Volume 276, number 1,2, 219-222 FEBS 09217 December 1990

Characterization of a protein that appears in the nervous system of the moth *Manduca sexta* coincident with neuronal death

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Received 27 September 1990

Two-dimensional gel electrophoresis was used to locate potential neuronal death-related proteins in the moth *Manduca sexta*. Protein patterns of ganglia of pharate adult moths (taken prior to adult ecdysis) compared with protein patterns of one-day-old adults revealed reproducible changes in protein patterns. An acidic protein of approximately 40 000 Da was present in all samples from adult moths undergoing neuronal death and essentially absent from pharate adult samples.

Neuronal death; Gel electrophoresis, 2-D; Cell death; Metamorphosis; Protein synthesis; Abdominal ganglia; *Manduca sexta*

1. INTRODUCTION

Selective neuronal death is a feature of development in both vertebrate and invertebrate nervous systems; it is also characteristic of certain pathological states, such as Huntington's disease and Alzheimer's disease [1]. The three unfused abdominal ganglia of the moth *Manduca sexta* provide a highly enriched preparation for the study of neuronal death, because half of their nerve cells die shortly after the adult emerges [2–5]. The same neurons always die in the same sequence in each moth. It has previously been established that a steroid hormone, 20-hydroxyecdysone, plays an important role in controlling post-metamorphic neuronal death in the adult moth [6]. The intracellular mechanisms underlying this endocrine regulation of cell survival are unknown.

It appears that the site of hormone actin is at the level of the dying neurons themselves, because many of the neurons fated to die after adult emergence are themselves steroid hormone target cells (as indicated by their nuclear concentration and retention of radiolabeled ecdysteroid) [7], and because the degeneration of at least some of these neurons occurs in the same temporal sequence in isolated cultured ganglia [8]. Results from experiments using cycloheximide, puromycin, and actinomycin D imply that the continued synthesis of RNA and protein is needed to mediate this post-metamorphic cell death as treatment with such agents promotes neuronal survival [2,9]. In the intact animal, it seems likely that the humoral signal – the decline in levels of circulating steroid accompanying the end of development – acts to release an active program of cellular suicide that involves production of new RNA and the synthesis of proteins.

A direct approach to the identification of proteins present in the nervous system of *Manduca sexta* at the time of neuronal death is to use two-dimensional gel electrophoresis to separate proteins on the basis of charge in the first dimension and molecular weight in the second dimension [10–12]. Although such analysis of nervous tissue is often problematic because of the heterogeneous nature of tissue, we reasoned that the abdominal ganglia of the moth should constitute and appropriate source for neuronal death-associated proteins, since in each of the ganglia approximately 350 of the 700 neurons die during the first two days after the adult emerges [3,4]. At this time, the unfused abdominal ganglia contains a mixture of neurons that will survive during adult life, neurons that are already degenerating, and neurons that are committed to die but have not yet begun the process of degeneration [4,6]. Thus, any changes in protein pattern detected at this time could reflect changes in protein expression in any of these three cell populations.

2. MATERIALS AND METHODS

Manduca sexta (Lepidoptera: Sphingidae) were reared in the laboratory according to the method described by Bell and Joachim [13]. Individual developing adult moths were staged with reference to a previously described set of cuticle pigmentation markers [4]. The pharate adult group consisted of moths judged to be 36 h away from ecdysis on the basis of the appearance of a set of spots along the distal margin of the wing (Stage 6 of Schwartz and Truman [14]). No loss of neurons occurs in the unfused abdominal ganglia at this time [4]. The adult group consisted of moths that had emerged 18–20 h prior to dissection. Time of emergence was confirmed by observation, and moths were held in paper bags at 26°C until needed. For preparation of the gel samples, the ventral nerve cord was rapidly dissected from the abdomen. The three unfused abdominal ganglia were then further

