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Engrailed homeoprotein secretion is a regulated process

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SUMMARY

Chicken Engrailed 2 homeoprotein is transported between cells in culture. This intercellular transfer is based on unconventional secretion and internalisation mechanisms: Engrailed 2 has access to vesicles but lacks a signal sequence for secretion and is internalised by a non-endocytic process. We show that phosphorylation of a serine-rich domain within Engrailed 2 by the protein kinase CK2 specifically inhibits Engrailed 2 secretion. The availability of the serine-rich domain to CK2 is highly increased when it is displaced from its normal position to the C terminus of Engrailed 2, leading to a constitutive blockage of Engrailed 2 intercellular transfer. This indicates that intercellular transfer of Engrailed 2 is a highly regulated process.

Key words: Engrailed, Homeoprotein, Unconventional secretion, Phosphorylation, Regulation

INTRODUCTION

Homeoproteins are a class of transcription factors that are structurally and functionally conserved during evolution and involved in a wide range of biological processes, from embryonic development to terminal differentiation. They are defined by the presence of a 60 amino acid long DNA-binding domain: the homeodomain. In accordance with their role as transcriptional regulators, homeoproteins are predominantly localised in the nucleus. It has been demonstrated that chick EN2 homeoprotein (cEN2) expressed in COS-7 cells is secreted and subsequently internalised by neighbouring cells with no apparent degradation (Joliot et al., 1998). Homeoprotein internalisation requires the presence of a 16 amino acid motif corresponding to the third helix of the homeodomain (Derossi et al., 1994). Internalisation occurs both at 37°C and 4°C by a non-endocytic mechanism (Chatelin et al., 1996) and does not show any cell type specificity or require the presence of a specific chiral receptor (Derossi et al., 1996). Because homeoproteins are devoid of a classical signal sequence, their intercellular transfer also involves an unconventional secretion pathway. Although mainly nuclear, cEN2 homeoprotein is also detected both in cytosolic and membrane fractions of transfected COS-7 cells (Joliot et al., 1997). The latter membrane fraction has features characteristic of caveolae or microdomains. A small fraction (5%) of membrane-associated cEN2 pool is located in intra-luminal compartments (Joliot et al., 1997). Endogenous rat Engrailed homeoproteins expressed in the mes/metencephalic region show a similar intracellular distribution, arguing for a physiological significance of these observations (Joliot et al., 1997). Deletion of a short 11 amino acid sequence within cEN2 homeodomain inhibits both secretion and access to luminal intra-vesicular compartments (Joliot et al., 1998). The predominant role of the homeodomain for both secretion and internalisation may suggest a conservation of these properties among homeoproteins. Supporting this hypothesis, maize Knotted 1 homeoprotein shows both non cell-autonomous activity (Sinha and Hake, 1990) and intercellular transfer in vivo (Lucas et al., 1995), although not necessarily requiring protein transfer across the plasma membrane.

Protein phosphorylation is widely used to regulate their subcellular distribution, both in nucleo-cytoplasmic exchanges and in membrane targeting. It is a common post-translational modification found in homeoproteins. Among homeoprotein kinases identified, the serine/threonine protein kinase 2 (CK2) is ubiquitously expressed and phosphorylates murine Cut (Coqueret et al., 1998), Hox-b6 (Fienberg et al., 1999) and NKX2.5 (Kasahara et al., 1998), as well as *Drosophila melanogaster* Antennapedia (Jaffe et al., 1997), Engrailed (Bourbon et al., 1995) and Even-skipped (Li and Manley, 1999) homeoproteins. CK2 is a holoenzyme composed of two catalytic subunits (α and/or α') and two regulatory subunits (β), which adopt tetrameric $\alpha\alpha'/\beta 2$ or $\alpha 2/\beta 2$ structures. CK2 is essential for cell viability (Padmanabha et al., 1990) and is involved in multiple cellular functions (reviewed by Allende and Allende, 1995). We demonstrate a crucial role for CK2 activity in the regulation of the intercellular transfer of cEN2. CK2-dependent phosphorylation of a serine-rich domain in cEN2 drastically impairs cEN2 secretion. These results establish for the first time that intercellular transfer of homeoproteins is a regulated event.

MATERIALS AND METHODS

Plasmids, constructs and mutagenesis

Expression plasmids for all CK2 constructs are described in Heriche et al. (Heriche et al., 1997). The pTLcEN2Δ(1-180) and pTLcEN2Δ(1-142) plasmids were obtained from pTLcEN2 (Joliot et al., 1997). Synthetic oligonucleotides corresponding to the SRD sequence (SGAELSVSSDSDSSQAG), SRD5A (SGAELSVAADA-DAAQAG) or SRD5E (SGAELSVEEDEDEEQAG) were cloned within either cEN2 or downstream of the CDS. Eucaryotic GST-cEN2 fusion was obtained by subcloning GST upstream of cEN2. Details on plasmids constructs are available upon request.

Cell culture and electroporation

COS-7 cells were cultured and transfected by electroporation as previously described (Joliot et al., 1998). Rat embryonic neurones were prepared and cultured as previously described (Chamak and Prochiantz, 1989; Joliot et al., 1998).

Immunofluorescence and microscopy

COS-7 cells (5×10^3) were co-cultured with freshly dissociated rat E16 cortical neurones (2×10^5) for 48 hours and fixed as previously described (Joliot et al., 1998). Rabbit anti-Engrailed serum (1/1000) and rat monoclonal anti HOXC8 antibody were kind gifts from S. Saule and N. Peyreiras, respectively. Detection of NCAM was performed by a mouse monoclonal antibody (OB11, Sigma). Following secondary antibodies were used: Cy3-conjugated goat anti-mouse antibody (1/500, Jackson), biotinylated goat anti-rabbit antibody (1/200, Vector) and biotinylated donkey anti-rat antibody plus a DTAF-conjugated streptavidin (1/200, Jackson). All images were obtained using a digital camera (Spot, Diagnostic Instrument). Confocal microscopy was performed on a Leica system. All pictures were acquired using constant integration parameters and processed using Adobe Photoshop.

Quantification of intercellular transfer

Confocal sections (low magnification) were processed with NIH Image 1.6.2 (density slice tool). Briefly, cEN2 staining was quantified (Area×Intensity) in neurones and COS-7 cells. The ratio neurone/COS cEN2 staining was expressed as a percent of the condition showing maximum intercellular transfer.

cEN2 internalisation

Recombinant cEN2 was incubated with 10 units of CIP (New England Biolabs) for 30 minutes at 30°C or was phosphorylated (see below) by CK2 in 10 mM Tris pH 7.5 and 10 mM $MgCl_2$. The protein was diluted to 1μM in culture medium and incubated with 2×10^5 neurones for 1 hour at 37°C.

