Induction of proteins in response to cold acclimation of rainbow trout cells

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Abstract The in vitro translation of poly(A)-RNA isolated from control and cold-treated cells showed that low temperatures induced changes in the population of translatable mRNAs. When cellular proteins extracted from cold-treated cells were subjected to two-dimensional gel electrophoresis, the 70 kDa protein was found to be synthesized during the cold treatment. N-terminal sequence analysis showed that the 70 kDa cold-inducible protein was a homolog of the mammalian valosin-containing protein and yeast CDC48p. The changes in mRNA and protein contents during cold acclimation may result from the expression of genes involved in the adjustment of cellular metabolism to low temperature or the induced proteins may be directly involved in the cold acclimation.

Key words: Cold acclimation; Valosin-containing protien; CDC48; (Rainbow trout); (RTG-2 cell)

1. Introduction

Fish cells can generally grow in a wide range of temperatures, and the culture temperature is correlated with the body temperature in the fish's natural habitat. For example, since rainbow trout can adapt to a temperature range of 0–22°C, cultured trout cells usually grow in the range of 0 to 24°C with an optimum at 22°C [1]. On the other hand, in the case of mammalian cells, the permissive growth temperature range is known to be 36–39°C, with an optimum at 37°C [2].

When cells are exposed to heat shock and other stresses, such as heavy metals and UV radiation, several stress proteins are induced [3]. This induction has been shown to be mediated by a stress-activated heat shock factor, which can recognize specific heat shock promoters. In the case of rainbow trout cells, when cell cultures are transferred from 22°C to 28°C, the heat shock proteins have been reported to appear transiently [4].

On the other hand, the phenomenon connected with cold acclimation has been well studied in rainbow trout. Changes in the isozymes of acetylcholinesterase [5], and desaturation of membrane phospholipids [6] were reported for the cold acclimated fish. These phenomena were found to occur within 1–4 weeks of acclimation [5,6].

This study is an attempt to characterize the molecular and cellular mechanisms of the cold acclimation of fish cells. A cold response was observed with rainbow trout cell line RTG-2 when the culture temperature was shifted from 22°C to 4°C. It was found that both induction and repression of protein synthesis took place in the trout cells during the cold acclimation. The major cold-inducible protein in the trout cells was identified as a homolog of the mammalian valosin-containing protein (VCP) and yeast CDC48p.

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Abbreviations: VCP, valosin-containing protein; CDC48p, cell division cycle gene CDC48 protein; RTG-2, rainbow trout gonadal cell line RTG-2 cells; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; cold70, major cold-inducible 70 kDa protein

2. Materials and methods

2.1. Cell culture

Rainbow trout RTG-2 cells were maintained in Leibovitz L-15 medium containing 8% fetal bovine serum at 22°C [1]

2.2. In vitro translation

Poly(A)-RNA was obtained by oligo(dT)-Sepharose chromatography using a Microprep RNA kit (Pharmacia). Poly(A)-RNA (1 μg) was translated in vitro in a 25 μl volume with rabbit reticulocyte lysate (Stratagene) at 37°C for 30 min with L-[^{35}S]methionine (approx. 1100 Ci/mmol; New England Nuclear) as described by the manufacturer.

2.3. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cells were harvested and lysed with lysis buffer (0.15 M NaCl, 0,1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5) containing protease inhibitors, 1 μg/ml leupeptin, 1 μg/ml chymostatin, 1 μg/ml E-64, 1 μg/ml pepstatin A, and 1 mM PMSF). The lysate was clarified by centrifugation at 13000×*g*. The soluble fraction thus obtained was separated by 2D-PAGE according to the method of O'Farrell [7]. The first dimensional run was using an equilibrium isoelectric focusing gel (ampholines, pH 3–10, 1.5% (w/v); pH 5–8, 0.5%), and the second one used a 10% SDS-PAGE gel. The gels were stained with Coomassie Blue R-250, followed by staining with a silver stain kit (Wako). For autoradiography, the gels were fixed, dried, and autoradiographed for 1 week.

2.4. Protein blots and determination of the N-terminal amino acid sequence

The cell lysate was subjected to 2D-PAGE as previously described. The separated protein was electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad) [8]. The membrane was then stained for protein with Coomassie Blue, the 70 kDa protein spot was excised from the blot, and the stained membrane fragment was applied to an Applied Biosystems model 476 protein sequencer.

2.5. Immunoprecipitation

For immunoprecipitation, a rabbit polyclonal antibody was raised against a synthetic peptide, GILLYGPPGTGKMLIWGAVAN, corresponding to amino acid residues 1–21 of the N-terminal sequence of the trout cold 70 kDa protein.

