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The *Drosophila engrailed* protein is phosphorylated by a serine-specific protein kinase

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ABSTRACT

The *engrailed* gene is required during embryogenesis of *Drosophila melanogaster* for normal segmental development and for differentiation of posterior compartments. The protein encoded by the *engrailed* gene contains a homeodomain, has sequence specific DNA binding activity, and has been proposed as a transcriptional regulator. We show here that the *engrailed* protein, isolated from both cultured cells and embryos, has been modified by a serine-specific protein kinase. This is the first report that homeobox proteins are post-translationally modified. Phosphorylation of the *engrailed* protein may directly or allosterically modify its function, and offers the possibility that the *engrailed* protein becomes phosphorylated in response to extracellular, mitogenic or positional stimuli.

INTRODUCTION

The *engrailed* gene is one of several *Drosophila melanogaster* genes whose expression during embryogenesis is necessary for the subdivision and determination which ultimately elaborate the compartments and segments of the adult insect (1,2). Expression of the *engrailed* gene initiates prior to the ninth embryonic nuclear division cycle, within 1 1/2 hr after fertilization (3). By the cellular blastoderm stage (the 14th nuclear division cycle), the *engrailed* transcript and protein are localized into fourteen stripes, 1-2 cells wide, spaced along the anterior-posterior axis of the embryo (4,5). These iterated stripes of *engrailed* expression define the anlagen of the posterior compartment of each segmental unit, and *engrailed* expression is absolutely required for the maintenance of these developmental subdivisions (2,4,6).

The primary structure of the *engrailed* protein is unusual. It has stretches of polyalanine and polyglutamine, and towards the C-terminal end there is a highly conserved region, the homeobox domain, also found in

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several other Drosophila proteins involved in segmentation (7). Homeoboxes are also encoded in the genomes of vertebrate species, including human (8). The *engrailed* protein mediates sequence-specific DNA binding in vitro to numerous sites in both Drosophila and bacteriophage λ DNA (9). The biochemical properties of these interactions remain poorly characterized and their functional significance unproven. Yet, these data, taken together with genetic evidence (10, 11) and the observed nuclear localization of *engrailed* protein (5), suggests that the *engrailed* gene directly orchestrates a subset of other genes whose role is to elaborate the developmental program of a cell.

RESULTS

The *engrailed* protein can be detected by immunofluorescence in nuclei of early Drosophila embryos. To investigate the structure and function of the *engrailed* protein, we wished to determine whether this protein is modified post-translationally. As the *engrailed* protein is expressed at very low levels in Drosophila embryos, we developed a more accessible source of the protein by placing the *engrailed* coding sequences under the control of the hsp70 promoter and stably transfecting this DNA into the Schneider 2 line of Drosophila tissue culture cells. Heat shocking the resulting cell line (termed HS-EN) at 37° C resulted in the accumulation of high levels of *engrailed* protein in the cell nucleus (Figure 1); untransfected Schneider 2 cells do not express *engrailed*.

To determine whether the *engrailed* protein produced in these cells is modified by the action of protein kinases, HS-EN cells were heat shocked and labeled with $^{32}PO_4$, and a soluble protein extract was prepared. With a few differences, the pattern of total phosphorylated proteins in these cells is similar to those produced in untransfected Scnneider 2 cells (Figure 2a,b). However, immunoprecipitation of the ^{32}P-labelled HS-EN cell extract with antibodies directed against the *engrailed* protein produced a single labelled species (Figure 2d). This protein had a mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) indistinguishable from that of *engrailed* protein produced in E. coli. Immunoprecipitation of the parental Schneider 2 cell extract yielded no such phosphoprotein (Figure 2c).