GST co-purification experiments

COS-7 cells (2.5×10^5) co-transfected with a HA-tagged CK2 subunits and GST fusions proteins were cultured for 24 hours on polyornithine-coated 60 mm culture dishes. After washing with PBS, cells were lysed and GST fusion protein was purified as described (Chatton et al., 1995).

Phosphorylation assays

Recombinant oligomeric CK2 or its isolated monomeric CK2α subunit was expressed in Sf9 cells and purified to homogeneity (Filhol et al., 1991). CK2 assays were performed using a specific CK2 peptide substrate as previously described (Bojanowski et al., 1993). GST or GST-cEN2 (3 μg) was incubated at 22°C with [γ-^{32}P] ATP or 10 μM ATP, and 10 mM $MgCl_2$ in the presence of 0.3 μg of either oligomeric CK2 or 1.0 μg monomeric CK2α subunit. After 15 minutes, the phosphorylated proteins were analysed by SDS electrophoresis and autoradiography.

Metabolic labelling and immunoprecipitation

COS-7 cells (2.5×10^5) expressing indicated constructs, were cultured for 24 hours on polyornithine-coated 60 mm culture dishes. After washes with phosphate-free medium (ICN), cells were incubated for 30 minutes in the same medium containing 250 μCi of [^{32}P] H_3PO_4 inorganic phosphate (HCl free, ICN biomedicals), and 10% (v/v) foetal calf serum dialysed overnight against TBS. Cells were lysed in boiling 2% SDS, scraped, boiled for 5 minutes and centrifuged for 15 minutes at 4°C. The supernatant was pre-cleared on protein A-sepharose for 30 minutes at room temperature and immunoprecipitated by an anti-Engrailed serum as described (Rousselet et al., 1988). Radioactivity was quantified by phosphoimaging.

Preparation of cell extracts for bi-dimensional electrophoresis

COS-7 cells (2.5×10^5) co-expressing indicated combination of constructs were cultured for 24 hours on polyornithine-coated 60 mm culture dishes. Cells were washed twice in PBS, scraped in 500 μl of PBS containing a protease inhibitor cocktail (Complete, Boehringer) and 1 mM sodium orthovanadate (except for CIP treatment) and sonicated. Extracts were incubated in 100 mM Tris pH 9.2 with 10 U of CIP (New England Biolabs) for 30 minutes at 30°C with or without 200 mM NaH_2PO_4 as a competitor. Rat mesencephalon was immediately resuspended in 8 M urea after dissection.

Gel electrophoresis and western blotting

SDS-PAGE was performed according to classical methods. Two-dimensional PAGE was performed according to O'Farrel (O'Farrell et al., 1977) and Larcher (Larcher et al., 1992) with minor modifications. First dimension separations were performed at basic non-equilibrium pH gradient electrophoresis (NEPHGE) for 1.5 hours at 500 V in 4% polyacrylamide gel containing 2% ampholine (1.6% pH 3.5-10.0 and 0.4% pH 5.0-8.0). Western blots were performed as described previously (Joliot et al., 1998). Rabbit polyclonal anti-Engrailed (1/25000) or AF24 rabbit polyclonal anti-Engrailed (1/1000) were used for Engrailed detection. Rat monoclonal anti-HA antibody (3F10, Boehringer) was used at 1/100 dilution.

RESULTS

EN2 is a phosphoprotein

Control cells or COS-7 cells transfected with cEN2 expression vector were incubated with ^{32}Pi and crude cell extracts were immunoprecipitated with an anti-Engrailed antiserum. A ^{32}P-labeled band migrating with the apparent molecular weight of cEN2 was detected specifically in transfected COS-7 cells (Fig. 1A), and was lost after calf intestine phosphatase (CIP) treatment of cell extracts (not shown), indicating that cEN2 was phosphorylated. To separate the different isoforms, the cell extracts from cEN2 expressing COS-7 cells were analysed by 2D gel electrophoresis. After separation, six spots were detected with the anti-Engrailed antibody (Fig. 1B): an abundant group of poorly resolved basic isoforms (1-3) and three well separated more acidic isoforms (4-6). When the extracts were treated with CIP, the pattern was reduced to the two most basic isoforms (Fig. 1B, CIP). Post-translational modification was investigated by separating in 2D electrophoresis crude mesencephalon extracts from E18.5 mouse embryos. As shown in Fig. 1C, endogenous mouse EN2 was resolved in multiple isoforms, most of which were sensitive to CIP