Rainbow trout RTG-2 cells at 80–90% confluence in a 24 cm^2 dish were rinsed three times with phosphate-buffered saline, incubated in the labeling medium (minimum essential medium minus methionine plus 8% dialyzed fetal calf serum) for 30 min prior to the addition of radioisotopes, and then incubated with 1 ml of the same medium containing 200 μCi of a ^{35}S-labeled methionine and cysteine mixture (approx. 1100 Ci/mmol; New England Nuclear) for 6 h. The cell lysate was precleared by incubation with normal rabbit serum and protein A-Sepharose beads (50% slurry) at 4°C for 1 h. The samples were then incubated with antiserum against the cold 70 kDa protein and with

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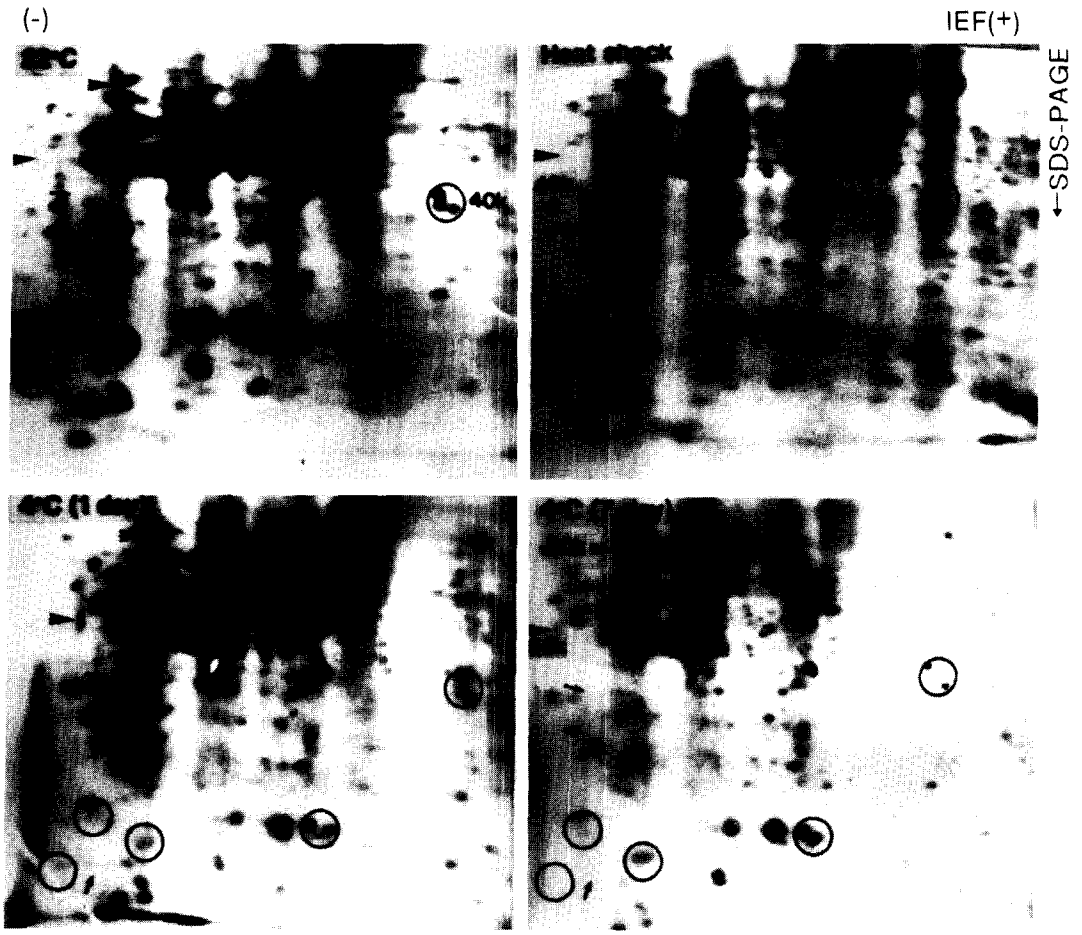

Fig. 1. Change in the mRNA populations of rainbow trout RTG-2 cells with a shift from 22°C to 4°C. The autoradiograms were obtained for gels of in vitro translation products of poly(A)-RNA labeled with [^{35}S]methionine before cold treatment (22°C), or after cold temperature (4°C) or heat shock (30°C) treatment. Arrowheads indicate polypeptides that increased in amount upon cold treatment, while the open circles are those that decreased. Arrows indicate those that increased upon heat shock treatment.

protein A-Sepharose beads at 4°C for a total of 2 h. The recovered antigen-antibody complexes were washed five times in the wash buffer (0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% SDS). The immunoprecipitated materials were separated by SDS-PAGE and detected by autoradiography of the dried gel.