The immunoprecipitated *engrailed* protein was hydrolyzed, and the resulting phosphoamino acids were analyzed (12). The phosphorylated amino acids of the *engrailed* protein isolated from HS-EN cells proved to

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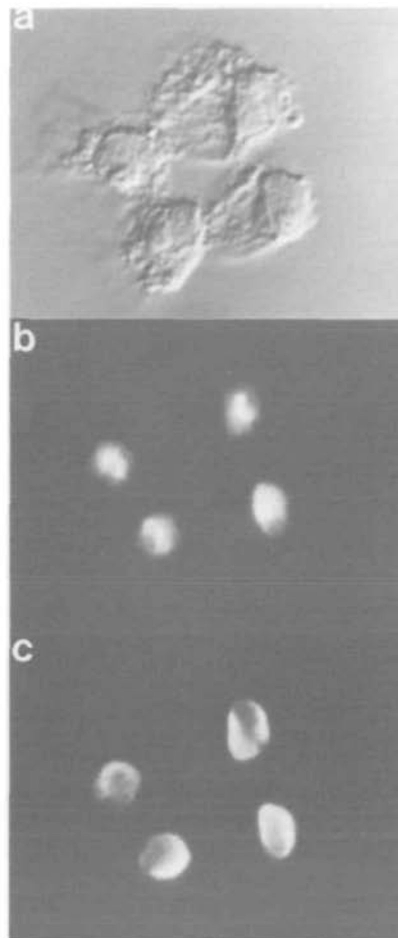

Figure 1 Expression of *engrailed* protein in HS-EN cells.
HS-EN cells, containing a gene fusion composed of a portion of the Drosophila hsp-70 promoter, the coding sequences of the *engrailed* gene, and the *engrailed* polyadenylation site, were heat shocked to induce expression of the *engrailed* protein, fixed, and stained for indirect immunofluorescence. Shown are four representative HS-EN cells after heat shock treatment that have been visualized by (a) differential interference contrast optics, (b) fluorescence optics to show the presence of the DNA binding dye 4,6-diamidino-2-phenylindole (DAPI) to localize nuclei, and (c) fluorescence optics to show binding of anti-*engrailed* antibody and rhodamine-coupled secondary antibody to localize *engrailed* protein.

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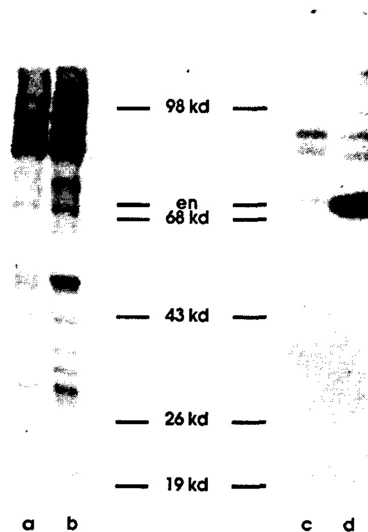

Figure 2 In vivo labelling of HS-EN and Schneider 2 cells and purification of phospho-*engrailed* protein by immunoprecipitation.
Cells were incubated with $^{32}PO_4$, and labelled protein was separated in a 12.5% SDS polyacrylamide gel and monitored by autoradiography. (a) Soluble protein extract of Schneider 2 cells. (b) Soluble protein extract of HS-EN cells. (c) Products of immunoprecipitation of Schneider 2 cell extract with anti-*engrailed* antibody. (d) Products of immunoprecipitation of HS-EN cell extract with anti-*engrailed* antibody. Protein molecular mass standards were run in adjacent lanes and detected by staining with Serva G. en = E. coli produced *engrailed* protein.

be mostly phosphoserine, with a trace of phosphothreonine (Figure 3). Phosphotyrosine was not detected.

The observed phosphorylation of the *engrailed* protein in HS-EN cells may be a consequence of heat shock, a non-physiological artifact of abnormal actions by protein kinases, or an artifact of overproduction of the *engrailed* protein in the tissue culture cell line. To show that the *engrailed* protein is also modified by phosphorylation in normal developing embryos, we used a modified octane permeablization technique to incorporate $^{32}PO_4$ into living embryos. Many proteins of a soluble protein extract of the embryo were labelled by this method (Figure 4a). Immunoprecipitation with *engrailed* antibodies resulted in predominantly a single phosphoprotein (Figure 4b) with a mobility identical to that of

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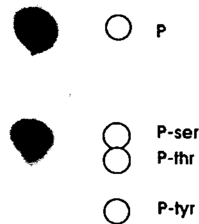

Figure 3 Phosphoaminoacid analysis of HS-EN produced phospho*engrailed* protein.
^{32}P-labelled phospho-*engrailed* protein prepared by immunoprecipitation and gel purification was hydrolyzed with 6M HCl. Hydrolysis products were separated by thin layer electrophoresis in an H_2O: acetic acid: pyridine (945:50:5) pH 3.5 buffer (12.). Standards (indicated by circles) were visualized with ninhydrin and the ^{32}P radiolabelled phosphoamino acids were localized by autoradiography.