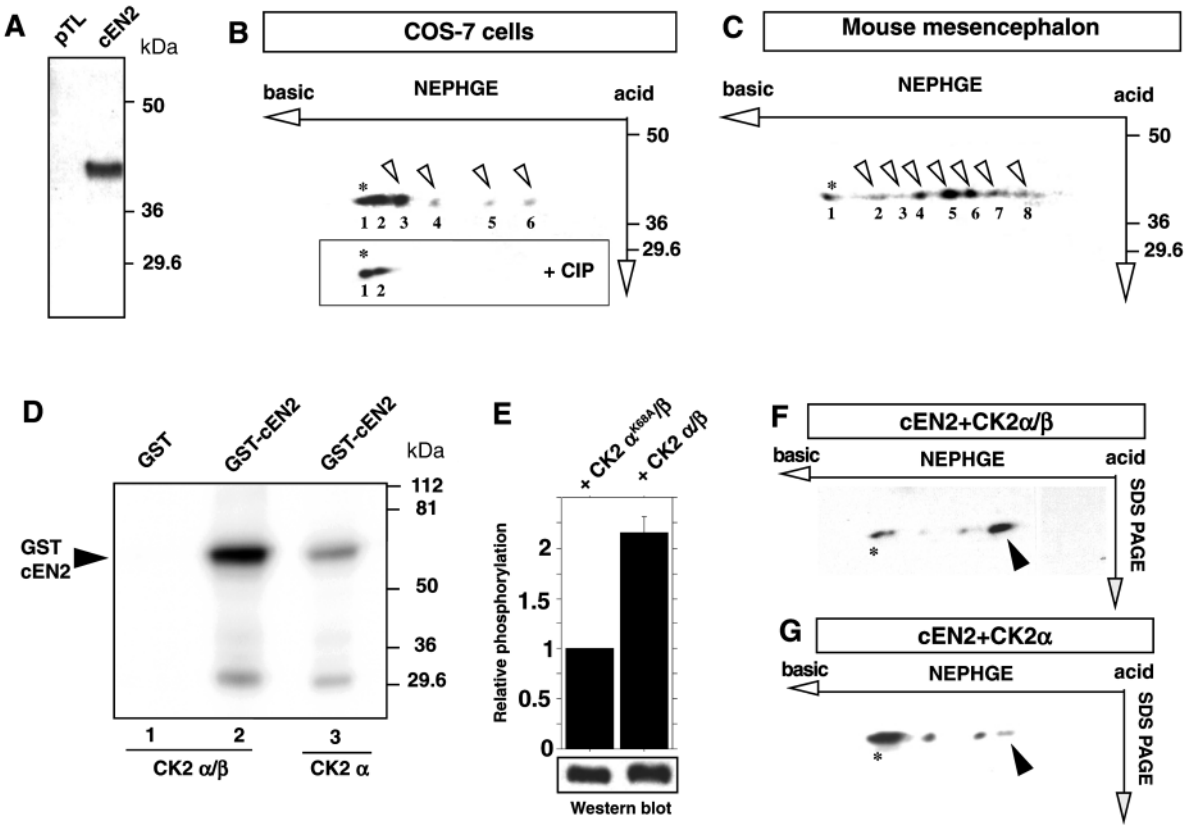

Fig. 1. cEN2 is phosphorylated at multiple sites both in COS-7 cells and is a substrate for CK2. (A) COS-7 cells were transiently transfected with control plasmid (pTL) or cEN2 expression plasmid (cEN2). Twenty-four hours later, cells were labelled with ^{32}Pi for 30 minutes and the cellular content was immunoprecipitated using an anti-Engrailed serum. Immunoprecipitated fractions were analysed by SDS-PAGE and autoradiography. (B) Crude cell extracts from COS-7 cells transiently expressing cEN2 were either non-treated or treated with CIP (CIP). Proteins were separated on 2D gels and subjected to immunoblotting with the anti-Engrailed anti serum. cEN2 is resolved into six isoforms (1-6). Arrowheads indicate isoforms selectively lost upon CIP treatment. (C) Crude cell extracts from E18.5 mouse mesencephalon were separated on 2D-gels and subjected to immunoblotting with an anti-Engrailed anti serum. EN2 is resolved in many isoforms. Arrowheads indicate isoforms selectively lost upon CIP treatment of the extracts (not shown). Two dimensional gels have been aligned using an invariant standard (bacterially expressed GST-cEN2). The asterisk indicates the position of the most basic isoform of cEN2 and mouse EN2; NEPHGE, non equilibrium pH gradient electrophoresis. (D) cEN2 is phosphorylated by CK2. Recombinant GST or GST-cEN2 were incubated with [γ-^{32}P]ATP in the presence of CK2 holoenzyme (lanes 1, 2) or of recombinant CK2α alone (lane 3). Phosphoproteins were resolved by SDS-PAGE and autoradiographed. GST is not phosphorylated and phosphorylation of cEN2 by CK2α is increased fourfold in presence of CK2β. (E) cEN2 phosphorylation is increased by CK2 transfection in COS-7 cells. COS-7 cells co-transfected with cEN2 and either wild-type CK2 (+CK2α/β) or a catalytically deficient mutant (+CK2α^{K68A}/β) were incubated with [^{32}P] H_3PO_4. [^{32}P] incorporation in cEN2 was quantified following anti-Engrailed immunoprecipitation. The relative phosphorylation of cEN2 upon CK2α/β versus CK2α^{K68A}/β co-transfection is 2.15±0.17 (mean±s.e.m, n=4). The lower panel indicates that equal amounts of cEN2 are immunoprecipitated. (F,G) cell extracts of COS-7 cells expressing the indicated combination of proteins were resolved by 2D gel and probed with anti-Engrailed antiserum. A dramatic increase in the hyperphosphorylated isoform (black arrowhead) is observed only upon CK2α/β overexpression. The asterisk (*) indicates the position of the most basic isoform of wild-type cEN2

treatment (not shown). Most of the endogenous protein is phosphorylated. Taken together, these experiments demonstrate that EN2 is a phosphoprotein.

cEN2 is a substrate for CK2 in vitro and in vivo

Among candidate kinases for cEN2 phosphorylation, CK2 is ubiquitously expressed in all eucaryotic cells and phosphorylates different homeoproteins, including *Drosophila* Engrailed protein (Bourbon et al., 1995). Kinase assays were performed on purified GST and GST-cEN2 fusion protein with recombinant chick CK2 catalytic subunit CK2α or the CK2α2/β2 holoenzyme. GST-cEN2 but not GST was strongly phosphorylated by the CK2 holoenzyme (Fig. 1D, lanes 1, 2). The phosphorylation of cEN2 by CK2α subunit alone was fourfold weaker than phosphorylation by the CK2 holoenzyme (lane 3).

CK2-dependent cEN2 phosphorylation was then analysed in a cellular context. As its 2D pattern indicates, the protein was poorly phosphorylated by endogenous kinases in COS-7 cells (Fig. 1B). cEN2 phosphorylation was increased by co-transfection with CK2 expression vectors. COS-7 cells co-transfected with vectors coding cEN2, CK2β and either CK2α or a kinase inactive mutant (CK2α^{K68A}) were incubated with ^{32}Pi and cell extracts were immunoprecipitated with the anti-Engrailed antibody. cEN2 phosphorylation was increased by twofold in CK2α/β compared to CK2α^{K68A}/β co-transfected

cells (Fig. 1E, upper panel), although cEN2 was expressed at similar levels (lower panel). The 2D pattern of cEN2 was shifted toward the most acidic isoform by CK2α/β co-transfection (Fig. 1F). Co-transfection with CK2α alone (Fig. 1G) or CK2α^{K68A}/β (not shown) did not modify cEN2 phosphorylation pattern.

