 30°C, a temperature that induces maximal heat-shock responses in D. discoideum (Loomis & Wheeler, 1980), the accumulation of the same ubiquitin transcripts as during starvation was induced or enhanced (Fig. 1, second lane). No substantial heat-shock effect was observed for the 0-6- and 0-7-kb ubiquitin transcripts.

Since the effects of heat shock in Drosophila and mammalian cells are mimicked by other stresses including exposure to heavy metals (Ashburner & Bonner, 1979; Ananthan et al. 1986; Burdon, 1986), we tested the influence of cadmium, zinc and cobalt on ubiquitin gene expression in D. discoideum cells. Treatment with 100μM-Cd(NO₃)₂ for 30 min induced, similar to heat shock, the 0-9- and 1-2-kb transcripts and strongly enhanced accumulation of the 1-4-, 1-5- and 1-9-kb species (Fig. 1, third lane). No effect was seen under the same conditions with 1 mM-ZnSO₄ or 0-8 mM-CoCl₂.

The heat-shock effect on ubiquitin gene expression was fast; accumulation of the induced mRNAs began within 15 min and reached a maximum within 30 min (Fig. 2A). After shifting the cells back to 21°C, they returned within 30 min to about the initial state of mRNA expression (Fig. 2A). After 1 and 2 h of recovery at 21°C the cells expressed even less of the heat-shock-induced 1-5- and 1-9-kb mRNA than they had expressed before the heat shock (zero time in Fig. 2A).
Fig. 1. Ubiquitin transcript accumulation after heat shock, cadmium treatment and initiation of development by starvation. RNA isolated from growth phase cells of *D. discoideum* was extracted at the same time from control cells kept in nutrient medium at 21°C (C at 0 h), from cells incubated for 30 min at 30°C (Hs), and from cells treated for 30 min with 100 μM-Cd²⁺ at 21°C (Cd). Alternatively, cells were starved in phosphate buffer and harvested after 5 h of development at 21°C (C at 5 h). Northern blots were labelled with the ubiquitin-specific cDNA probe UB1U.

Microscopic examination indicated that the cells remained intact, excluding the possibility that the lower expression of these mRNAs was due to lysis. Furthermore, as an internal control, the expression of the mRNA for α-actinin was used. This mRNA encodes a cytoskeletal protein that is known to be present in all stages of growth and development (Witke *et al.* 1986). No significant change in the amount of this RNA was found during the heat shock or within the recovery period (Fig. 2B).

When growing cells were shifted from 21°C to 4°C, the same ubiquitin transcripts accumulated as in heat-shocked cells (Fig. 3). The accumulation was slower and less extensive during the cold shock than after a heat shock. The cold-shock response is not a unique feature of polyubiquitin genes; a similar accumulation upon heat and cold shock has been observed for another developmentally regulated transcript of *D. discoideum* (Maniak & Nellen, 1988).

Fig. 2. Ubiquitin transcript regulation during recovery from heat shock. Growth-phase cells were incubated at 30°C for the indicated times (heat shock) and shifted back after 60 min to the normal growth temperature of 21°C (recovery). Northern blots were either labelled with the UB1U ubiquitin-specific probe (A), or with an α-actinin-specific cDNA probe (B).

*Overexpression of ubiquitin transcripts by the inhibition of protein synthesis*

The inhibition of protein synthesis was tested as a potential stress factor and was found to enhance strongly ubiquitin transcript accumulation. Starved cells were incubated with three different concentrations of cycloheximide to compare accumulation of ubiquitin transcripts with the inhibition of protein synthesis. After 2 h of incubation a substantial increase in the amounts of transcripts was observed at all cycloheximide concentrations tested (Fig. 4). At the lowest concentration, 50 μg ml⁻¹, [³⁵S]methionine incorporation was reduced by 68% and accumulation of ubiquitin mRNAs was clearly detectable. This increased accumulation was specific for the five larger
ubiquitin transcripts. These mRNAs were maximally expressed with 250 μg ml⁻¹ cycloheximide, which inhibited [³⁵S]methionine incorporation by 90%. The same dramatic accumulation of ubiquitin mRNAs was observed with 500 μg ml⁻¹ cycloheximide, a concentration that caused 98% inhibition of [³⁵S]methionine incorporation (Fig. 4).

Other elongation inhibitors of protein synthesis showed the same pattern of ubiquitin mRNA induction (Fig. 5): 40 μM-anisomycin and 500 μM-emetine inhibited [³⁵S]methionine incorporation by 84% and 77%, respectively, and showed the same or slightly weaker ubiquitin mRNA accumulation as 50 μg ml⁻¹ cycloheximide which inhibited incorporation by 68%.

Accumulation of the ubiquitin transcripts was not due to a general stabilization of mRNA by cycloheximide (Kelly et al. 1987). The amounts of ubiquitin mRNAs remained high for at least 6 h in the presence of cycloheximide (Fig. 6A), but a reference mRNA encoding another protein disappeared in the presence of cycloheximide (Fig. 6B). The mRNA used here as a reference encoded a cytoskeletal protein, the 120K gelation factor (Condeelis et al. 1982), and was present throughout growth and development in untreated cells (A. Noegel and M. Schleicher, unpublished results).

Discussion

Ubiquitin as a putative mediator of stress responses

Ubiquitin genes have previously been shown in yeast (Finley et al. 1987) and chicken (Bond & Schlesinger, 1985) to be expressed in response to heat shock. The results obtained with D. discoideum show that this effect is more general. Evidence for a function of ubiquitin in protecting cells against heat shock is provided by the increased sensitivity against heat shock of a yeast mutant in which a gene encoding polyubiquitin is disrupted (Finley et al. 1987). Strong expression of heat-shock proteins at non-heat-shock temperature in the ts 85 mouse cell line, which carries a mutation responsible for thermolability of the ubiquitin-activating enzyme E1, suggests that the E1-ubiquitin complex is involved in the suppression of heat-shock genes at normal temperatures (Finley et al. 1984).

