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In vivo membrane trafficking role for an insect *N*-ethylmaleimide-sensitive factor which is developmentally regulated in endocrine cells

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Summary

The hexameric ATPase, *N*-ethylmaleimide-sensitive factor (NSF) is implicated in the release of neurotransmitters and in mediating fusion between intracellular membranes. Due to the conservation of proteins in constitutive and regulated membrane fusion reactions, NSF and its downstream targets have been predicted also to participate in fusion reactions underlying endocrine function, but there is little experimental evidence to support such a role for NSF in insect neuroendocrine secretion. Here we have characterized the NSF orthologue (MsNSF) from the endocrine model for development *Manduca sexta*. MsNSF is developmentally regulated in endocrine organs of the protocerebral complex. Enrichment of MsNSF in corpora cardiaca (CC) and not in corpora allata (CA) indicates that it might play a preferential role in releasing hormones produced in CC. Endocrine/paracrine cells of the enteric system in *M. sexta* exhibit selective MsNSF enrichment. Together the data point to a more selective participation of MsNSF in development of *M. sexta* by its involvement in a subset of factors, whereas other as-yet-unidentified homolog(s) might regulate secretion from CA and a large set of endocrine/paracrine cells. We further characterized the *in vivo* role of MsNSF by heterologous expression. In contrast to vertebrate NSF, MsNSF is functional in yeast membrane fusion *in vivo*. MsNSF rectifies defects in *SEC*18 (yeast NSF homologue) at nearly all discernible steps where Sec18p has been implicated in the biosynthetic route. This underscores the utility of our approach to delineate functional roles for proteins from systems that are not currently amenable to *in vitro* reconstitution.

Key words: *N*-ethylmaleimide-sensitive factor, NSF, membrane trafficking, neuroendocrine secretion, development, *Manduca sexta*.

Introduction

Membrane fusion events govern key cellular processes. Genetic and biochemical approaches have helped to understand intracellular membrane fusion in general terms by uncovering a large set of factors that is conserved in evolution (Pryer et al., 1992; Rothman, 1994). Among the conserved proteins, the *N*-ethylmaleimide-sensitive factor (NSF) has received considerable attention due to the central role it plays in most, if not all, membrane fusion reactions (Ikonen et al., 1995; Rothman, 1994; Wilson, 1995). NSF is recruited to membrane anchors by its cofactor, soluble NSF attachment protein (α-SNAP) (Weidman et al., 1989; Whiteheart et al., 1992). The intrinsic ATPase activity of NSF is stimulated by α-SNAP that is bound to its membrane receptors, SNAREs (Barnard et al., 1997; Morgan et al., 1994). The ATPase activity of NSF is thought to induce conformational changes in SNAREs that disassemble SNARE pairs formed between different intracellular membranes, subsequently leading to membrane fusion (Hanson et al., 1997; Söllner et al., 1993a). NSF-mediated SNARE disassembly has been implicated in many cellular fusion reactions, in both constitutive and regulated processes (for reviews, see May et al., 2001; Rothman, 1994; Whiteheart et al., 2001), but the stage at which such disassembly is required is not entirely clear. NSF is thought to act at a pre-fusion stage (Banerjee et al., 1996), possibly to prime docked vesicles (Golby et al., 2001; Kawasaki et al., 1998; Tolar and Pallanck, 1998), or at a post-fusion stage to break apart SNARE bundles to recycle components for future rounds of fusion (Littleton et al., 1998, 2001) or in the fusion stage itself as originally proposed (Söllner et al., 1993a,b).

NSF belongs to a superfamily of ATPases that are used in several cellular contexts (Patel and Latterich, 1998). Mutations in the yeast NSF homologue, *SEC*18, affect several steps in yeast biosynthetic and endocytotic pathways (Graham and Emr, 1991; Hicke et al., 1997; Riezman, 1985). Among these fusion reactions, purified Sec18p has been shown to reconstitute endoplasmic reticulum (ER)–Golgi (Barlowe, 1997), Golgi–ER (Spang and Schekman, 1998), and vacuole–vacuole fusion (Mayer et al., 1996). Functional conservation of NSF and Sec18p has been demonstrated by the ability of yeast cytosol overexpressing Sec18p to mediate fusion in mammalian *in vitro* systems employing

N-ethylmaleimide (NEM)-treated membranes (Wilson et al., 1989; Woodman et al., 1996). Sec18p is more resistant to NEM than to NSF (Steel et al., 1999) and, given the presence of other NEM-sensitive factor(s) participating in several fusion reactions (Goda and Pfeffer, 1991; Rodriguez et al., 1994), these data show only indirectly that Sec18p is a functional homologue of NSF. Thus far, purified Sec18p has been shown to function in a heterologous environment only in permeabilized chromaffin cells (Steel et al., 1999).

Yeast cytosol overexpressing Sec18p could impart transport competence to Chinese hamster ovary (CHO) Golgi membranes *in vitro* only when its cofactor Sec17p was present. α-SNAP (a cofactor for NSF) does not substitute for Sec17p in these reactions (Clary et al., 1990; Clary and Rothman, 1990; Wilson et al., 1989). Importantly, CHO NSF does not complement yeast *SEC*18 defects *in vivo* (Griff et al., 1992), neither is it known to be functional in Sec18p-mediated *in vitro* reactions. Cdc48p, the yeast homologue of p97 (related to NSF by sequence), participates in ER membrane fusion. Although vertebrate cytosol contains a potent yeast ER fusion activity, purified p97 is inactive in these assays and p97 does not complement *CDC*48 defects *in vivo* (Latterich et al., 1995). A simple interpretation for this lack of complementation is that vertebrate NSF and p97 would require their corresponding SNAP(s) (Clary et al., 1990) and p47 (Kondo et al., 1997) as cofactors to be functional in yeast.

Information converging from different experimental approaches indicates that homologues of participants in constitutive membrane trafficking might play similar roles in regulated exocytosis of neurotransmitters (Lin and Scheller, 2000) and hormones (Burgoyne and Morgan, 1998), but not enough is known about the molecular machinery involved in hormone and neuroendocrine secretion in insect model systems including *Drosophila melanogaster*. Our interest is in understanding the role and function of homologues of transport proteins in endocrine regulation of the developmental model, *Manduca sexta*. This system offers several tangible advantages compared to *Drosophila*; in particular, the neuroendocrine system and factors produced in it have been studied to a greater degree than in any other developmental model (Copenhaver and Truman, 1986b).

We have begun an analysis of factors involved in neuroendocrine function of *M. sexta*, borrowing from the expanding wealth of information in yeast and mammalian cell-free systems. We have previously cloned the *M. sexta* ortholog of NSF, MsNSF (Pullikuth and Gill, 1999). Here we characterize the *in vivo* function of MsNSF by using yeast as a tractable model system. Our results show that an animal NSF could genetically replace yeast Sec18p and does not require *M. sexta* α-SNAP-like protein(s) to be coexpressed to do so. Using an antibody specifically raised against MsNSF, we identified a novel, developmentally regulated role for MsNSF in neuroendocrine cells. Moreover, the selective enrichment of MsNSF in corpora cardiaca (CC) and enteric-endocrine cells of *M. sexta* suggests that MsNSF plays a preferential role in the secretion of selective hormones, while secretion of others would either be NSF-independent or might prefer as-yet-unidentified NSF homologue(s).

Materials and methods

Plasmids, strains and antibodies

The *SEC18* gene (pSEY8-SEC18) (Eakle et al., 1988) was kindly provided by Drs E. C. Gaynor and S. D. Emr, (University of California, San Diego, USA). Wild-type and *sec18* strains were obtained from Drs E. C. Gaynor and S. D. Emr, R. Schekman (University of California, Berkeley, USA) and H. Riezman (University of Basel, Switzerland) (Gaynor and Emr, 1997; Hicke et al., 1997; Rothblatt and Schekman, 1989) (Table 1). Antibodies to invertase, carboxypeptidase and α-factor were provided by Drs E. C. Gaynor, S. D. Emr, T. Graham (Vanderbilt University, Nashville, TN, USA), H. Reizman and T. H. Stevens (University of Oregon, Eugene, OR, USA). HSP150 antibody, which works best in immunoblots, was obtained from Dr M. Makarow (University of Helsinki, Helsinki, Finland) (Russo et al., 1992). Antibodies to Sec18p and Sec17p were gifts from Dr W. Wickner (Dartmouth Medical School, NH, USA) (Mayer et al., 1996).

Yeast growth conditions

Strains were either grown in rich medium (1 % yeast extract, 2 % peptone) supplemented with 2 % glucose (YPD) or 3 % galactose (YPG) or in minimal synthetic medium [YNB: 0.17 % yeast nitrogen base without amino acids and $(NH_4)_2SO_4$] supplemented with 0.5 % $(NH_4)_2SO_4$ and amino acids, depending on the selection required. Cells for metabolic labeling were grown in minimal medium [containing 200 μmol l^{-1} $(NH_4)_2SO_4$] with selective supplements lacking methionine and uracil, from a saturated culture in rich medium.