CK2 overexpression inhibits cEN2 secretion and intercellular transfer

The influence of cEN2 phosphorylation on its intercellular transfer was then investigated. COS-7 cells co-transfected with cEN2 and either wild-type CK2α/β or the catalytic deficient mutant CK2α^{K68A}/β expression vectors, were co-cultured with primary neurones (Joliot et al., 1998). After 48 hours, cells were fixed and immunodetection of cEN2 was performed. When expressed alone, cEN2 was transferred from the producing COS-7 cells to the 'recipient' neuronal cells, as illustrated by the presence of cEN2-positive nuclei in neuronal cells (characterised by NCAM staining) (Fig. 2A). When co-transfected with wild type CK2α/β, cEN2 intercellular transfer was dramatically impaired as shown by the reduced number of cEN2-positive neuronal nuclei localised in the vicinity of producing COS cells (Fig. 2B). Co-transfection of the catalytic-defective holoenzyme CK2α^{K68A}/β (Fig. 2C) or CK2β subunit alone (not shown) had no effect on cEN2 intercellular transfer. The kinase activity of CK2 is therefore absolutely required for the inhibition of cEN2 intercellular transfer. In addition, the co-transfection of CK2α alone, which poorly phosphorylates cEN2, had no effect on the intercellular transfer of the protein (Fig. 2D). Intercellular transfer was quantified by measurement of cEN2 staining in neurones normalised by the staining measured in producing COS-7 cells (≥15 COS cells analysed for each condition). Co-transfection with CK2α/β resulted in a 75% reduction of cEN2 intercellular transfer compared to CK2α^{K68A}/β (Fig. 2E).

Intercellular transfer involves two successive steps of secretion and internalisation. To investigate which step was affected by cEN2 phosphorylation, bacterially produced cEN2 was pretreated either with CK2 or CIP and incubated with embryonic neurones (1 hour, 37ºC). Protein internalisation was monitored by immunochemistry after fixation. As shown in Fig. 2I,J, cEN2 phosphorylation by CK2 did not affect its internalisation by neurones. The phosphorylation state of the internalised protein was analysed 1 hour after ^{32}P-cEN2 addition. Before extraction, the culture was treated with CIP to eliminate all extracellular phosphorylated cEN2. Although CIP treatment efficiently dephosphorylates cEN2 located in the medium (Fig. 2K, lane 3), ^{32}P-cEN2 was recovered from the cells (lane 4). Therefore, phosphorylation does not block cEN2 internalisation.

Fragment 146-169 of cEN2 is necessary for both phosphorylation and CK2-induced inhibition of intercellular transfer

To determine which part of the protein was involved in this inhibition, nested N-terminal deletions of cEN2 were constructed and tested for their sensitivity to CK2α/β co-transfection in the co-culture assay. Deletion of the 142 N-terminal amino acids did not affect CK2-induced intercellular transfer inhibition (compare Fig. 3A with 3B). By contrast, the

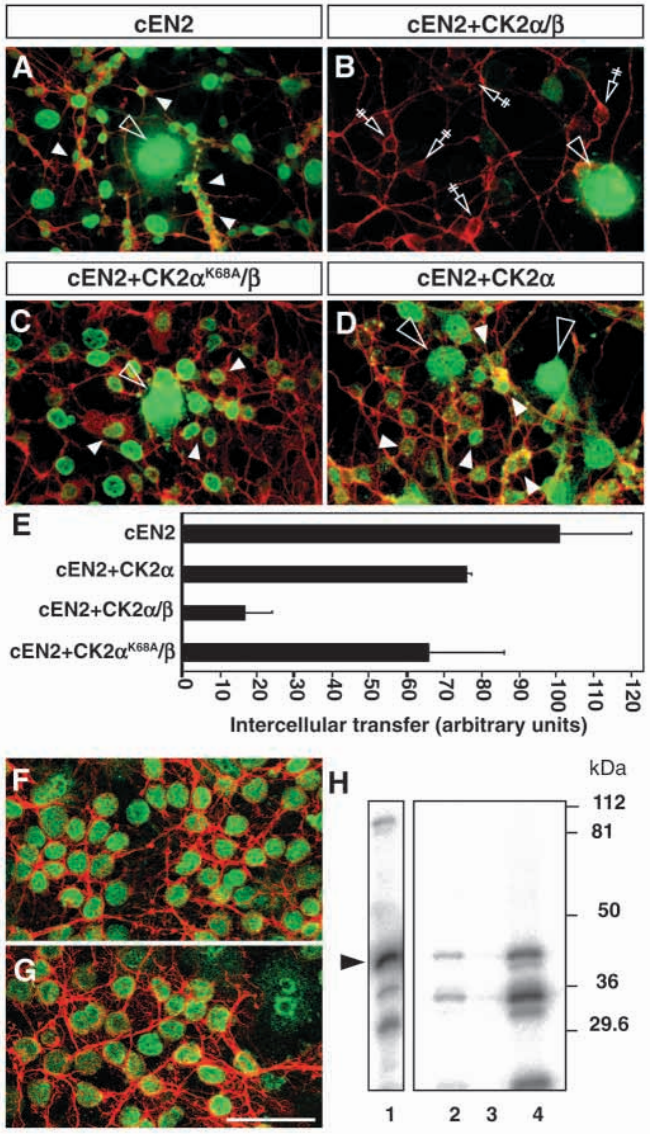

Fig. 2. CK2 inhibits intercellular transfer of cEN2. COS-7 cells transfected with cEN2 alone (A), or co-transfected with cEN2 and CK2α/β (B), CK2α^{K68A}/β (C) or CK2α (D) were co-cultured with rat embryonic neurones for 48 hours. After fixation cEN2 (green) and NCAM (red) were immunodetected. cEN2 transfer from COS-7 cells (open arrowheads, NCAM negative) to recipient neurones (NCAM positive) was monitored. cEN2 accumulation in recipient neurones (A-D, white arrowheads) is specifically inhibited upon CK2α/β overexpression (B, broken arrows). (E) Quantification of cEN2 intercellular transfer shown in A-D. (F,G) Bacterially produced cEN2 was phosphorylated by recombinant CK2 holoenzyme (G) or treated with CIP (F) and incubated with cultured rat embryonic neurones for 1 hour at 37°C. In both cases, cEN2 (green) is immunodetected on confocal sections within the nucleus of neurones (NCAM positive, red). Scale bar: 30 μm. (H) Bacterially produced cEN2 was phosphorylated by recombinant CK2 holoenzyme in presence of [γ-^{32}P]ATP (lane 1) and incubated with neurones for one hour at 37°C. Half of the internalisation medium was collected and immunoprecipitated with the anti-Engrailed serum (lane 2). The remaining medium (lane 3) and neurones (lane 4) were incubated with CIP for 15 minutes at 37°C and immunoprecipitated. Immunoprecipitates were resolved on SDS-PAGE and detected by autoradiography. ^{32}P-cEN2 is immunoprecipitated from CIP-treated cell extracts (lane 4), but not from CIP-treated medium (lane 3).

transfer of cEN2Δ(1-180) (lacking the 180-first amino-acids) was not inhibited by CK2α/β co-transfection (Fig. 3C). Fragment 142-180, was further subdivided and it was found that deleting amino acids 146-169 [cEN2Δ(146-169)] was sufficient to confer resistance to CK2-induced intercellular transfer inhibition (Fig. 3D). When transfected alone, all the deleted proteins were efficiently transferred between cells (not shown).