engrailed protein synthesized in E. coli (Figure 4c). The very small amount of phospho-*engrailed* protein that can be purified from Drosophila embryos makes it impractical to identify and localize the modified amino acids in this material. In addition to the phospho-*engrailed* protein, a number of minor phosphoprotein species were present in the immunoprecipitate (Figure 4b). These other bands may represent proteins that share antigenic determinants with the *engrailed* protein (for example other homeobox containing proteins).

DISCUSSION

The *engrailed* protein contains 79 serine residues, 64 of which are conserved in the *engrailed* protein of a distantly related fruit fly, *Drosophila virilis* (13). The homeobox domain contains five serines, one of which, Ser 462, is conserved or substituted with threonine in all known homeobox sequences (Figure 5). Several groups of serine/threonine phosphorylating protein kinases have been characterized in mammalian

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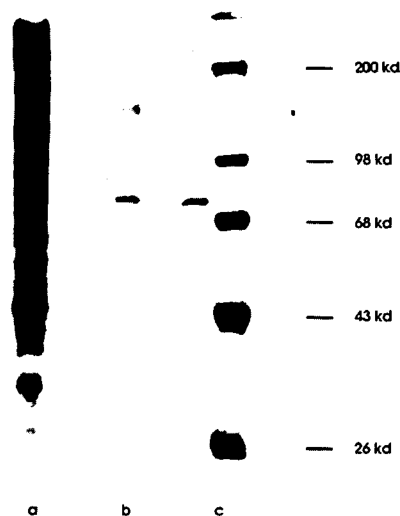

Figure 4 In vivo labelling of Drosophila embryos.
Embryos were labelled with $^{32}PO_4$ 2-6 hr after egg laying and *engrailed* protein was purified by immunoprecipitation. The immunoprecipitate was separated in a SDS polyacrylamide gel, and the gel was dried and autoradiographed for 10 days with an intensifying screen. (a) Soluble ^{32}P labelled embryo total protein extract. (b) Immunoprecipitation of ^{32}P-labelled embryo extract with anti-*engrailed* antibodies. (c) E. coli produced *engrailed* protein (left) and protein molecular mass marker.

cells, and for two such groups of kinases, a primary amino acid sequence specificity has been recognized (14). In the case of cAMP- and cGMP-dependent protein kinases, a basic dipeptide two or three residues to the amino side of the phosphorylated amino acid is required, whereas the Ca^{+2} dependent group exemplified by phosphorylase kinase requires an arginine residue two amino acids on the carboxy-terminal side of the modified amino acid. In the *engrailed* protein, no sequence that conforms to the former consensus is found, but Ser 182, 367 and 432 have an appropriate C-neighboring arginine residue (Figure 5). None of these is in the homeodomain, but all three are conserved in the *D. virilis engrailed* protein. Ser 432 is also conserved in the *D. melanogaster invected* protein and in the *engrailed*-related mouse proteins (15, 16).

Based on other examples, phosphorylation of the *engrailed* protein is likely to be of functional significance by causing a direct or allosteric

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MALEDRCSPQ	SAPSPITLQM	QHLHHQQQQQ	QQQQQQMQHL	HQLQQLQQLH	50
QQQLAAGVFH	HPAMAFDAAA	AAAAAAAAAA	AHAHAAALQQ	RLSGSGSPAS	100
CSTPASSTPL	TIKEEESDSV	IGDMSFHNQ	HTTNEEEEAE	EDDDIDVDVD	150
DTSAGGRLPP	PAHQQQSTAK	PSLAFSISNI	LSDRFGDVQK	PGKSIENQAS	200
IFRPFEANRS	QTATPSAFTR	VDLLEFSRQQ	QAAAAAATAA	MMLERANFLN	250
CFNPAAYPRI	HEEIVQSRLR	RSAANAVIPP	PMSSKMSDAN	PEKSALGSLC	300
KAVSQIGQPA	APTMTQPPLS	SSASSLASPP	PASNASTISS	TSSVATSSSS	350
SSSGCSSAAS	SLNSSPSSRL	GASGSGVNAS	SPQPQPIPPP	SAVSRDSGME	400
SSDDTRSETG	STTTEGGKNE	MWPAWVYCTR	YSDRPSSGPR	YRRPKQPKDK	450
TNDEKRPRTA	FSSEQLARLK	REFNENRYLT	ERRRQQLSSE	LGLNEAQIKI	500
WFQNKRAKIK	KSTGSKNPLA	LQLMAQGLYN	HTTVPLTKEE	EELEMRMNGQ	550
IP					