Yeast expression construct

The coding sequence from MsNSF cDNA (approx. 5.5 kbp) (Pullikuth and Gill, 1999), was amplified with primers corresponding to the start and stop codon with *Kpn*I and *Not*I restriction sites, respectively. Upstream (5′-TCCTGGTACC-GGTCCATGTCTTCTATGCGTATGAAGGGAGGA-3′) and downstream primers (5′-TTATGCGGCCGCTCATTCTTATT-GAATAGTAGTGCCTAGATCTAG-3′) [underlined sequences correspond to coding region of the native open reading frame (ORF)] were used in amplification with Long Template® polymerase chain reaction (PCR) system (Roche Biochemicals). The amplified product was cloned downstream from the GAL1 promoter in the yeast expression vector, pYES2 (*URA*3, *Amp*^{r}, 2 μm ori) (Invitrogen) to yield the plasmid P_{GAL}-MsNSF. This introduced a 55 bp spacer between the GAL1 promoter and the initiator codon of MsNSF. The entire coding sequence of MsNSF in P_{GAL}-MsNSF was sequenced to confirm that no PCR errors were incorporated. A *Bam*HI/*Hin*dIII fragment containing the entire reading frame and 5′ untranslated region of the yeast *SEC*18 (in pSEY8) was cloned into *Bam*HI/*Hin*dIII digested pBlueScript (SK+) to yield the construct pASH-SK18. A *Bam*HI/*Xho*I fragment from

Table 1. *Yeast strains used in this study*

Strain	Genotype	Source
X2180-1B	*MAT*α *gal2 SUC*2 *mal mel CUP*1 (wild type)	YGSC[1]
RSY 248	*MAT*α *his4-619* (wild type)	R. Schekman[2]
RSY 249	*MAT***a**, *his 4-619, ura3-52* (wild type)	R. Schekman
SEY 5186	*MAT*α *sec*18-1 *ura3-52 leu2-3, 112*	E. C. Gaynor and S. D. Emr[3]
ASHY 1896-1	*MAT*α *sec*18-1 *ura3-52 leu2-3, 112* (pYES2::*URA*3)	This study
ASHY 1896-2	*MAT*α *sec*18-1 *ura3-52 leu2-3, 112* (P_{GAL}-MsNSF::*URA*3)	This study
ASHY 1896-5	*MAT*α *sec*18-1 *ura3-52 leu2-3, 112* (*SEC*18::*URA*3)	This study
ASHY 1896-7	*MAT*α *sec*18-1 *ura3-52 leu2-3, 112* (P_{GAL}-*SEC*18::*URA*3)	This study
RSY 271	*MAT*α *sec*18-1 *ura3-52 his4-619*	R. Schekman
ASHY 1896-9	*MAT*α *sec*18-1 *ura3-52 his4-619* (pYES2::*URA*3)	This study
ASHY 1896-10	*MAT*α *sec*18-1 *ura3-52 his4-619* (P_{GAL}- MsNSF::*URA*3)	This study
RSY 319	*MAT*α *sec*18-*2 leu2-3 112*	R. Schekman
RSY 272	*MAT***a**, *his 4-619, ura3-52, sec*18-1	R. Schekman
ASHY 18a-11	*MAT***a**, *his 4-619, ura3-52, sec*18-1 (pYES2::*URA*3)	This study
ASHY 18a-12	*MAT***a**, *his 4-619, ura3-52, sec*18-1 (*SEC*18::*URA3*)	This study
ASHY 18a-13	*MAT***a**, *his 4-619, ura3-52, sec*18-1 (P_{GAL}-*SEC*18::*URA*3)	This study
ASHY 18a-14	*MAT***a**, *his 4-619, ura3-52, sec*18-1 (P_{GAL}-MsNSF::*URA*3)	This study
SEY 5188	*MAT*α *sec*18-1 *ura3-52 leu2-3 112 suc* 2Δ9	E. C. Gaynor and S. D. Emr
ASHY 18-1	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 suc* 2Δ9 (pYES2::*URA*3)	This study
ASHY 18-2	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 suc* 2Δ9 (P_{GAL}- MsNSF::*URA*3)	This study
ASHY 18-3	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 suc* 2Δ9 (*SEC*18::*URA3*)	This study
ASHY 18-4	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 suc* 2Δ9 (P_{GAL}-*SEC*18::*URA*3)	This study
EGY 1181-5	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 his*3*suc* 2Δ9	E. C. Gaynor and S. D. Emr
ASHY 1181-1	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 his*3*suc* 2Δ9 (pYES2::*URA*3)	This study
ASHY 1181-2	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 his*3*suc* 2Δ9 (P_{GAL}- MsNSF::*URA*3)	This study
ASHY 1181-3	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 his*3*suc* 2Δ9 (*SEC*18::*URA3*)	This study
ASHY 1181-4	*MAT*α *sec*18-1 *ura3-52 leu2-3,112 his*3*suc* 2Δ9 (P_{GAL}-*SEC*18::*URA*3)	This study
RH 448	*MAT***a** *ura3, leu2, his4, lys2, bar1-1*	H. Riezman[4]
RH 1737	*MAT***a** *sec18-20, ura3, leu2, his4, bar1-1*	H. Riezman

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[4]University of Basel, Basel, Switzerland.

pASH-SK18 was cloned into pYES2 to yield the plasmid pASH18GAL, (PGAL-*SEC18*). Yeast strains were transformed by the lithium acetate method (Ausubel et al., 1994).

Immunoprecipitation of carboxypeptidase Y (CPY) and MsNSF from yeast cells

Cells were grown overnight at 24 °C in YNB with 5 % glucose and supplements, omitting uracil and methionine. Cells equivalent to an A_{600} of about 50 were pelleted and grown at 24 °C in induction medium (2 % galactose) for 2–8 h as indicated in the figure legends. Prior to labeling, cells were preincubated for the times indicated in the figure legends to impose a complete block of *sec*18-1. Metabolic labeling was done with *trans*-^{35}S (ICN Biochemicals) at 25 μCi (925 kBq)/1 A_{600} equivalent of cells. Labeling was stopped by the addition of a chase mixture containing 20 mmol l^{-1} each of methionine, cysteine and $(NH_4)_2SO_4$. Chase portions (5–10 A_{600} units) were precipitated with 10 % trichloroacetic acid (TCA) on ice. TCA precipitates were pelleted and washed twice with acetone (prechilled at –20 °C) and dried under vacuum. Acetone-dried pellets were disrupted with glass beads and immunoprecipitation carried out as previously described (Gaynor and Emr, 1997) using Protein-A sepharose CL4B to sediment the immunocomplexes. After the final wash, the beads were dried under vacuum, resuspended in SDS–PAGE sample buffer and analyzed by SDS–PAGE. Fluorography was performed with Entensify® solutions (DuPont).

Labeling cells for media proteins

About 10 A_{600} equivalents of cells grown as above were concentrated and resuspended in fresh medium and preincubated for 15 min at 37 °C. Bovine serum albumin (500 μg ml^{-1}) and α2-macroglobulin (0.05 units ml^{-1}, Roche Biochemicals) were added prior to preincubation. Cells were labeled and chased as above. The chase was stopped by the addition of NaN_3–NaF on ice at a final concentration of 20 mmol l^{-1} each. The culture medium was separated from cells by centrifugation at 14 000 ***g*** for 5 min. Cell pellets and media were precipitated separately with 10 % TCA on ice.

TCA precipitates were washed with acetone and dried as described above. Pellets were resuspended by sonication in 50 µl of TETx (10 mmol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA, 0.5 % Triton X-100), an equal volume of SDS–PAGE sample buffer (with 5 % 2-mercaptoethanol) was added, and the samples were heated at 85 °C for 15 min. Denatured samples were clarified at 12 000 ***g*** for 5 min and equal A_{600} equivalents of the supernatants analyzed by SDS–PAGE. Acetone-washed cell pellets were lysed with glass beads and divided into two portions. One portion was immunoprecipitated with α-MsNSF and the other with α-CPY to check for any block of CPY in *sec*18-1 mutants.

Analysis of HSP150 trafficking

Cells were grown as for labeling medium proteins but in rich medium, YPG (3 % galactose). Equal portions were washed twice in water, once in fresh medium and resuspended in prewarmed medium (37 °C) and incubated at 37 °C for 30 min. Cycloheximide was added to a final concentration of 20 µg ml^{-1} (from a stock of 2 mg ml^{-1} in 95 % ethanol). Samples were removed just before (0 min chase) or 30 min after the addition of cycloheximide (see Fig. 3C legend). Cells and media were transferred to 20 mmol l^{-1} NaN_3–NaF on ice and separated by centrifugation (14 000 ***g***, 5 min). Cell and media proteins were TCA-precipitated, acetone-washed and air-dried. Precipitates were separated by SDS-PAGE, immunoblotted with anti-HSP150 antibody (1:5000) and detected with anti-rabbit horseradish peroxidase (HRP) antibody (diluted 1:2500) using ECL (Amersham Pharmacia).