Bi-dimensional separation revealed that cEN2Δ(146-169) pattern was reduced to the 3 most basic isoforms whether or not CK2α/β was co-transfected (Fig. 3E,F). The link between CK2 and cEN2 was further assessed by testing their interaction. When co-expressed with GSTcEN2 in COS-7 cells, both CK2 subunits were efficiently retained on GST affinity columns (Fig. 3G, lane 1). Deletion of amino acids 146-169 (lane 3) or omission of CK2β subunit (lane 2) totally abolished this interaction. Using purified recombinant proteins we have observed that CK2β directly interacts with cEN2 (not shown).

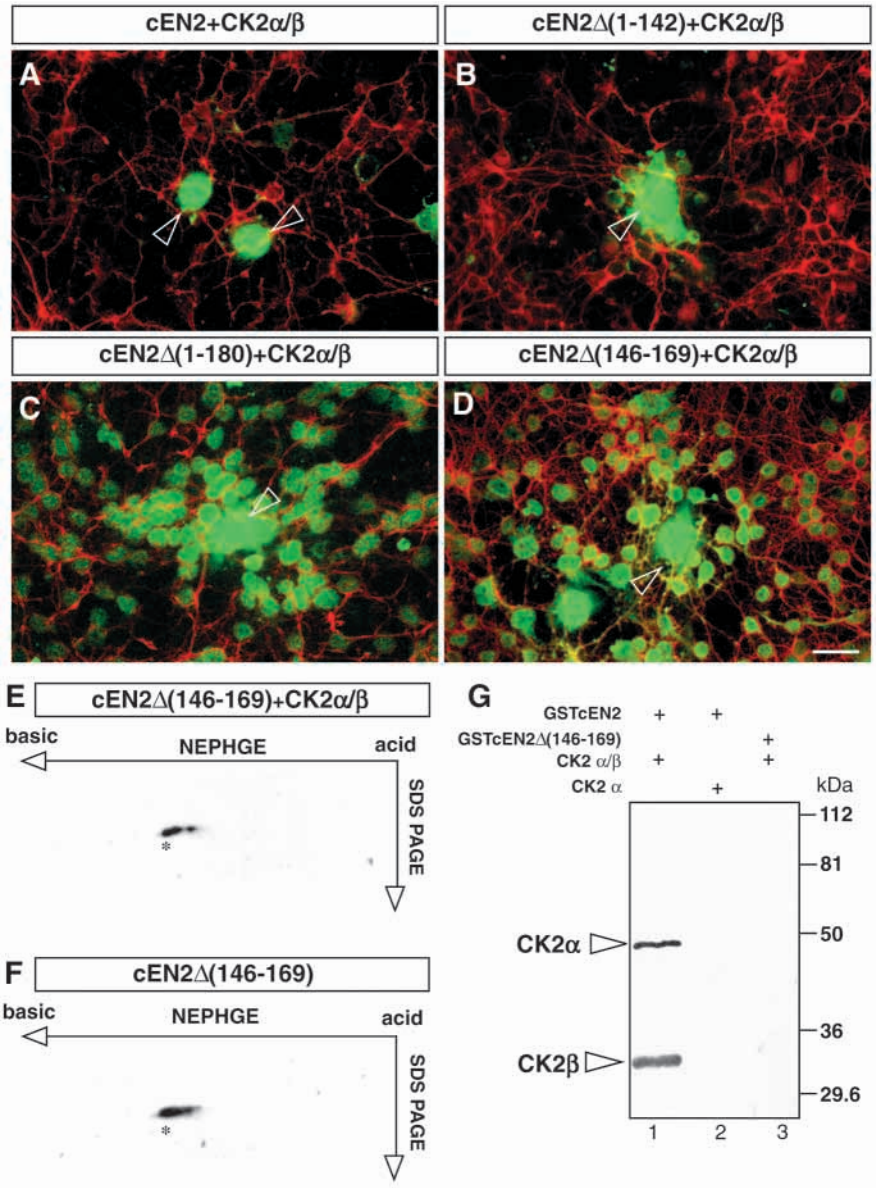

Fig. 3. Residues 142 to 169 of cEN2 are required for CK2-induced inhibition of intercellular transfer and phosphorylation. (A-D) Residues 142 to 169 are required for CK2-induced inhibition of transfer. Intercellular transfer of deletion mutants of cEN2 co-transfected with CK2α/β was analysed as in Fig. 2. CK2α/β overexpression inhibits intercellular transfer of both cEN2 (A) and cEN2Δ(1-142) (B), but does not affect intercellular transfer of cEN2Δ(1-180) (C) or cEN2Δ(146-169) (D). Arrowheads indicate transfected COS-7 cells. Scale bar: 30 μm. (E-G) Residues 142 to 169 of cEN2 are required for CK2 phosphorylation. (E,F) Extracts of COS-7 cells expressing cEN2Δ(146-169) alone (E) or together with CK2α/β (F) were separated on 2D gels and immunoblotted with the anti-Engrailed serum. cEN2Δ(146-169) 2D pattern is restricted to the three most basic spots (E) and no additional acidic spots are detected when CK2α/β is overexpressed (F). The asterisk indicates the position of the most basic isoform of wild-type cEN2. (G) Total cellular extracts of COS-7 cells co-expressing the indicated proteins were loaded on GST affinity columns. Bound material was analysed by western blot using an anti-HA antibody. cEN2 interaction with CK2 required the presence of both residues (146-169) and CK2β subunit.