3. Results

As the first step toward the identification of cold-inducible proteins in trout cells, translatable mRNA populations from cold-treated cells were analyzed by in vitro translation and 2D-PAGE of the protein products. This experiment revealed mRNAs encoding prominent polypeptides of 50, 70 and 80 kDa which appeared during the cold treatment at 4°C for 1 day and accumulated during additional 7 day cold treatments (Fig. 1, see arrowheads). In addition to the appearance of these mRNA species, mRNAs encoding 18–20 and 40 kDa polypeptides completely disappeared during the cold treatment for 1 day (Fig. 1, see open circles). On the other hand, the heat shock treatment of the trout cells induced the expression of 18 and 40 kDa polypeptides (Fig. 1, see arrows). However, the heat shock stress did not affect the accumulation and disappaearance of the cold-induced polypeptides (Fig. 1, see arrowheads).

To identify the cold-inducible proteins in the trout cells, cellular proteins were also applied to 2D-PAGE followed by Coomassie Blue and silver staining. A 70 kDa protein spot gradually appeared during the cold treatment using Coomassie Blue staining (Fig. 2A, arrowheads). Although the 70 kDa protein spot stained by Coomassie Blue was destained by silver staining, the presence of 10, 18, 20, 40, 50 and 60 kDa proteins was demonstrated by silver staining (Fig. 2B, arrowheads). On the other hand, the disappearance of 18–20 kDa proteins during the cold treatment was shown by silver staining (Fig. 2B, open circles) and corresponded to the in vitro translation products which disappeared during the cold treatment for 1 day (Fig. 1A, see open circles).

However, the other protein spots found by the protein stainings did not correspond to the polypeptides detected by the in vitro translation assays. It is possible that unique mRNAs exist for these proteins, but were not detected in the in vitro translation assays. Alternatively, it is possible that these additional spots are polypeptides that have been post-translationally modified, some of which are derived from the 50, 700 and 80 kDa polypeptides found in the in vitro translation assays.

The amino acid sequence of the N-terminal region of the 70 kDa protein (tentatively named 'cold70') which accumulated in the cells cold treated for 7 days was determined using a