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

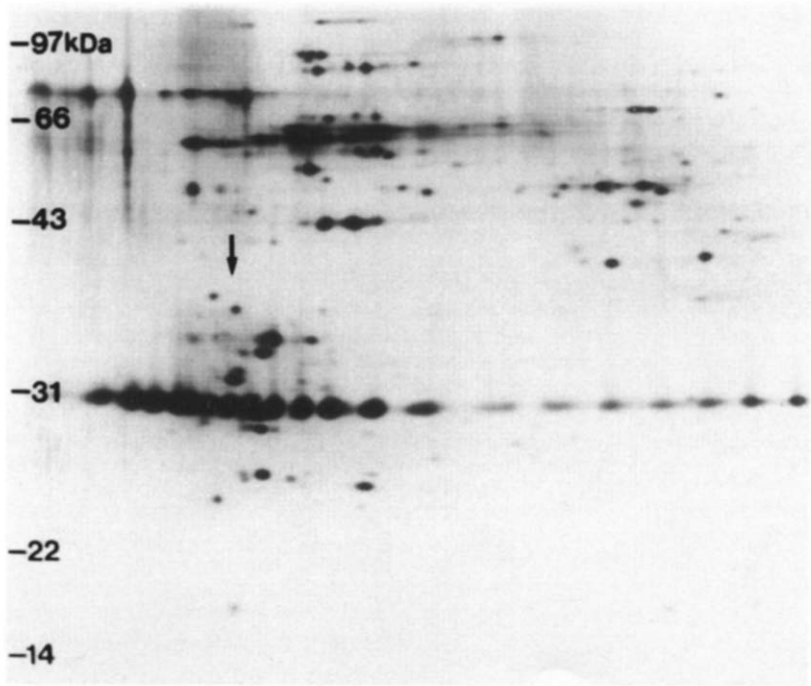

Fig. 1. In this two-dimensional gel, pharate adult abdominal ganglia proteins have been separated between 10–100 kDa (vertical axis) and 4.2–8.5 p*I* (horizontal axis). The gel was stained with silver stain for maximum visualization of protein. The sample load on the gel was 25 μg. The train of spots seen across the bottom third of the gel are commercial carbamylate standards used to assess reproducibility of the pH gradient. The arrow highlights the absence of the protein which is observed in the adult sample in Fig. 2.

isolated by the removal of nerves and interganglionic connectives. Samples were sonicated in 9 M urea solubilization buffer containing 5.5% BioRad carrier ampholytes (3/10 and 5/7 in equal amounts), 4% NP-40 and 2% β-mercaptoethanol. Aliquots of homogenate were used to determine the protein concentration of each sample [15].

Ampholyte polyacrylamide tube gels were prefocused for 1 h at 200 V to set up the pH gradient. A constant 25 μg of sample protein accompanied by Pharmacia carbonic and hydrase carbamylyte was loaded per gel. Gels were run for a total of 30 000 V·h. The gels were extruded and incubated with SDS-equilibration buffer to neutralize