Phosphorylation state of cEN2 serine rich domain controls intercellular transfer

The N-terminal part of cEN2 fragment 146-169 contains a domain highly enriched in serine residues (seven serines, SGAELSVSSDSDSSQAG). To verify that CK2-induced effects were mediated through this serine-rich domain, we performed S→A and S→E substitutions in the SSDSDSS sequence. These mutations should mimic unphosphorylated and phosphorylated serine residues, respectively. First, five serine to alanine substitutions were introduced in the sequence. The resulting protein (cEN2/5A) was co-expressed either with the active or the inactive CK2 holoenzyme and its 2D pattern was analysed. When co-transfected with CK2α/β, cEN2/5A pattern was reduced to the most basic isoforms (Fig. 4B), and was almost identical to cEN2Δ(146-169) pattern (Fig. 3E). As expected, the same pattern was observed in extracts from cells co-transfected with the inactive CK2α^{K68A}/β (Fig. 4A). Concomitantly, intercellular transfer of cEN2/5A became insensitive to CK2 co-transfection (Figs. 4C,D). When the five serine residues were then substituted by glutamates, cEN2/5E intercellular transfer was constitutively inhibited (Fig. 4E). Intercellular transfer of both cEN2/5A and cEN25E becomes independent of the presence of active CK2 (Fig. 4F). S→E substitution resulted in a 85% inhibition of cEN2 intercellular transfer compared with S→A substitution. The opposite effects of alanine and glutamate substitutions clearly demonstrate that the phosphorylation of these serines is directly responsible for the CK2-induced inhibition of intercellular transfer.

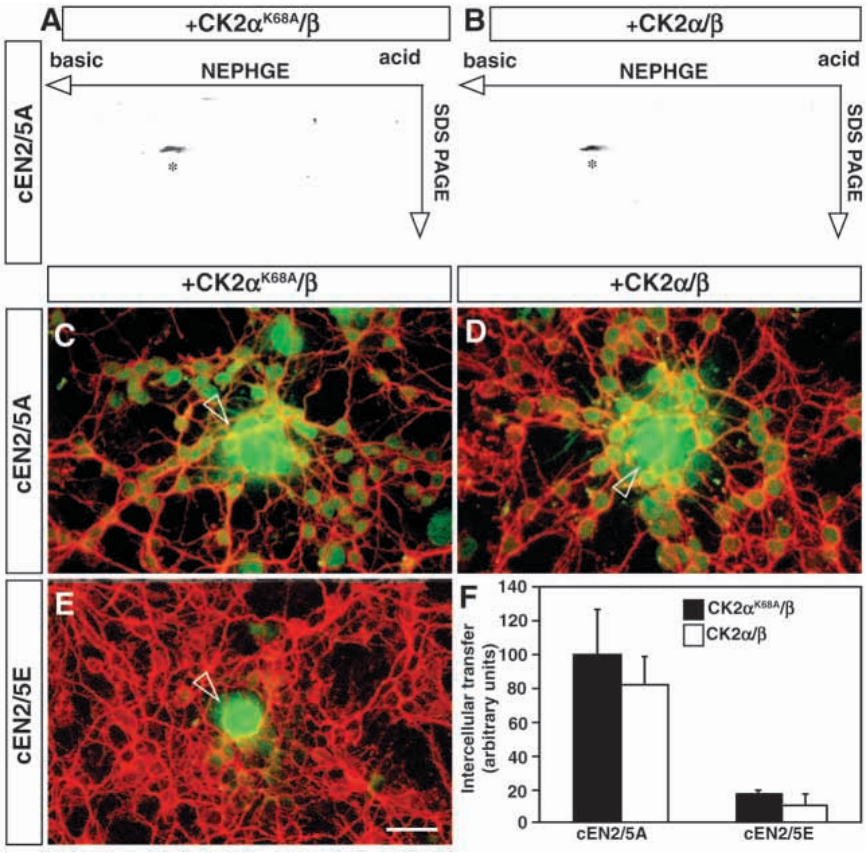

Fig. 4. cEN2 SRD phosphorylation controls intercellular transfer. (A-B) Serine to alanine substitutions within SRD abolishes CK2-induced phosphorylation. Extracts of COS-7 cells expressing cEN2/5A together with CK2α^{K68A}/β (A) or CK2α/β (B) were separated on 2D gel and immunoblotted with anti-Engrailed serum. cEN2/5A 2D pattern is restricted to the three most basic spots (A) and no additional acidic spots are detected when functional CK2 is expressed (B). The asterisk indicates the position of the most basic isoform of wild-type cEN2. (C-F) Serines substitution within SRD can mimic CK2-induced inhibition of transfer. Intercellular transfer of 5S→5A (C,D) or 5S→5E (E) substitution mutants of cEN2 co-expressed with either CK2α^{K68A}/β (C,E) or CK2α/β (D) was analysed as in Fig. 2. Although both mutants are insensitive to CK2 overexpression, cEN2/5A transfers between cells (C,D) and cEN2/5E does not transfer (E). (F) Quantification of cEN2 intercellular transfer. Arrowheads indicate transfected COS-7 cells. Scale bar: 30 μm.