Protein extraction, immunoblotting and immunohistochemistry

M. sexta tissue dissection and protein extraction have been described previously (Pullikuth and Gill, 1999). Insects were staged and tissues dissected under ice-cold PBS (20 mmol l^{-1} $NaPO_4$, 250 mmol l^{-1} NaCl, pH 7.6), then immediately fixed in Bouin's fixative (1:3:0.1, formaldehyde (37 %):water-saturated picric acid:glacial acetic acid) or in PBS-buffered 4 % paraformaldehyde at 4 °C overnight. Fixed tissues were processed for whole-mount histochemistry or paraffin sectioning as described (Pullikuth, 1997). Briefly, for whole-mounts, the neurilemma was manually removed, rinsed several times in PBST (PBS with 1 % Tween-20) and blocked in PTS (PBST containing 10 % normal goat serum) for at least 5 h at 4 °C. Blocked tissues were incubated with the appropriate antibodies in PTS for 3 days at 4 °C with gentle rocking. Secondary antibodies conjugated to Cy3 (goat anti-rabbit, for polyclonal primary antibody) or Cy2 (goat anti-mouse, for monoclonal primary antibodies) (Jackson ImmunoResearch Laboratories, West Groove, PA, USA) were used at a dilution of 1:200 (from a stock diluted 1:2 in 50 % glycerol) in PBST. Fluorescence observations were made with a Zeiss Axiophot fluorescence microscope fitted with the appropriate filters. For thin-section histochemistry, paraffin ribbons (7–10 µm thick) were adhered to poly(lysine)-coated slides treated with gelatin (2 %), deparaffinated in xylene (descending series)/ethanol (ascending series) followed by rehydration to 20 % ethanol. All other steps were the same as above except that antibody incubations were done in a humid chamber overnight and solutions contained 0.1 % Tween-20.

Antibodies to insect neuropeptides and hormones

Affinity-purified α-MsNSF antibodies have been described (Pullikuth and Gill, 1999). Antibodies to insect hormones and peptides were generous gifts from the following sources: diuretic hormone and leucokinin (J. Veenstra, University of Bordeaux, France), eclosion hormone, C terminal (Drs T. G. Kingan and M. E. Adams, University of California, Riverside, CA, USA), ecdysis triggering hormone (Drs D. Zitnan and M. E. Adams), proctolin and FMRFamide (T. G. Kingan), bombyxin (D. Zitnan), *M. sexta* prothoracicotropic hormone, PTTH (Dr W. Bollenbacher, University of North Carolina, NC, USA), small cardioactive peptide B, SCP_B (Dr D. Willows, University of Washington, WA, USA), silkmoth period and PTTH (Drs I. Sauman, S. Reppert and T. Gotter) and *Drosophila* synaptotagmin (Dr Hugo Bellen, Baylor College of Medicine, Texas, USA). Monoclonal antibody (HPC-1) to syntaxin was purchased from Sigma (St Louis, MO, USA).

Results

Expression constructs

The consensus sequence surrounding the initiation codon in yeast, A/pyAA/UAUGUCU, differs from that in the vertebrate CA/GCCAUGG (Romanos et al., 1992). The native MsNSF cDNA contains an excellent consensus around the initiation codon (AAAAATG TCT TCT) for expression in yeast. Our *in vitro* manipulation introduced a T residue at –3 (with respect to ATG) and retained the critical TCT TCT (second and third codon) and TAA (stop) without altering any other sequence in the ORF. *In vitro* translation of this construct yielded a doublet of 81 and 83 kDa similar to the native 5.5 kbp clone in pSPORT (Pullikuth and Gill, 1999) and to *in vitro* and *in vivo* translated Sec18p (Eakle et al., 1988). Introduction of this expression construct P_{GAL}-MsNSF into yeast produced immunoprecipitable protein when induced with galactose (see below). Additional MsNSF constructs were made by cloning the 540 bp 5′ region upstream from the *SEC18* initiator codon to MsNSF ORF in pYES2 (P_{GAL}-5′-SEC18-MsNSF) and pSEY8. These constructs did not yield significant MsNSF expression in yeast and, in the case of former, expression was detected only when induced with galactose (not shown). Thus the P_{GAL}-MsNSF construct was used in the experiments described here. Initial experiments were done with both *sec*18-1 (Novick et al., 1980) and *sec*18-20 (Hicke et al., 1997) alleles (Table 1). Under restrictive conditions growth phenotype and transport of CPY were similar in both strains with different genetic backgrounds, thus MsNSF in the *sec*18-1 allele was characterized further.

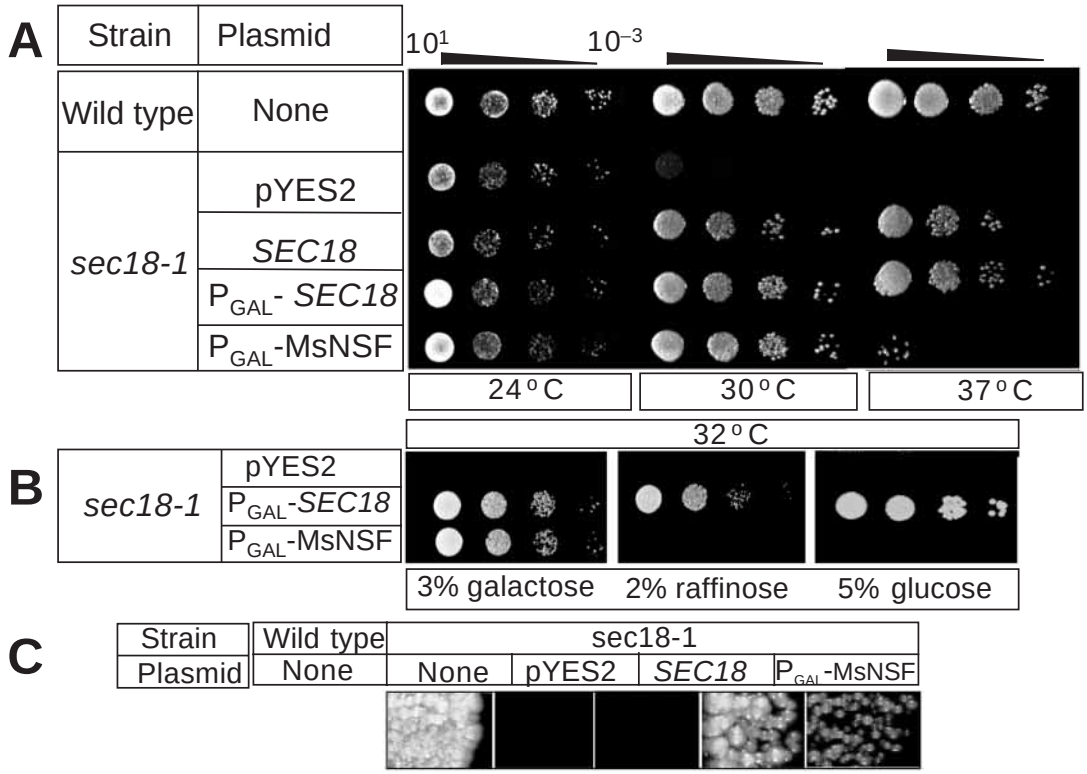

Fig. 1. *In vivo* expression of MsNSF rescues the temperature-sensitive growth defect in sec18-1 yeast mutant. (A) Wild-type (RSY 248) or strains derived from *sec*18-1 (SEY 5186) (Table 1) containing the 'empty' vector, pYES2 (ASHY1896-1), wild-type *SEC18* gene (ASHY1896-5) or *SEC18* (ASHY1896-7) and MsNSF (ASHY1896-2) cloned downstream from the *GAL1* promoter (P_{GAL}) were grown at 24 °C. Serial dilutions (indicated above panels) were spotted on to rich agar medium containing 3 % galactose and incubated at 24 °C (left), 30 °C (middle) or at 37 °C (right) for 3 days. (B) Cells were incubated at 32 °C in rich agar medium containing 3 % galactose (left), 2 % raffinose (middle) or 5 % glucose (right) as carbon source. (C) Wild-type (X2180-1B) or cells derived from *sec*18-1 containing various constructs were incubated at 37 °C in a humid chamber for 8 days and returned to 22 °C for 4 days.

Rescue of sec*18-1 temperature-sensitive growth defect by heterologous expression of MsNSF*

Drosophila has two NSF homologues (Boulianne and Trimble, 1995; Ordway et al., 1994; Pallanck et al., 1995b); however, neither is known to be involved in intra-Golgi transport *in vitro* or *in vivo*, much less to be functional in yeast. The mutant allele *sec*18-1 exhibits temperature-sensitive growth inhibition at ≥28 °C, whereas its growth is similar to wild type at 24 °C (Fig. 1A). To test if MsNSF possesses Sec18p-like activity, MsNSF was expressed under the regulation of the GAL1 promoter, which could be induced to high levels with galactose (Table 2). *Sec*18-1 cells containing P_{GAL}-MsNSF or *SEC18* gene with its native 5′ region, or P_{GAL}-*SEC18*, were spotted on galactose-containing medium and grown at permissive and non-permissive temperatures (Fig. 1A,B). The temperature-sensitive growth was rescued by MsNSF expression in *sec*18-1 cells, which was comparable to wild-type or *SEC18*-carrying cells at 30 °C (Fig. 1A). Without prior induction of MsNSF, complementation was only modest at 37 °C (Fig. 1A).