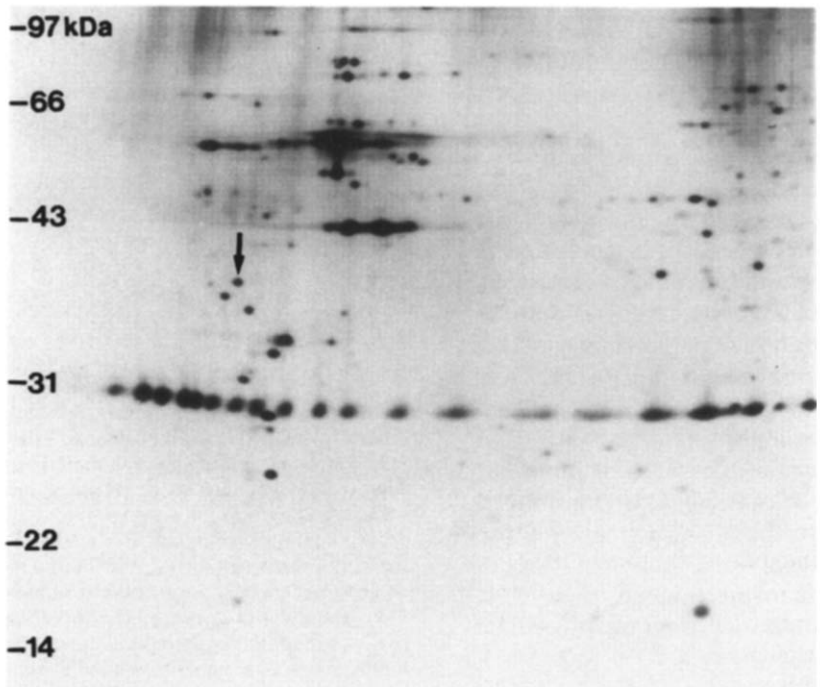

Fig. 2. The arrow in this silver-stained gel indicates the presence of a protein observed in gels prepared with adult tissue samples; this spot is not seen in gels run with pharate adult samples. The apparent molecular mass of the protein is approximately 40 kDa and the p*I* is 5.2–5.6.

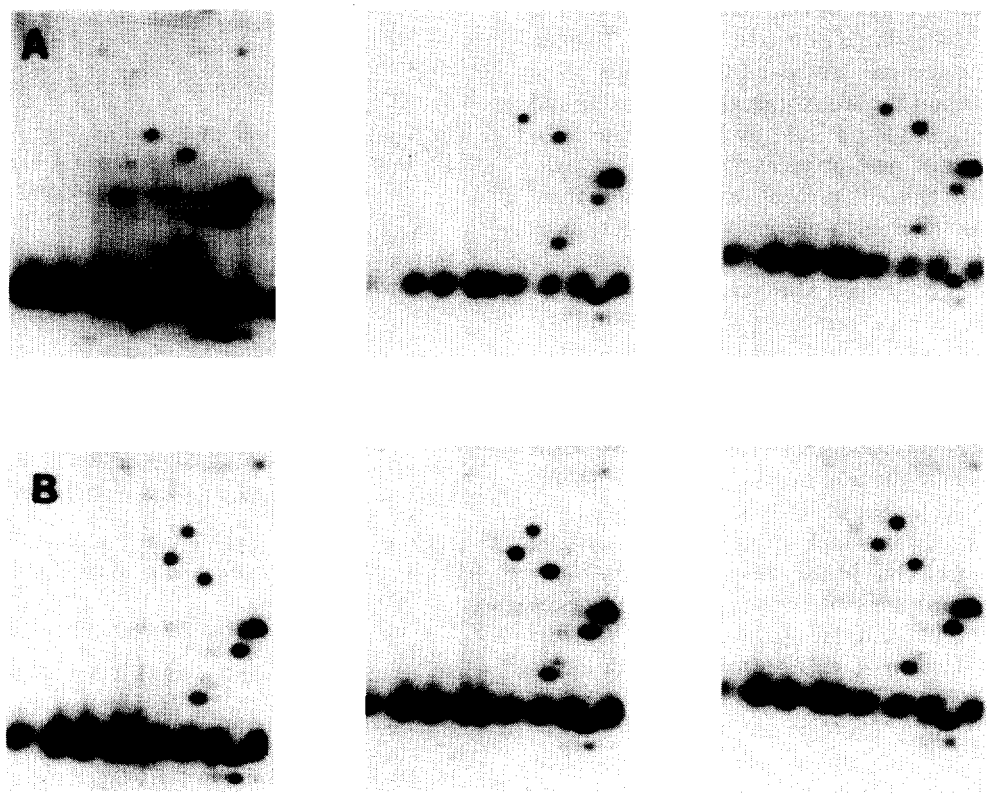

Fig. 3. (A) Pharate adult protein patterns from 3 separate samples (B) Adult protein patterns from three separate samples. Note the appearance of a 40 kDa protein which is absent from the pharate adult protein patterns in (A). The location of this protein in the uncut gel is indicated by an arrow in Fig. 2.

charge, then frozen to -70°C. The second dimension separation was obtained with a linear gradient slab gel (9–17% acrylamide). Tube gels were placed onto slabs oriented with the acid end to the left. BioRad low range molecular weight standards were run at the acid end of several gels. Slab gels were run in a chilled tank at 150 V and ≤0.6 A for approximately 18 h. Gels were then fixed and silver-stained [16]. Positive black-and-white transparency image transfers were made directly from the silver-stained gels onto Kodak X-OMat duplicating film. From the positive image transfers, negatives and prints were made.