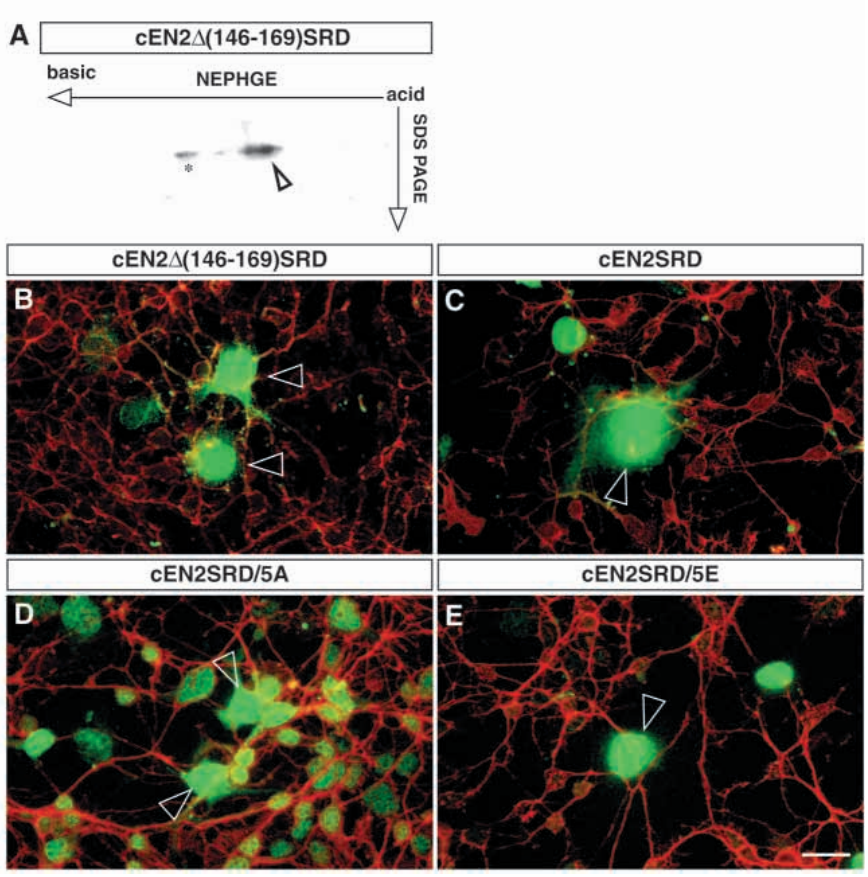

Fig. 5. SRD phosphorylation by endogenous CK2 inhibits homeoproteins intercellular transfer. (A) Extracts of COS-7 cells expressing cEN2Δ(146-169)SRD alone were separated by 2D gel and immunoblotted with anti-Engrailed serum. The acidic spot characteristic of wild-type cEN2 hyperphosphorylation is found (arrowhead). The asterisk indicates the position of the most basic isoform of wild-type cEN2. (B-E) Intercellular transfer of cEN2Δ(146-169)SRD (B) or cEN2SRD (C), cEN2SRD/5A (D), cEN2SRD/5E (E) expressed alone was analysed as in Fig. 2. Intercellular transfer of is inhibited independently of kinase overexpression (B). Ectopic addition of cEN2 SRD at the C terminus of wild-type cEN2 has the same inhibitory effect on intercellular transfer (C). S→A substitutions within the ectopic SRD restores transfer (D) but S→E substitutions does not (E). Arrowheads indicate transfected COS-7 cells. Scale bar: 30 μm.

Table 1. cEN2 is a substrate for endogenous CK2

Constructs	% of total CK2 activity co-purifying	Fold activation	
GST	0.407±0.040 (*n*=4)	1	
GST-EN2	1.720±0.213 (*n*=6)	4 (*P*=0.0078)	*P*=0.0265
GST-EN2Δ(146-169)	0.899±0.215 (*n*=6)	2 n.s.	
GST-EN2Δ(146-169)SRD	10.99±0.607 (*n*=4)	27 (*P*<0.0001)	

COS-7 cells expressing the indicated constructs were lysed and GST containing fusion proteins were purified. CK2 activity associated with these fusion proteins was quantified using a synthetic peptide substrate. The amount of CK2 activity is expressed as a fraction of total activity present before purification. Both cEN2 and cEN2Δ(146-169)SRD fusion proteins significantly interact with CK2, although with different efficacy (4× and 27×, respectively). Deletion of the SRD within cEN2 strongly decrease this interaction.

Autonomous action of cEN2 SRD on intercellular transfer

CK2 displays a high basal activity, raising the question of the poor efficacy of cEN2 phosphorylation by endogenous CK2. We asked whether SRD accessibility within cEN2 could be influenced by protein context or protein conformation. To this end, the SRD was shifted at the C terminus of cEN2Δ(146-169). In absence of overexpressed CK2, the 2D pattern of the protein was characterised by the predominance of one very acidic isoform (Fig. 5A), which was sensitive to CIP treatment (not shown). Unlike cEN2, the 2D pattern of cEN2Δ(146-169)SRD was not modified when CK2 was co-transfected (not shown). These results indicate that the new position of SRD increases its phosphorylation by endogenous kinases.

To investigate the contribution of the endogenous pool of CK2, we determined the amount of CK2 activity co-purified with cEN2 or cEN2Δ(146-169)SRD. Cell extracts from COS-7 cells transfected with GST, GST-cEN2 or GST-cEN2Δ(146-169)SRD expression vectors were loaded on GST-affinity columns. Endogenous CK2 activity retained on the column was quantified with a specific peptide substrate in the presence of [γ-^{32}P] ATP (Filhol et al., 1992). CK2 activity co-purified with cEN2Δ(146-169)SRD was dramatically increased (15-fold), compared with cEN2 activity (Table 1). This demonstrates that shifting SRD to cEN2 C terminus facilitates CK2-cEN2 interactions. In addition, endogenous CK2 activity co-purified with cEN2 is significantly higher than the one co-purified with GST alone (4×). Involvement of the native SRD was further assessed with GST-cEN2Δ(146-169). This small deletion provoked a 50% reduction in the amount of co-purified CK2 activity compared with wild-type protein (Table 1). These results clearly demonstrate that cEN2 interacts with endogenous CK2 through its SRD.

As the position of the SRD within cEN2 greatly influences its phosphorylation, we investigated its influence on cEN2 intercellular transfer. In the absence of overexpressed CK2, cEN2Δ(146-169)SRD intercellular transfer was undetectable (Fig. 5B). Similar results were obtained by grafting SRD at the C terminus of wild-type cEN2 (Fig. 5C). The role of serine phosphorylation within the ectopic SRD was further analysed by serine residues substitution. Alanine substitutions (cEN2SRD/5A) restored intercellular transfer (Fig. 5D), but glutamate substitutions (cEN2SRD/5E) did not (Fig. 5E).

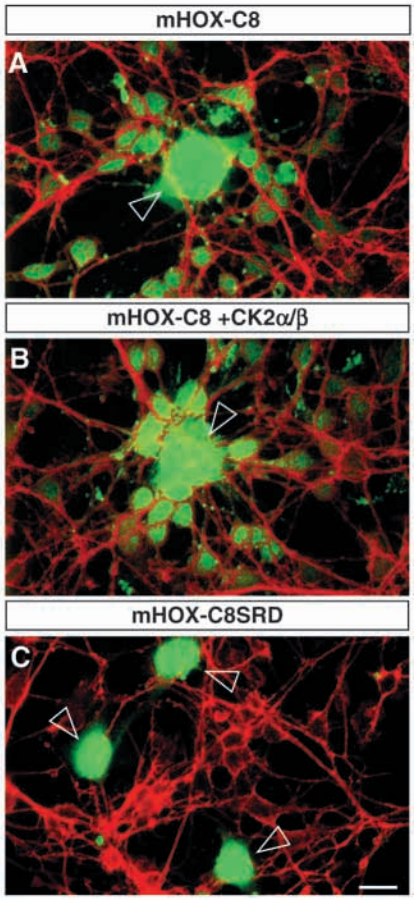

Fig. 6. Intercellular transfer of HOXC8 is insensitive to CK2. (A-C) Intercellular transfer of HOXC8 expressed alone (A), HOXC8 co-expressed with CK2α/β (B) or of HOX-C8SRD expressed alone (C) was analysed as in Fig. 3. Intercellular transfer of HOXC8 is not inhibited by kinase overexpression (B). Ectopic addition of cEN2 SRD at the C terminus of HOXC8 has a strong inhibitory effect on intercellular transfer (C). Arrowheads indicate transfected COS-7 cells. Scale bar: 30 μm.