Glucose suppresses GAL1 promoter activity. Raffinose neither suppresses nor induces GAL1 promoter, suggesting that the GAL1 promoter is constitutively active when raffinose alone is provided as carbon source. Cells were grown as above and spotted on medium containing different carbon sources. As shown in Fig. 1B, *sec*18-1 failed to grow at 32 °C, but this was alleviated by MsNSF expression when induced with galactose. This rescue was completely suppressed by glucose and raffinose, which indicates that the growth phenotype was a

Table 2. *Expression levels of MsNSF and Sec18p*

Plasmid	Carbon source (%)	Induction time (h)	Expression level*
pSEY8-SEC18	Glucose (5)	18	31±2
	Raffinose (2)	16	27±4
	Galactose (2)	4	29±6
		16	30±8
P_{GAL}-SEC18	Raffinose (2)	16	30±2
	Galactose (2)	4	100±11
		16.5	>375
P_{GAL}-MsNSF	Glucose (5)	18	ND
	Raffinose (2)	16	ND
	Galactose (2)	4	65±17
		16.5	>225

*Representative of three experiments.
ND, below the detection limit of antibody.

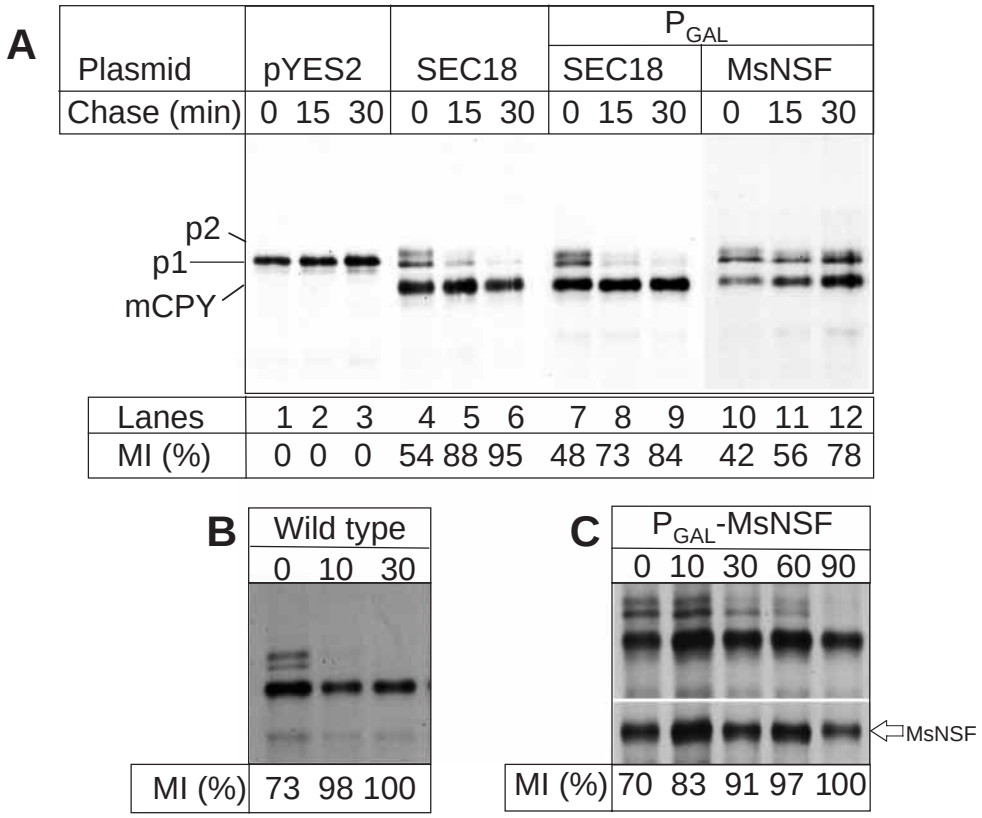

Fig. 2. Progression and maturation of CPY at non-permissive temperature is promoted by MsNSF. (A) Expression of MsNSF in *sec*18-1 removes the transport block for CPY. Cells were grown to a density of 1 A_{600} unit ml^{-1} in minimal medium lacking methionine and uracil, supplemented with 3% galactose and 200 μmol l^{-1} $(NH_4)_2SO_4$ for 2 h (A) or 6 h (B,C) to induce expression. Cells were resuspended in fresh medium (lacking sulfate), incubated at 33 °C for 30 min and labeled with trans-^{35}S for 30 min at 33 °C. Cells (10 A_{600} units) were removed at the indicated time intervals and analyzed by CPY immunoprecipitation (IP). Wild-type (B) and MsNSF (C) cells were induced for 6 h before temperature-shift and pulse-chase, and CPY IP was done as in A. Images from three independent experiments were digitized from various exposures and analyzed by NIH image. The CPY maturation index (MI) was calculated from quantified values as [mCPY/(p1+p2+mCPY)]×100. The coefficient of variation was <6%. Also shown in C is MsNSF (83 kDa) immunoprecipitated with α-MsNSF antibody after CPY-IP. The strains used are the same as in Fig. 1 (see Table 1 for genotypes). The relevant portions of autoradiographs are shown. p1 (67 kDa), p2 (69 kDa) and mCPY (61 kDa) refer to ER, Golgi and vacuolar forms of CPY, respectively.

direct effect of MsNSF expression. Under constitutive levels of expression with raffinose, MsNSF failed to rescue the temperature-sensitive phenotype (Fig. 1B), strongly suggesting that the rescue was dose-dependent. It is noteworthy that P_{GAL}-*SEC*18 could not be completely suppressed by glucose or raffinose, suggesting that it is constitutively expressed due to the presence of 5′-region of SEC18 in the construct. Constitutive levels of Sec18p are thus sufficient to rescue temperature-sensitive growth in *sec*18-1 mutants.

Several of the *sec* mutants accumulate intra-cellular structures and lyse after prolonged exposure to non-permissive temperatures (Novick et al., 1980; Riezman, 1985). Cells were incubated at 37 °C for 8 days, returned to 22 °C and incubated for 4 days. MsNSF-containing cells remained viable and were capable of growth after this prolonged exposure to restrictive temperature, while *sec*18-1 cells as expected were unable to revive growth (Fig. 1C). These data clearly demonstrate that an animal NSF is capable of rescuing the temperature-sensitive phenotype when expressed in *sec*18 mutant, and does not require the activity of animal SNAPs to do so.

To obtain biochemical support for the above data, cells grown at various induction conditions were analyzed by immunoblotting lysates with α-MsNSF and α-Sec18p antibodies (Table 2). Induction time and carbon source affected the level of expression of MsNSF. Twice as much MsNSF was expressed after induction for 4 h in galactose, compared to Sec18p expressed from its native 5′ region; this level only marginally rescued the temperature-sensitive defect at 37 °C. However, rescue was complete up to 32 °C (Fig. 1). MsNSF levels needed to be eight- to ninefold higher than native Sec18p for complete complementation. Due to large variations between experiments it was not possible to conclusively identify the minimal level of MsNSF required for rescue, and the levels reported here might be overestimated.

Rescue of transport defect by expression of MsNSF

In yeast, *SEC*18 is an early acting gene, and defects in it cause secretory proteins to arrest in the ER-modified forms when shifted to restrictive temperatures. Additional defects in *sec*18-1 mutant were detected in intra-Golgi and Golgi-plasma membrane routes of transport (Graham and Emr, 1991). The data shown above clearly indicate the feasibility of using yeast as a model system to understand the *in vivo* role(s) of proteins from heterologous systems where assays to understand such roles are not available. To analyze the role of MsNSF in intracellular protein transport, we chose three well-characterized markers, carboxypeptidase Y (CPY), alpha factor (α-F) pheromone and heat shock protein 150 (HSP150).