Figure 5 Potential phosphorylation sites in the *engrailed* protein sequence.
Serine residues are in bold type and those not conserved in *D. virilis engrailed* protein are italicized. Three residues with appropriately positioned C terminal arginine residues are outlined. The homeobox domain is underlined.

activation, inactivation, or modification of *engrailed* protein function. Histone H1 becomes modified when the cell is committed to mitosis (18); the degree of phosphorylation of the large T antigen of SV 40, a nuclear phosphoprotein involved in the regulation of viral transcription and initiation of viral replication, is correlated with an increase in site-specific DNA binding affinity (19), and phosphorylation of the NS phosphoprotein of vesicular stomatitis virus potentiates its role as an activator of viral RNA synthesis (20).

The reversible activation of substrate specific serine/threonine protein kinases is known to mediate the transduction of a variety of extracellular signals. One example is the G protein-mediated activation of phospholipase C by agonist receptor interactions resulting in the production of two second messengers by the hydrolysis of

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phosphatidylinositol 4,5 triphosphate to 1,2 (sn) diacylglycerol and inositol 1,4,5 triphosphate. Diacylglycerol directly activates the serine/threonine specific protein kinase C and inositol 1,4,5 triphosphate mobilizes intracellular Ca+2, a second activator of protein kinase C's (21). These are a family of closely related kinases, ubiquitous in animal cells, and implicated in the mitogenic effects mediated by many effector molecules (22-25). Similarly, the receptor- mediated activation of adenylate cyclases results in the synthesis of cAMP, an activator of the cAMP-dependent protein kinases. The cdc 2 gene product of Schizosaccharyomyces pombe, which is required to commit a cell to the mitotic cycle (26), is related to these mammalian cAMP-dependent enzymes. Thus, phosphorylation of the *engrailed* protein may modify its function in response to external stimuli, such as embryonically specified positional information. Alternatively, the stimuli may be mitogenic, and the *engrailed* protein function could be modulated in a cell cycle dependent manner. In this regard, a protein kinase C homolog which has recently been isolated from Drosophila contains a metal/DNA binding domain very similiar to that found in Xenopus TFIIIA and several other DNA-binding proteins (26). Sequence-specific DNA binding of the kinase homolog might facilitate phosphorylations of adjacent bound factors, such as *engrailed* protein.

MATERIALS AND METHODS

Cell and embryo culture and labeling

Plasmid phs-en is a derivative of plasmid pcopneo (27) which carries the bacterial gene encoding G418 resistance under the control of a copia transposable element promoter. phs-en was constructed by eliminating the single EcoRI site in pcopneo, and inserting into its single BamHI site a 450 bp BamHI-EcoRI fragment containing the Drosophila HSP-70 promoter (a gift from H. Stellar) that was fused to a 2 kb genomic EcoRI-BamHI fragment containing the *engrailed* polyadenylation site. A 2 kb EcoRI fragment containing coding sequences from an *engrailed* cDNA clone, c2.4 (7), was then inserted into the EcoRI site separating the heat shock promoter from the *engrailed* genomic sequences. The resulting plasmid, phs-en was introduced into Schneider line 2 cells by calcium phosphate mediated transfection, and a stable cell line, HS-EN, was selected for resistance to antibiotic G418 (Gibco). The parental Schneider 2 cells or the HS-EN cells were grown in spinner culture or on plates at 25° C in Schneider's Drosophila medium (Gibco).