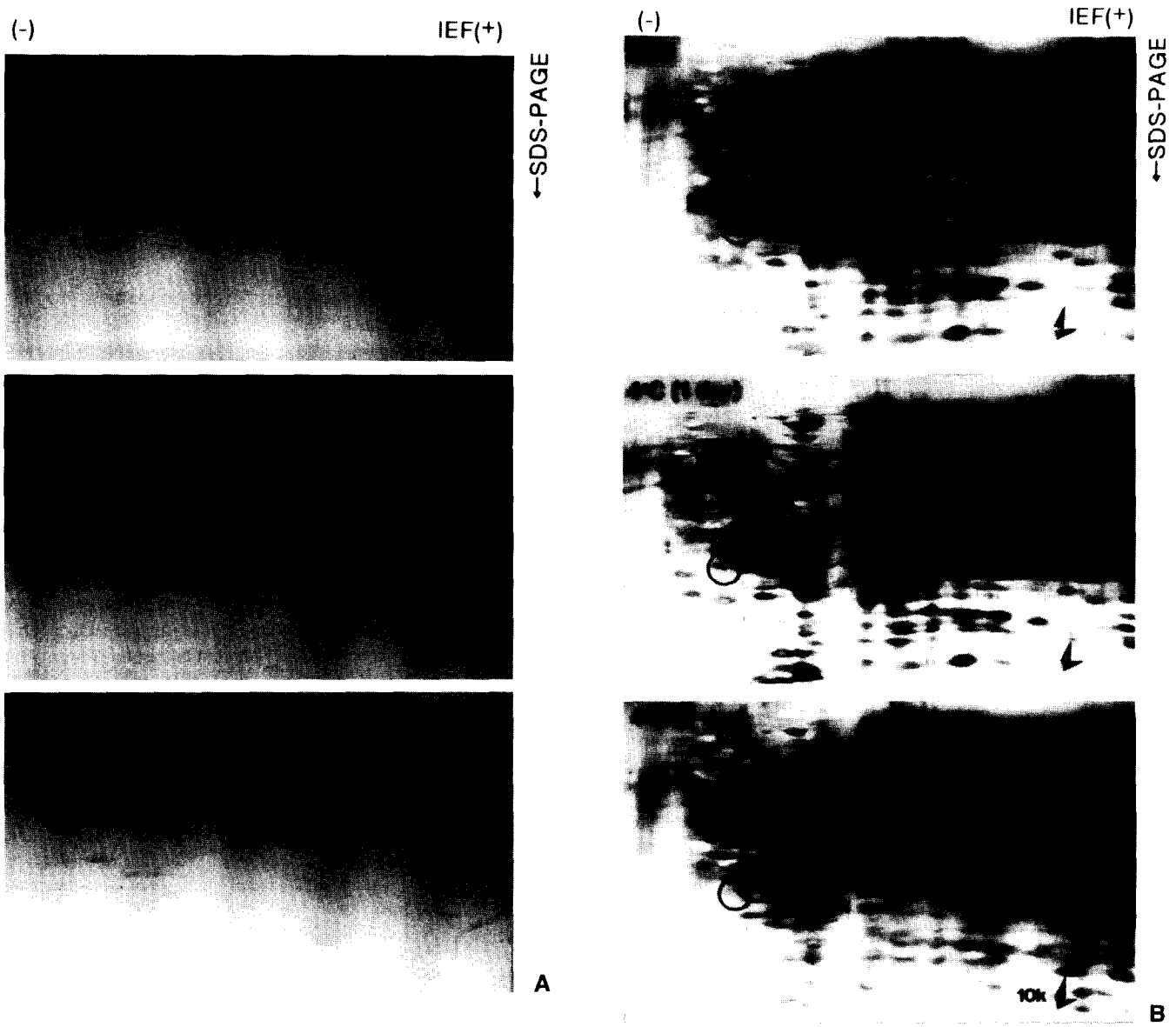

Fig. 2. Changes in proteins of rainbow trout RTG-2 cells with a shift from 22°C to 4°C. A cell culture growing at 22°C was transferred to 4°C for 1 and 7 days. Each culture was harvested and the cytoplasmic fraction was subjected to 2D-PAGE. The gel was stained with Coomassie blue dye (A) and then silver-stained (B). Arrows indicate polypeptide species that increased upon cold treatment, while the circles are those that decreased. Arrowheads indicate those that increased upon heat shock treatment.

blotted sample. The amino acid sequence at the first 21 residues of the protein was compared with those in the protein sequence databases using the Fasta Search Program [9] (Fig. 3). The sequence was found to exhibit extensive homologies with yeast CDC48p protein [10] and mammalian VCP [11,12], and 18 of the first 21 residues were identical to the sequence of the ATP binding motif of CDC48p [10].

To characterize the cold-inducible biosynthesis of cold70, antiserum was raised by immunizing a rabbit with a synthetic peptide corresponding to the first 21 residues of the N-terminal amino acid sequence. This antiserum specifically immunoprecipitated 130 and 70 kDa proteins in RTG-2 cell lysates (Fig. 4). The levels of the immunoreactive 70 and 130 kDa proteins started to increase during the first 4 days of cold treatment and remained at a high level throughout the duration of the 7 day acclimation period. The expression of cold70 is highly responsive to a temperature change; when the cells were returned to the control experimental condition of 22°C, the amounts of the 70 and 130 kDa proteins markedly decreased within 1 day. A band of 43 kDa was also noted, and since the band was not detected by immunoblotting (data not shown), it may be due to non-specific immunoreactivity with the antibody or co-precipitation with cold70.

4. Discussion

This study demonstrated that rainbow trout RTG-2 cells synthesized various proteins and grew when acclimated to 4°C. Several specific proteins were found to accumulate in the cold-treated cells, and the production of other proteins was repressed by cold treatment of the cells. Changes in mRNAs upon in vitro translation assaying became apparent within 1 day of cold treatment. This suggests that their transcription may be regulated by the cold. Another possibility can be considered

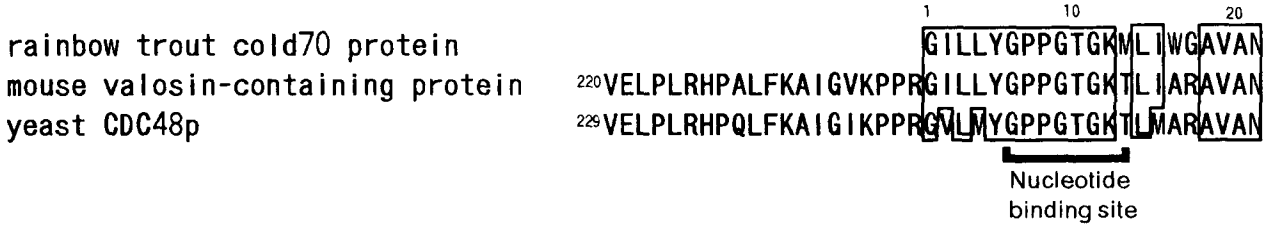

Fig. 3. N-terminal amino acid sequence of the rainbow trout cold70. The sequence of cold70 was used to search the protein databases. A matching of 86% was found with the mouse valosin-containing protein, and 71% of the sequence coincided with that of yeast CDC48p.