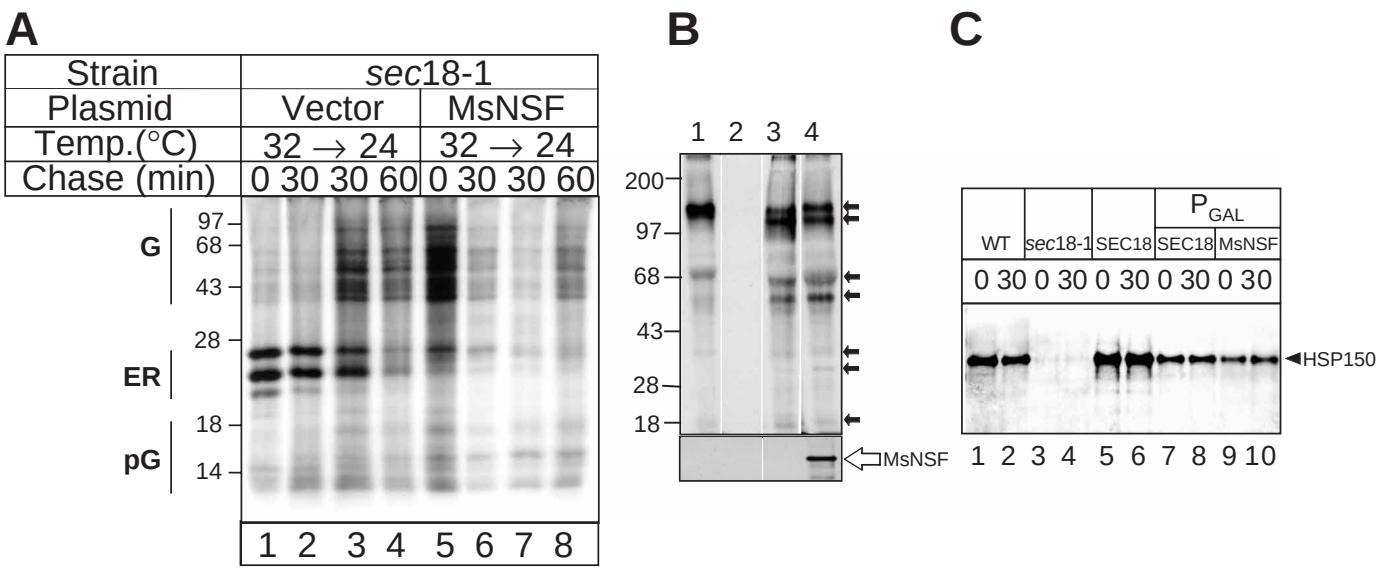

Fig. 3. Restoration of intra-Golgi and post-Golgi trafficking by MsNSF expression *in vivo*. (A) Cells were grown in induction medium for 4 h, shifted to 32 °C for 30 min and labeled for 30 min. Cell and medium portions were separately precipitated with TCA and analyzed by immunoprecipitation with anti-α factor antibody. After 30 min of chase time at 32 °C, cells were shifted to 24 °C to follow the blocked forms to maturation. Cells were derived from EGY1181-5 (*sec*18-1) (Table 1), ASHY1181-1 (vector only) (lanes 1–4), and ASHY 1181-2 (P_{GAL}-MsNSF) (lanes 5–8). During 0 min chase a substantial amount of α-factor already resides in the Golgi (lane 5) in MsNSF cells, and this is rapidly matured and secreted (lane 6, 30 min chase). In *sec*18-1 cells the ER form predominates all through the chase time (compare lanes 1 and 2), and is only processed more slowly when returned to the permissive temperature (30 and 60 min chase at 24 °C, lanes 3 and 4). The mature peptide was not immunoprecipitated with this antibody. Thus maturation of α-factor is inferred from the disappearance of the Golgi (G)-modified form during the chase. ER, G and pG denote the expected positions of ER, Golgi and post-Golgi forms of α-factor. The positions of molecular mass markers (kDa) are shown on the left. (B) Analysis of proteins secreted into the medium at restrictive temperatures. Expression of MsNSF in *sec*18-1 mutants restores transport of proteins secreted into the medium at the restrictive temperature of 37 °C. The band at 150 kDa corresponds to an extensively *O*-glycosylated heat shock protein, HSP150. Wild-type (RSY 248, lane 1) or cells derived from *sec*18-1 (as mentioned in Fig. 1) were incubated at 37 °C for 15 min, labeled and chased for 30 min each at 37 °C. Arrows indicate seven predominant proteins that are secretion-blocked in *sec*18-1 cells. Medium from 0.5 A_{600} cell equivalents was analyzed by autoradiography. Cell pellets were immunoprecipitated with MsNSF antibody to confirm the expression of MsNSF (bottom). (C) Immunoblot of secreted HSP150 at 37 °C. Cells were grown overnight in rich medium (supplemented with 3% galactose) to a density of 1 A_{600} unit ml^{-1}. Cells (25 A_{600} units) were concentrated and washed twice in water and once in medium and resuspended in prewarmed medium (37 °C) to a density of 10 A_{600} units ml^{-1}. Cells (1 ml) were incubated at 37 °C for 30 min and equal portions (0 min chase, odd-numbered lanes) were transferred to NaN_3–NaF (20 mmol l^{-1}) on ice. The remainder of the culture was washed in prewarmed medium to remove pre-existing proteins and resuspended in prewarmed medium containing cycloheximide (20 μg ml^{-1}); proteins were chased for 30 min (even-numbered lanes). Samples equivalent to 3 A_{600} units of cells were analyzed by immunoblotting with HSP150 antibody. WT, wild type.

MsNSF restores CPY transport to the vacuole in the sec*18-1 mutant*

CPY is a 61-kDa subunit enzyme of which 10 kDa is contributed by four *N*-linked oligosaccharide modifications. In wild-type cells, CPY targeted to the ER is modified by the addition of core oligosaccharides (p1 form, 67 kDa), and is further modified in the Golgi (p2 form, 69 kDa). The p2 form is sorted to the vacuole where it is proteolytically processed to yield the mature form of the enzyme (mCPY, 61 kDa) (Hasilik and Tanner, 1978; Stevens et al., 1982). Mutations in *SEC*18 affect the transit of CPY to the distal Golgi compartment (Graham and Emr, 1991). If MsNSF could replace the defective Sec18p *in vivo* then the ER-arrested form (p1) should be matured to mCPY under restrictive conditions. Fig. 2 shows the comparative maturation of CPY in *sec*18-1, MsNSF and *SEC18* cells. After induction for 2 h, when cells were labeled after incubation at 33 °C for 30 min, *sec*18-1 cells accumulated p1 CPY, which failed to mature during the chase period (Fig. 2A, lanes 1–3). This transport block was rescued by the expression of MsNSF, which yielded the mature vacuolar form, mCPY (lanes 10–12). MsNSF mediated rapid vacuolar transport since 42% mCPY was recovered at 0 min of chase (lane 10). The above data was quantitated from three experiments by densitometry of digitized images. In MsNSF-, *SEC*18- and P_{GAL}-*SEC*18-expressing cells, mCPY maturation, after a 30 min chase, was approx. 78%>95% and 84%, respectively (Fig. 2A). Since expression levels under longer induction showed phenotypic rescue, cells were induced for 6 h before temperature shifts and analyzed for CPY maturation (Fig. 2B,C). Consistent with the phenotypic data (Table 2, Fig. 1), higher levels of MsNSF expression facilitated mCPY formation, but was kinetically slower than wild type (Fig. 2, compare lane at 30 min in B with corresponding lane in C). From these results we conclude that MsNSF expression could