Taken together, these results indicate that CK2-phosphorylated SRD autonomously blocks cEN2 intercellular transfer.

Specificity of CK2-induced intercellular transfer inhibition

Although S→E substitutions within the native SRD efficiently inhibit cEN2 intercellular transfer in the absence of overexpressed CK2, we could not ruled out an indirect effect of CK2 overexpression on the intercellular transfer of homeoproteins. Similar to cEN2, HOXC8 homeoprotein is transferred between cells (Fig. 6A) but is not a CK2 substrate (Fienberg et al., 1999). It was therefore a good candidate to investigate the specificity of CK2-induced intercellular transfer inhibition. As shown in Fig. 6A,B intercellular transfer of HOXC8 was insensitive to CK2 overexpression. However, grafting SRD to the C terminus of the protein led to constitutive inhibition of intercellular transfer (Fig. 6C), strongly suggesting that the effects of CK2 (overexpressed or not) on intercellular transfer absolutely requires the presence of CK2 sites within the homeoprotein.

DISCUSSION

Several Engrailed 2 orthologues in *Drosophila* (Bourbon et al., 1995) and mouse (this study) are phosphorylated. Because cEN2 homeoprotein overexpressed in COS-7 cells is phosphorylated at multiple sites, we have explored the influence of the phosphorylation of cEN2 on its intercellular transfer. COS-7 cells represent a good cellular model for the analysis of cEN2 intercellular compartmentalisation. We had previously demonstrated that identical amounts of cEN2 are present in a secretory compartment in vivo and in transfected COS-7 cells (Joliot et al., 1997). We demonstrate that the overexpression of CK2 holoenzyme (which efficiently phosphorylates cEN2) increases cEN2 phosphorylation in transfected cells and antagonises its intercellular transfer in a COS/neurone co-culture model. cEN2 internalisation is not affected by CK2 phosphorylation, indicating that intercellular transfer is inhibited through the secretion step. This report is the first demonstration of a regulation of homeoprotein intercellular transfer and secretion.

The inhibitory effect of CK2 on cEN2 intercellular transfer tightly correlates with cEN2 phosphorylation. Both phenomena require the presence of the regulatory CK2β subunit and intercellular transfer of HOXC8 (which is not phosphorylated by CK2) is insensitive to CK2 overexpression. Three different approaches have identified a cluster of five serine residues (amino acids 150-156 of cEN2) as the main phosphorylated target. First, the deletion of residues (146-169) in the cEN2 homeoprotein or S→A substitutions in the serine cluster, abolishes both CK2-induced cEN2 intercellular transfer inhibition and cEN2 phosphorylation. Second, S→E substitution of the same serine residues efficiently mimics the effect of CK2 on intercellular transfer. Third, shifting SRD (143-159) to the C terminus of cEN2 strongly increases phosphorylation of the serine cluster by endogenous kinases and provokes a constitutive inhibition of cEN2 intercellular transfer. It can be noticed that the serine cluster characterised in cEN2 is conserved in all vertebrate Engrailed 2 orthologues, both in term of sequence and position relative to the homeodomain.

cEN2 is poorly phosphorylated in COS-7 cells and the inhibition of its intercellular transfer is observed only when CK2 is overexpressed. Using a highly sensitive test for CK2 interaction, we have shown that cEN2 specifically interacts with endogenous CK2, requiring the presence of cEN2 SRD (Table 1). It should be mentioned that the deletion of the SRD as well as the S→A substitution impairs endogenous phosphorylation of cEN2 (compare Fig. 1B with Fig. 3E, Fig. 4B). SRD shift at the C terminus of the protein greatly enhances an interaction with endogenous CK2 (6,7×) that correlates with its phosphorylation in the absence of overexpressed CK2. These results strongly suggest that, in its native position, SRD accessibility to CK2 is downregulated, possibly by protein conformation or protein/protein interactions. Masking/unmasking the SRD and making it accessible to phosphorylation would thus be a primary event in intercellular transfer regulation.

Our study raises the question of the molecular mechanism of cEN2 intercellular transfer inhibition. SRD phosphorylation may promote interactions with regulators of intercellular transfer, either through direct interactions (supported by the autonomous activity of ectopic SRD) or through indirect effects on other domains of the protein. In the latter case, the homeodomain would be a potent candidate as it is the major determinant of homeoprotein subcellular distribution (Derossi et al., 1994; Joliot et al., 1998; Maizel et al., 1999). Indeed, the cEN2 homeodomain is efficiently transferred between cells (A. M. and A. J., unpublished), indicating that it contains all the sequences required for this process. In addition, HOXC8, which shares only the homeodomain in common with cEN2, becomes unable of intercellular transfer upon addition of cEN2 SRD.

Regulation of homeoprotein intercellular transfer could have important consequences on their physiological functions, as it could restrict this process spatially and temporally and limit it to well defined situations. The predominance of phosphorylated forms of Engrailed in physiological situation (Fig. 1C), may indicate that most of the protein is kept in an incompetent state for intercellular transfer. Such regulation could be specific to each class of homeoproteins, as the regulatory domain identified in cEN2 localised in a portion poorly conserved among homeoproteins.

Protein intercellular transfer is not a unique feature of homeoproteins. Indeed, several other proteins, although unrelated both in sequence and function, present a similar behaviour (reviewed by Prochiantz, 2000). The mechanisms involved in this process could be partly similar. In particular, intercellular transfer does not provoke irreversible modifications of these proteins, such as cleavage of a secretion signal sequence. In all cases, the transfer of these proteins between cells necessitates their accumulation in distinct subcellular compartments. Phosphorylation status may represent an important mechanism co-ordinating and/or regulating protein movements. Interestingly, the phosphorylation of HSV-1 VP22 protein has been proposed as a possible regulator of its intercellular transfer (Elliott et al., 1999; Pomeranz and Blaho, 1999).

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