that *cis*-elements in the mRNAs might relate to post-transcriptional regulation, such as translation efficiency and stability of the mRNA. Furthermore, protein spots, which newly appeared in the 2D-PAGE pattern, and become more intense 7 days after cold treatment. These findings indicate that a cold responsive gene expression is induced within 1 day after the shift of the temperature to 4°C, and that the induced proteins are accumulated in the cold-treated cells within several days.

Since animal cells synthesize a specific set of proteins when they are heat-shocked [3,4], the possibility that the cold-induced mRNAs might be related to the heat shock protein of rainbow trout was examined in this study. Upon exposure to heat shock conditions, the trout cells synthesized specific mRNA species corrsponding to 40 and 18 kDa polypeptides (Fig. 1). These polypeptides were not similar in electrophoritic properties to most of the polypeptides induced or repressed by cold treatment. This indicates that the change in the population of mRNAs during cold acclimation is a different response from that to heat shock [3,4]. It also suggests the presence of an additional type of temperature response in animal cells.

Upon N-terminal amino acid sequence analysis, the major cold-inducible 70 kDa protein, 'cold70', was found to exhibit apparent homology with CDC48p [10] and VCP [11,12], and thus is considered to bo a rainbouw trout homolog of these proteins. The finding that the N-terminal sequence of cold70 was essentially identical to residues 240–260 of porcine VCP and residues 249–269 of yeast CDC48p suggests that the trout protein is a truncated form of a 100 kDa protein like VCP and CDC48p. In porcine tissues, VCP was found to be synthesized as a 90 kDa polypeptide and then to be processed into a 43 kDa form in vivo [11]. Several processing products of murine VCP have also been reported [12]. In the case of the cold-treated trout cells, immunoprecipitation analysis showed that the 130 and 70 kDa proteins were reactive with the antiserum against cold70. Therefore, these findings suggest that the trout cold70 is a processing product derived from a mature 130 kDa protein in vivo in the cold-treated cells. A possible processing protease may be responsible for the formation of cold70 during the cold acclimation process in the cells.

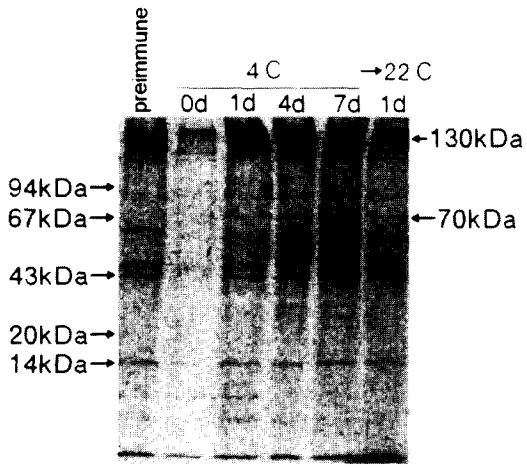

Fig. 4. Induction of cold70 during the cold acclimation of trout cells. Cell cultures growing at 22°C were transferred to 4°C. The trout cells were radiolabeled and cell lysates were subjected to immunoprecipitation with antiserum against the trout cold70.

According to the results of studies on cold-sensitive mutants of yeast [10], CDC48p is found to be essential for the yeast to survive at temperatures under 10°C. Furthermore, VCP is considered to act as a substrate for a protein kinase related to mitosis and regulating protein interaction through a chaperone-like activity [12,13]. The fact that the homologous proteins, CDC48p and VCP, are associated with cell division and mitosis [10,12,13] suggests that the accumulation of the trout cold70 might participate in the metabolic compensation for the delay in cell cycling due to cold temperature.

Exposure of trout cells to a temperature of 4°C alters the relative abundance of specific mRNAs and proteins. The changes in the mRNA and protein contents during the cold treatment may result from the expression of genes involved in the adjustment of the cellular metabolism to a low temperature, or else the induced proteins may be directly involved in the acclimation and growth of the trout cells.

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