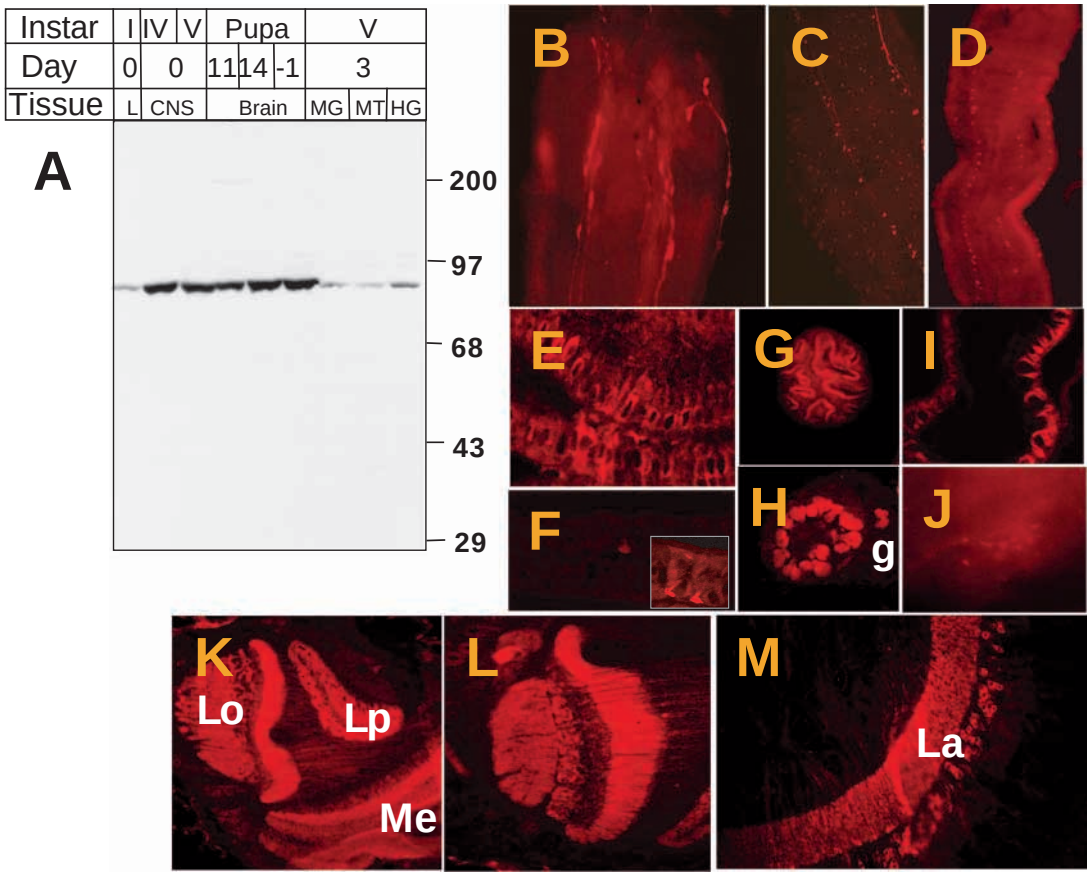

Fig. 4. Expression and localization of *M. sexta* NSF (MsNSF). (A) Expression of MsNSF is enriched in neuronal tissues of larvae, pupa and developing adults. Midgut (MG), hindgut (HG) and Malpighian tubules (MT) contain reduced reactive species. Detergent-solubilized protein extracts (50 μg lane^{-1}) were separated by SDS–PAGE and analyzed by immunoblotting with affinity-purified α-MsNSF antibodies. The positions of molecular mass markers (kDa) are shown. Neonate larvae (B–D) or fifth-instar midguts (E,F) were stained with α-FMRFamide (B,C) or α-MsNSF antibodies (D-F) and detected by Cy3-conjugated secondary antibodies. Enteric neurons and their axons and a large set of enteric endocrine cells could be marked by FMRFamide reactivity (B,C). Enriched MsNSF immunoreactivity (eNSF-IR) is not present in any enteric neurons or their axons. Six rows of enteric endocrine cells express eNSF-IR only in the median midgut (D), while staining in regions anterior and posterior was unremarkable (not shown). Pediculate cells of midgut stain for MsNSF either singly (F) or in twos (F, inset) per invagination. MsNSF expression predominates in the basolateral membrane of globet cells (E) and plasma membrane of Malpighian tubules (G,I). MsNSF expression is also detected in the glomeruli (g; H) in the antennal lobe of adult and synaptic boutons of larval protocerebrum (J). (K–M) MsNSF exhibits enrichment in photoreceptors, structures of the optic lobe and the antennal lobe of *M. sexta*. MsNSF is expressed in photoreceptors, mostly localizing to the cytoplasm (M) and granular structures (K,L) of the optic lobe. Lamina (La), medulla (Me), lobula (Lo) and lobula plate (Lp) intensely stain for MsNSF, indicating that MsNSF might function in the visual system of *M. sexta*. Whole-mount (B–D,J) and paraffin section histochemistry were performed as described in Materials and methods.

substitute for a mutant Sec18p *in vivo* in mediating intracellular trafficking of CPY. Functional substitution by MsNSF does not require any other fusion-promoting factors such as Sec17p or Rab homologues from *M. sexta* to exert its action in yeast. This is the first demonstration of an *in vivo* expression-dependent function for an animal NSF in intra-Golgi transport.

Intra-Golgi transport of yeast pro-alpha-factor is restored by MsNSF in sec18 mutants

In *sec*18 cells, the mating pheromone α-F is predominantly in the ER-modified form under non-permissive conditions. Transfer to the Golgi results in the addition of complex sugars in a compartment-specific manner, producing the hyperglycosylated form. In a distal Kex2p-containing compartment the prohormone is processed to the mature 13-amino-acid-residue active pheromone that is secreted into the medium. The addition of different sugars at distinct levels of Golgi organisation results in distinguishable forms of the prohormone that can be resolved by their migration on SDS–PAGE. The nature of sugar modification indicates the level of Golgi organisation that the protein has reached. In *sec*18-1 cells at 33 °C, only the core glycosylated ER form predominated, which failed to mature into the Golgi forms during the 30 min chase period at non-permissive temperature (Fig. 3A, lanes 1,2). The complete lack of α-1,6 and α-1,3

modified Golgi forms in the immunoprecipitates strongly suggested that, under our conditions, the core glycosylated prohormone was never delivered to the Golgi. In contrast, in MsNSF-expressing cells, even at 0 min chase, the predominant forms were Golgi-modified, corresponding to the sizes of both α-1,6 and α-1,3 mannose added forms (G; Fig. 3A, lane 5). These results suggested that the functionality imparted by active MsNSF is sufficient to provide rapid transport of α-factor through the compartment. After a chase for 30 min, cells were shifted to permissive temperature (24 °C) to allow for resumption of transport of ER-locked forms (lanes 3,4 and 7,8) and to demonstrate that α-factor in *sec*18-1 cells can acquire Golgi-specific modifications as expected for permissive conditions. It should be noted that MsNSF expression resulted in a rapid disappearance of Golgi-modified form, presumably by secreting the mature protein into the medium (Fig. 3A, lane 7; see legend). Together the data (Figs 2, 3A) suggest that MsNSF alleviates a Sec18p defect *in vivo* for two well-characterized secretory markers in intra-Golgi transport.

Golgi to plasma membrane and exocytosis

Consistent with the NSF interaction with neuronal SNAREs (Söllner et al., 1993b), Graham and Emr (1991) showed that Sec18p acts in the exocytotic pathway from post-Golgi to plasma membrane. However, purified Sec18p has not been shown to be required for this step. We used a secretion assay (Gaynor and Emr, 1997) to determine if MsNSF could functionally restore the late stage in exocytosis. This assay does not place bias on any particular protein but qualitatively assesses whether proteins in a size range are secreted into the medium after imposing a temperature block prior and during pulse-chase protocols. Cells were grown in induction medium and subjected to restrictive conditions for 30 min at 37 °C (Fig. 3B). After being subjected to a pulse-chase protocol, equal portions of labeled cultures were separated into medium and cell fractions, and analyzed by SDS-PAGE and fluorography.

Medium proteins were absent from *sec*18-1 mutants (Fig. 3B, lane 2). At least seven protein bands (arrows, Fig. 3B) were apparent in *sec*18-1 complemented with MsNSF (Fig. 3B, lane 4), similar to *SEC18*-carrying cells (Fig. 3B, lane 3). Among the predominant proteins whose secretion was blocked by *SEC18* mutation, the protein of approx. 150 kDa (Fig. 3B, lanes 1,3,4) corresponds to the well-characterized heat shock protein HSP150 (Russo et al., 1992).

HSP150 is induced approximately sevenfold under heat stress and is extensively *O*-glycosylated (Russo et al., 1992). Functional Sec18p is required for the secretion of HSP150 since in *sec*18-1 it is predominantly found in the ER form under restrictive conditions (Gaynor and Emr, 1997). Cells were incubated at 37 °C for 30 min, washed in prewarmed (37 °C) medium and incubated for 30 min with cycloheximide (Fig. 3C). Portions were removed prior to addition of cycloheximide (0 min chase, odd-numbered lanes). Cells were collected at 37 °C, washed in prewarmed medium twice, and incubation continued for 30 min with cycloheximide (Fig. 3C, even-numbered lanes). Medium proteins were separated by electrophoresis and analyzed by immunoblotting with HSP150 antibody. As expected in wild type (WT), HSP150 was efficiently secreted into the medium (Fig. 3C, lanes 1,2), whereas no detectable HSP150 was found in the medium from *sec*18-1 cells (Fig. 3C, lanes 3,4). Expression of MsNSF or *SEC*18 rescued this block of HSP150 (Fig. 3C, lanes 5–10). Sec18p has not been localized to fusion complexes formed during exocytosis, i.e. the exocyst complex, nor has purified Sec18p been shown to reconstitute this step *in vitro*. However, in *sec*18-1 mutants, components of this complex are localized to discrete sites in the plasma membrane, indicating a potential role for Sec18p in mediating exocytosis from the exocyst complex formed at the plasma membrane (Carr et al., 1999). The fact that MsNSF could relieve this late Golgi transport step suggests that MsNSF is indeed functional in post-Golgi trafficking, and could replace a mutant Sec18p defect precisely at that point of the exocytotic pathway.

Stage-, tissue- and cell-type-specific expression of M. sexta *NSF*

The level and pattern of MsNSF protein expression were analyzed with antibodies against the D1 domain of MsNSF. These preparations specifically recognized a 83kDa protein in immunoblots, consistent with the estimated size of MsNSF. MsNSF expression was highest in the central nervous system and the brain of larval and pupal stages of *M. sexta* (Fig. 4A), extending our previous observation (Pullikuth and Gill, 1999). Preimmune sera, or sera depleted by preincubating with the D1 domain of MsNSF, completely abolished the signal in immunoblots (not shown). Affinity-purified antibodies specifically immunoprecipitated *in-vitro*-labeled MsNSF (Pullikuth and Gill, 1999) and metabolically labeled MsNSF from cells carrying the MsNSF expression vector, but not from isogenic cells expressing Sec18p from a multicopy vector (pSEY8) or from an inducible expression vector (P_{GAL}) (Figs 2, 3B).