Denatured proteins have been shown to initiate heat-shock responses (Ananthan et al. 1986) and it has been supposed that it is the ubiquitin system that connects protein denaturation to the induction of heat-shock
Fig. 5. Comparison of cycloheximide, anisomycin and emetine effects on ubiquitin transcript accumulation. Growth phase cells were treated for 30 min in the presence of nutrient medium with 50 μg/ml of cycloheximide (CHX), 40 μM-anisomycin (AN), or 500 μM-emetine (EM). The rate of protein synthesis as determined by [35S]methionine incorporation in the inhibitor-treated cells is given as a percentage of synthesis in control cells (C).

genes (Finley et al. 1984; Munro & Pelham, 1985). The principal assumption is that denatured proteins compete with a heat-shock regulator protein for E1-activated ubiquitin, such that in the presence of denatured proteins more of the regulator protein exists in a non-ubiquitinated state. The regulator protein is thought to be inactive when ubiquitinated and to activate the heat-shock promoters in its free state. Relevant regulator proteins have been designated as heat-shock activator protein or heat-shock transcription factor HSTF (Zimarino & Wu, 1987).

Heat-shock-induced polyubiquitin mRNA accumulation

The results presented in this paper indicate that expression of a group of ubiquitin transcripts is increased in response to heat or cold shock, to Cd²⁺ treatment, or to induction of normal development by starvation. Even higher levels of expression were obtained with cycloheximide or other inhibitors of protein synthesis. The effect of all these treatments is restricted to the larger ubiquitin transcripts, which probably all encode tandem repeats of ubiquitin sequences (Giorda & Ennis, 1987). Transcripts of 0.6 and 0.7 kb, which were not regulated by these treatments, are too small to code for polyubiquitins. The 0.6-kb transcript encodes a mono-ubiquitin that is linked at its C-terminal end to a basic polypeptide (Westphal et al. 1986). The 0.7-kb transcript may code for a mono-ubiquitin with another C-terminal sequence or for a di-ubiquitin. These results indicate peculiar regulatory mechanisms for the polyubiquitin transcripts and suggest functions for the C-terminally extended mono-ubiquitin that are not involved in stress responses.

The parallel effects of heat shock or other stresses and of the initiation of Dictyostelium development by starvation (Westphal et al. 1986) suggest common steps in the pathways of ubiquitin gene expression. Moreover, polyubiquitin genes are not the only genes that are affected by heat shock and the initiation of development. A set of repeated sequences, which comprise an element homologous to the heat-shock promoter in Drosophila, is induced in D. discoideum both by heat shock and during induction of development by starvation (Zuker et al. 1983). Despite these similarities it is unlikely that development and the responses to various stresses including cold shock are all initiated by the same alterations within the cells.

The cycloheximide effect and its bearing on the regulation of polyubiquitin genes

The results shown in Fig. 6 together with those of Fig. 4 indicate that the amounts of polyubiquitin mRNAs continue to increase for several hours with negligible de novo synthesis of proteins. If activation of the ubiquitin genes is mediated by heat-shock activator protein, as discussed above, this protein should be either long-lived or continuously delivered from a large store or precursor pool. This conclusion is consistent with the results of Zimarino & Wu (1987), which indicate a fast reversible conversion of the activator protein from an inactive to an active form without a requirement for protein synthesis.

It has been suggested that cycloheximide indiscriminately stabilizes mRNAs in D. discoideum (Kelly et al. 1987). However, decay of the mRNA of a cytoskeletal protein, the 120K gelation factor, showed that mRNAs can be degraded in the presence of cycloheximide (Fig. 6B). Thus, accumulation in the presence of cycloheximide is not a general feature of D. discoideum mRNA. It is likely that cycloheximide affects the stability of polyubiquitin mRNA as well as the rate of transcription.

It is possible that cycloheximide stimulates ubiquitin gene expression by producing incomplete polypeptides that are readily ubiquitinated because they do not fold correctly. But if this were the only way in which cycloheximide causes ubiquitin gene expression, a maximal effect should be observed at intermediate cycloheximide concentrations that only partially inhibit protein synthesis. However, at concentrations that
inhibited protein synthesis almost completely, polyubiquitin transcripts were maximally expressed (Fig. 4).

Another mechanism of cycloheximide induction of polyubiquitin genes is suggested by the dual role of ubiquitin as a heat-shock-induced protein and a mediator of heat-shock responses. Therefore an autoregulatory cycle may exist in which a heat-shock activator protein would play a key role in negative feedback control of polyubiquitin genes (Finley et al. 1984; Munro & Pelham, 1985). On the basis of similar results in Drosophila, a loop for heat-shock proteins has been suggested with HSP 70 as a self-regulating protein (DiDomenico et al. 1982). Two results obtained with D. discoideum support negative feedback regulation of ubiquitin. First, during recovery of cells from a heat shock, an undershoot of ubiquitin transcripts is observed (Fig. 2). This result suggests inactivation of ubiquitin genes by the high concentrations of ubiquitin that result from ubiquitin overproduction during the heat shock. Second, the extraordinarily strong expression of ubiquitin genes is that is caused by cycloheximide (Figs 4–6) might be a consequence of disrupting a negative feedback cycle in which ubiquitin is involved. If ubiquitin is continuously consumed and no longer produced when protein synthesis is blocked, the ubiquitin genes would become fully activated and the untranslated transcripts would accumulate.

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