3. RESULTS AND DISCUSSION

We have used two-dimensional gel electrophoresis followed by silver staining to locate potential neuronal death-related proteins in the moth *Manduca sexta*. When protein patterns of ganglia taken prior to adult ecdysis were compared with protein patterns of one-day-old adults, a striking change in the protein pattern was readily detected (Figs 1,2). This extremely robust effect was the appearance of a new protein in early adulthood (Figs 2,3). This protein was observed at the acidic end of the gel, in the 5.2–5.6 p*I* range, as determined with carbamalyte standards. Its apparent molecular mass is approximately 40 kDa as determined by co-migration with molecular mass standards. In the first two replications of this experiment (pharate adult $n=7$, adult $n=7$), we observed this protein exclusively in the adult preparation. In the third replication of this experiment (pharate adult $n=8$, adult $n=5$), we soaked the gels in developer for approximately 20 min (4 times longer than the usual development time). At this long development time, we observed very faint evidence of this spot in 2 of the 8 pharate adult gels.

The concept that developmentally programmed cell death is an active process in which the dying cell effectively commits suicide was expressed in the 1960s [17]; it has recently received strong experimental support. A strikingly consistent picture emerges from work on a number of systems. Inhibitors of protein synthesis and RNA synthesis delay or block the normal post-metamorphic loss of neurons from the abdominal ganglia of *Manduca sexta* [2,9]. Martin et al. [18] have demonstrated that the same pharmacologic agents can block the death of cultured sympathetic ganglion cells that otherwise follows the withdrawal of nerve growth factor. Oppenheim and colleagues [19] have found the same result in their studies of the motoneuron death that occurs during the development of the chick spinal cord. Similar results have been found in non-neural tissues in vertebrates and invertebrates [17,20–22], suggesting that the ability to activate a protein synthesis-based process of self-destruction may be a characteristic of many classes of cells.

Although the above evidence suggests that newly-

synthesized proteins may kill cells fated to die during development or dying in response to loss of a trophic factor, there are few direct studies of the proteins being made by cells around the time of death. Lockshin and Wadewitz [23] and Schwartz and Kay [22] have described the appearance of new proteins visualized on two-dimensional gels at times that moth muscle is preparing to degenerate; at times of muscle stability, these proteins are absent. It is not yet known if these muscle death-associated proteins are also expressed in the nervous system. One of these appears to be ubiquitin [24], an intracellular protein often found in association with proteins prior to their degradation [25]. Other researchers have noted a significant increase in gene activity associated with testosterone-deprivation-induced death of epithelial cells in the rat prostate gland, with increases in the RNAs for the cellular oncogenes *c-fos* and *c-myc* and certain heat shock proteins [26]. Genetic analysis has revealed that in the nematode *Caenorhabdites elegans* the products of specific identified genes appear to trigger cell death during development [27,28]; again, it is not known if this form of cell death is analogous to that found in the insect nervous system, or whether related cellular proteins are involved.

In summary, we have demonstrated the existence of a developmentally regulated protein in the moth nervous system, the presence of which is temporally correlated with post-metamorphic neuronal death. Future identification of this protein and studies of its cellular distribution should yield insight into whether its expression is related to the functioning of neurons fated to die or to the continued existence of the survivors.

Acknowledgements: We thank Sandra Tollaksen and Anne Gemmell for technical assistance. This study was supported in part by NSF BNS 86-16777 to E.J.R. and by the US Department of Energy, Office of Health and Environmental Research under contract number W-31-109-ENG-38. M.E.M. is supported by NIMH Behavioral Neuroscience Postdoctoral Fellowship PHS 5 P32T32 MH18412. S.E.F. is an Alfred P. Sloan Research Fellow.

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