M. sexta is an excellent model to study neuroendocrine determinants in animal development as it has already been used as a system to identify and isolate several hormones and peptides involved in metamorphosis. The ontogeny and physiological roles of neuroendocrine cells in this system have been resolved to a far greater extent than in any other insect species (Copenhaver and Truman, 1986a,b). Although two isoforms of NSFs (dNSF1 and 2) have been isolated in *Drosophila*, their expression pattern at the protein level is not well understood. dNSF1 mRNA is expressed at higher levels in head and embryonic CNS (Ordway et al., 1994), whereas dNSF2 is ubiquitously expressed, suggesting a more generalized role in constitutive protein trafficking (Boulianne and Trimble, 1995; Golby et al., 2001; Pallanck et al., 1995b). Thus far, NSF protein has not been localized in endocrine organs in the brain. We focused on three aspects for further characterization. First, the enteric nervous system (ENS) was used as a model to understand the non-neuronal distribution. Second, we analyzed the anterior protocerebral neurosecretory

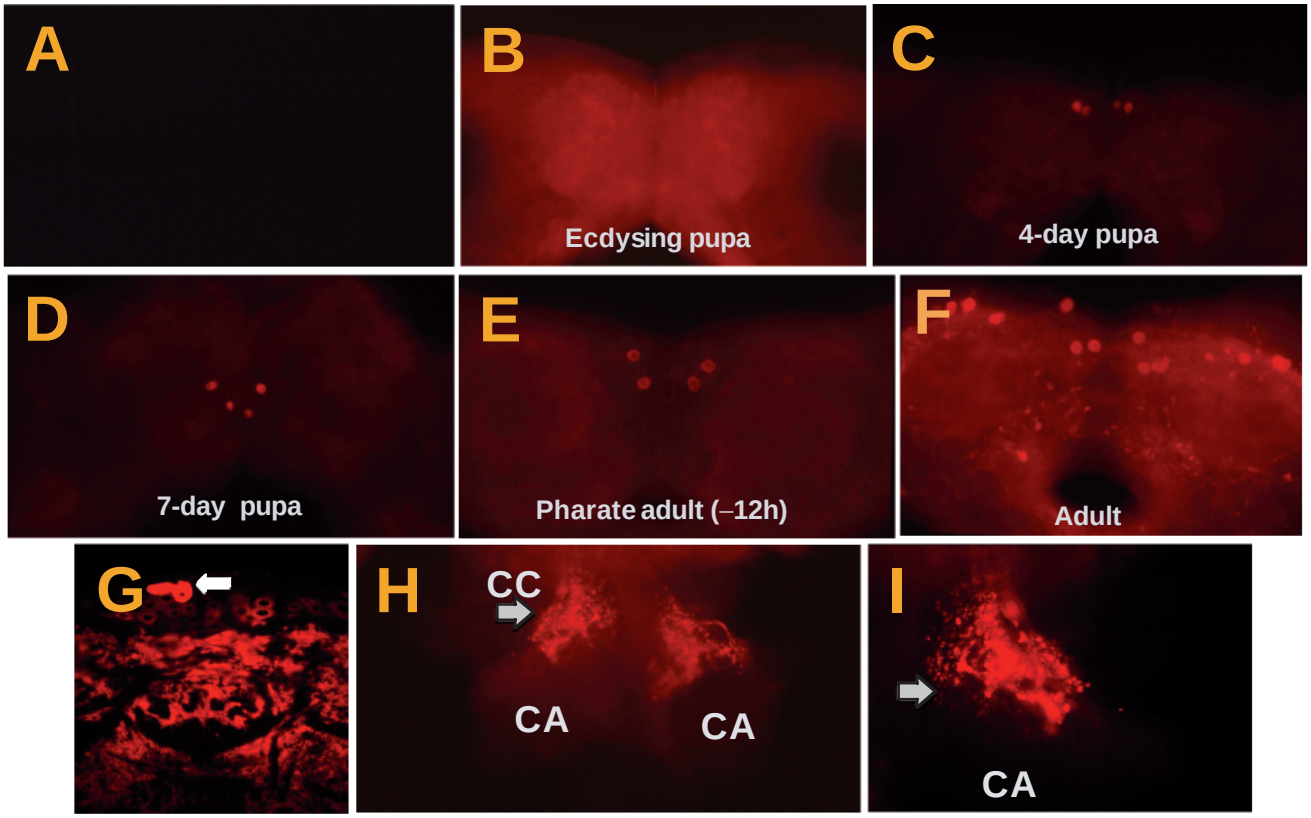

Fig. 5. Expression of MsNSF is developmentally regulated in a subset of neuroendocrine cells and is selectively enriched in specific subsites of hormonal release in *M. sexta* brain. Enriched MsNSF immunoreactivity (eNSF-IR) is present on or after day 3 of pupation and continues throughout adult development (B–E). FMRFamide staining detects most cells of the neurosecretory complex and various varicosities in the brain (F). (G) Paraffin section showing exaggerated Type I staining and eNSF-IR (arrow). The image was overexposed to show both eNSF-IR and Type I staining. eNSF-IR is present only in the corpora cardiaca (CC) (H,I) and excluded from the corpora allata (CA). (I) A higher magnification of eNSF-IR in varicosities of CC in neurohemal complex. Note that no other neurosecretory cells are stained in paraffin sections (G), ruling out problems of antibody penetration in our whole mounts. A representative staining with pre-immune IgG is shown in A, for comparison of specific signals with background staining.

complex during all developmental stages. Finally, we characterized a novel NSF reactivity in a specific set of neurosecretory cells that are regulated in development.

MsNSF in enteric nervous system (ENS)

M. sexta ENS consists of 80-100 neurons clustered in the enteric plexus in the anterior midgut (Fig. 4B). These neurons send axonal projections along the entire length of the organ (B) and innervate the proctodeal nervous system, terminating in varicosities indicative of hormone release sites (Truman, 1992). Apart from the ENS neurons, which modulate muscle activities of midgut, a slew of endocrine/paracrine cells (EPC) lines the entire length of midgut. These cells are peptidergic in nature and contain molecules antigenically similar to vertebrate neuropeptides and hormone. Further, the midgut is a rich source of ecdysteroids, which dictate the fate of the developing insect by rising and falling titers. ENS neurons and EPC could be marked by FMRFamide reactivities that stain a variety of peptides with FMRF epitopes (Fig. 4B,C) (Zitnan et al., 1993). Whole-mount immunohistochemistry was performed on isolated midguts from neonate larvae. Two distinct patterns of NSF reactivities were evident. A diffused staining pattern, apparent only with α-MsNSF antibodies, which we term as Type I NSF immunoreactivity (NSF-IR), was evident. This signal was specific since negative control experiments done in parallel produced little staining (not shown). Type I NSF-IR reflects the presence of MsNSF in most cells, consistent with its constitutive role(s). On the other hand, a pattern of enriched NSF-IR, which we term eNSF-IR, was restricted to perhaps six rows in the median midgut (Fig. 4D). FMRFamide reactivities were present in approximately 12 rows of EPC along the entire length of midgut (Fig. 4C). eNSF-IR was excluded from ENS neurons and axons, and from EPC of anterior and posterior midgut.

EPCs consist of two predominant types of cells, closed and open. Larval midgut consisted of one (Fig. 4F) or two (Fig. 4F, inset) eNSF-IR cells per invagination exclusively restricted to the closed cell type. We call them pediculate EPCs, since stalked processes project into the lumen of the midgut. No eNSF-IR was found in open EPCs. The eNSF-IR EPC cell body is exposed to hemolymph and thus strategically poised to relay hormonal information from the hemolymph to the midgut