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To label phosphoproteins, 2.5 x 10^7 HS-EN or Schneider 2 cells were collected by centrifugation (1500g, 5 min.) and washed twice in 25mM Tris-HCl pH7.2, 150mM NaCl. The cells were resuspended in 90% phosphate free Schneider Drosophila medium (Gibco), 10% dialyzed fetal calf serum and 2mCi of carrier free $^{32}PO_4$ (Amersham). The cells were then subjected to heat shock at 37° C for 45 min. and allowed to recover for 3 hrs. at 25° C. The cell were washed once with 3mM NaH_2PO_4, 7mM Na_2HPO_4, 150mM NaCl pH7.5 (PBS) and resuspended in 0.75 ml ice-cold lysis buffer (25mM Tris-HCl pH8, 150mM NaCl, 0.2% SDS, 0.5% NP40, 0.5% sodium deoxycholate and 1mM phenyl methyl sulphonyl fluoride). The lysate was passed twice through a 25 gauge syringe needle and insoluble material was removed by centrifugation (3 X 14,000g).

To label the embryo phosphoproteins, embryos were collected 2-6 hr after egg laying from corn meal agar plates, dechorionated by treatment with 1/2 strength bleach for 90 sec, and washed extensively with water. The dechorionated embryos were blotted gently with tissue paper to remove excess moisture and then approximately 500 were transferred to a 5ml glass tube. 1ml of octane was added and the embryos were mixed for 15 secs. 1ml of phosphate free Schneider medium was added, causing the embryos to aggregate at the interface. The octane was removed immediately with a pasteur pipette and residual solvent was blown off with compressed air (2-4 min.), causing the embryos to disaggregate and settle. The embryos were washed once with phosphate free medium (5ml), resuspended in 0.3ml of the same, and transferred to a 1ml glass pestle homogenizer into which 2.5mCi of $^{32}PO_4^{-3}$ had been dried. The embryos were incubated for 2 hours at 25° C with occasional mixing, then washed once with 5ml of PBS. 0.75ml of lysis buffer (see above) was added, the embryos were homogenized (25 strokes), and the lysate was passed twice through a 26 gauge syringe needle. Insoluble material was removed by centrifugation (3 X 14,000g), floating material was aspirated, and the soluble embryo protein extract was immunoprecipitated. After octane treatment a portion of the embryos was transferred to a vial and allowed to develop in the presence of mineral oil. Most of the embryos continued to develop and at least 10% hatched into first instar larvae. The method for labelling embryos was adapted from Inman, 1984 (28).

Anti-*engrailed* antibodies, immunoprecipitation, and histology

An *engrailed*-b-galactosidase fusion protein was generated in the vector pUR 278 (29) and consisited of the *engrailed* protein from residue 4 to the C-terminal end fused to b-galactosidase. The fusion protein was

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precipitated from extracts of E. coli with 60% ammonium sulphate, resuspended in PBS, and used to immunize rabbits. *engrailed* specific antibodies from rabbit sera were purified over a column of Affi-gel 10 (Biorad) to which E. coli produced *engrailed* protein (D. Rosen and T. Kornberg, unpublished) had been covalently linked. The column was washed with PBS and eluted with 0.1M glycine pH 3.0. The eluate was neutralized with a 1/20 volume of 1M Tris pH8 and dialyzed against PBS.

For immunoprecipitation reactions, 20 µl of anti-*engrailed* antibody was added to 300 µl of lysate and incubated at 4° C for 15 min. Then 75 µl of a 50% slurry of Sepharose-4B-coupled Protein A (Pharmacia) in PBS was added and the lysate incubated for a further 15 min. at 4° C. The immunoprecipitations were washed 5X5 min. with lysis buffer. The immunoprecipitated protein was removed from the beads by boiling for 5 min. in 100 µl of SDS-PAGE sample buffer (125mM Tris-HCl, pH6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol; 4M urea; .0025% bromophenol blue).

For immunostaining, HS-EN cells were allowed to settle onto glass cover slips in Schneider medium. Cells were heat shocked at 37° C for 30 min, followed by a 1 hr incubation at 25° C. The medium was removed and the cells were fixed in phosphate-buffered saline (PBS) containing 3.7% formaldehyde and 0.1% Triton-X100 for 15 min. After several washes with PBS, the cells were blocked for 30 min with a solution of PBS, 0.1% Triton-X100, 10% normal goat serum, and then stained with anti-*engrailed* antibody and a rhodamine-conjugated secondary antibody. After washing with PBS, the cells were treated with 1mg/ml 4'6-diamidino-2-phenylindole (DAPI) to visualize the DNA.

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