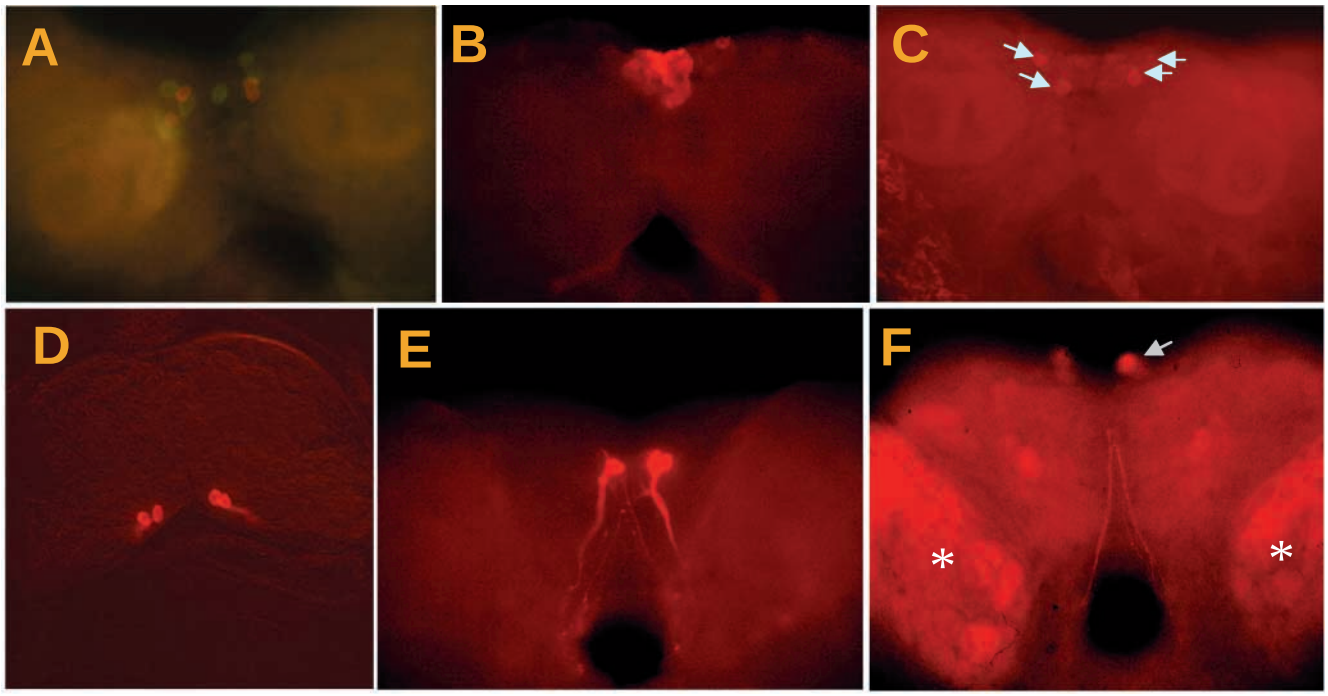

Fig. 6. Novel, developmentally regulated expression of MsNSF. Pharate adult brains were analyzed by staining with antibodies against MsNSF (A,C,F), diuretic hormone (DH) (B,C), eclosion hormone (EH) (D,E) or bombyxin (A). Bombyxin is synthesized in four pairs of cells in the anterior protocerebrum (A, green) that do not colocalize with enriched MsNSF immunorecativity (A, red). Diuretic hormone (DH) in later stages is localized to 80–100 neurosecretory cells (B), which lie medial to the eNSF-IR cells, as revealed by costaining with DH and MsNSF antibodies (C, arrows). EH in larvae is synthesized by paired ventro-medial cells (D). In later stages of development, EH-containing ventro-medial cells in the brain migrate anteriorly, to the proctocerebrum, and are positioned ventrally to the eNSF-IR cells (E). Axons of eNSF-IR run parallel to EH axons to end in the CC–CA complex (F). The posterior aspect of brains stained for EH (E) or MsNSF is shown (F). Note the axons of eNSF-IR from Type IIa_4 cells run ventrally from the dorsally situated cell bodies (outside the plane of focus, arrow) in F; asterisks denote Type I staining.

lumen. The spectra and identity of hormones produced in each type of EPC are not known, although many react to antibodies against vertebrate neuropeptides and hormones, and are thus thought to contain antigenically similar factors.

The midgut is composed primarily of two cell types, columnar cells and secretory goblet cells. An intermediate staining pattern, termed Type III, with a median midgut bias was found mostly restricted to the basolateral domain of goblet cells (Fig. 4E). Columnar cell staining was unremarkable. The localization pattern of MsNSF in the basolateral domain is consistent with studies performed *in vitro* where vertebrate NSF was shown to be required for transport from the TGN to the basolateral membrane. Diminished or lack of staining in most other cells points to the possibility that NSF-independent mechanisms, or other unidentified NSF isoforms, might be involved in trafficking in a majority of midgut cells. Exclusion of NSF-IR in enteric neurons and axons bolsters the claim that multiple MsNSF isoforms or functionally similar molecules would govern release processes in this important group of neurons, modulating various aspects of feeding, digestion and ecdysis. The selective expression of NSF is likely to exist in other species, as *Drosophila* midgut and gastric ceacae exhibited regional and cell-type-specific NSF enrichments with the same antibodies (A. K. P., M. Filippova and S. S. G., unpublished observations). In the Malpighian tubules, most NSF-IR was found restricted to the plasma membrane (Fig. 4G,I).

NSF reactivity in optic and antennal lobes of M. sexta

In the optic lobe most photoreceptors exhibited NSF-IR, largely restricted to cytoplasmic regions (Fig. 4M). Three granular structures of the optic lobe, namely lobula, medulla and lamina, stained for MsNSF (Fig. 4K,L), indicating a role for MsNSF in visual perception and integration of sensory information in *M. sexta*. No specific groups of cells of the optic lobe showed eNSF-IR. Distinct groups of cells containing neuropeptides and neurotransmitters have been localized in the optic lobe; however, the lack of specific enrichment in any of these cells suggests that MsNSF is expressed to similar extent in most types of peptidergic and neurotransmitter-containing cells of the optic lobe. In the antennal lobe, glomeruli (g) stain for MsNSF (Fig. 4H) where sensory inputs are integrated. Afferent nerves from glomeruli innervate the mushroom body, a critical structure implicated in learning and memory in insects.

NSF-IR in the neurosecretory complexes of M. sexta *brain*

Components of NSF-mediated complex have been predicted

to regulate exocytotic events, including hormonal secretion. However, to date, there is no direct evidence for the presence of NSF in insect neurosecretory cells or sites of hormone release. The protocerebral neurosecretory complex of *M. sexta* consists of several paired cells with distinct content catalogue and defined axonal ramifications (Copenhaver and Truman, 1986a,b; Zitnan et al., 1995a). Whole-mount immunohistochemistry was performed on brains from staged and sexed animals. The expression pattern of MsNSF was unremarkable in larval brain. Surprisingly, beginning with day 3 after pupal ecdysis, four cells in the anterior protocerebrum showed eNSF-IR (Fig. 5C) apart from the specific diffused Type I-IR found in all regions (Fig. 5B). eNSF-IR in these two pairs was sustained throughout later development and persisted after adult emergence (Fig. 5C–E). eNSF-IR in these cells was not sexually dimorphic (not shown), thus was unlikely to be involved in sex-specific traits. To rule out the possibility that the size of tissues in whole mounts might mask other reactivities, paraffin sections of pharate adult were examined with the same antibody. Confirming our results, only four cells expressed the eNSF-IR phenotype (Fig. 5G, arrow; only one from each pair in the plane of section shown). eNSF-IR was not due to problems of antibody penetration since α-FMRFamide stained several groups of cells and axons at all levels (Fig. 5F). Synaptotagmin and syntaxin antibodies stained all cells in the protocerebral complex equally, suggesting that these important components in membrane fusion are not preferentially expressed in these cells (data not shown).

Axonal processes from these eNSF-IR cells were weakly detected after 7 days of pupal ecdysis, gradually increasing in staining intensity toward adult development (Fig. 6F). Brain eNSF-IR is curiously poised within a cluster of cells constituting the major neurosecretory atrium in protocerebrum. Hormones involved in homeostasis, cellular differentiation, egg development, vitellogenesis and eclosion have all been localized to specific sets of cells within this complex. Peptides and hormones produced from this complex are released from either of the two major neurohemal organs, corpora cardiaca (CC) and corpora allata (CA), both posterior glandular structures connected to the brain through nervi corpora cardiaca (NCC). Despite both CC and CA being principal sites of peptide/hormone release, each is involved in releasing a subset of hormones during development. Given the putative role of NSF in mediating neurotransmitter release, we examined MsNSF expression in peptide/hormone release structures in *M. sexta*. CC–CA complexes from ecdysing pupa to adult insects were analyzed by whole-mount immunohistochemistry with affinity-purified α-MsNSF. Remarkably, only CC was found to produce eNSF-IR with distinct varicosities characteristic of hormone release sites (Fig. 5H,I). However, this pattern of eNSF-IR in CC was also present in larval stages (data not shown), albeit less intense. The preferential enrichment of NSF in one of the two major sites for hormone/peptide release suggests that it is involved in regulating the release of only a subset of factors produced in the brain. No change in eNSF-IR in CC was detected between larval–larval, larval–pupal, and pupal–adult ecdyses, ruling out the possibility that MsNSF is directly associated with ecdysis and eclosion.

To understand if eNSF-IR in *M. sexta* brain colocalized with distinct neuropeptides, immunostaining was performed with antibodies against eclosion hormone (EH) (Copenhaver and Truman, 1986a), PTTH (Westbrook et al., 1993), diuretic hormone (DH), leukokinin (Veenstra and Hagedorn, 1991), period (Sauman et al., 1996) and bombyxin (Zitnan et al., 1990, 1995b). Bombyxin, an insulin-related molecule that primes the prothoracic glands to secrete the ecdysis hormone, ecdysteroid, is produced in four pairs of cells. Since the location of bombyxin cells was similar to eNSF-IR cells, double immunostaining with α-MsNSF and α-bombyxin was performed. Signals were detected with anti-mouse-cy2 conjugated secondary antibody to visualize bombyxin reactivity (Fig. 6A, green), while anti-rabbit-cy3 conjugated antibody detected MsNSF-staining cells (Fig. 6A, red). These eNSF-IR cells did not coincide with any of the bombyxin-reactive cells as apparent from the lack of colocalization. Another candidate set of cells is those producing DH, which localize to the dorsal protocerebrum in larval stages. During pupal–adult development, DH production is shut off in these cells and production is shifted to a cluster of 8–10 smaller cells in early pupa and 80–100 cells in late pupa/adult (Fig. 6B), all probably arising from a common neuroblast in later stages (Veenstra and Hagedorn, 1991). The presence of these cells in later stages is controversial, since DH reactivity is the only known criterion for their identification. During adult metamorphosis these cells are thought to be lost or atrophied. However, α-MsNSF antibodies detect two pairs lateral to the cluster of 80–100 cells staining for DH (Fig. 6C). The sheer size difference and localization rules out any subset of DH cells to express eNSF-IR. However, it should be noted that these are the same cells that express DH in larva.

Eclosion hormone (EH) is one of the major determinants of ecdysis in *M. sexta* and directs its action through a cascade of hormonal signaling. EH in larva is produced by two pairs of ventro-medial (VM) cells (Fig. 6D). VM cells undergo migration concomitant with the gross rearrangement of the brain during pupal and adult development. Fusion of subesophageal ganglion with the brain coincides with VM cells being ventrally positioned in the anterior protocerebrum of late pupa/adult brain (Fig. 6E). EH in late stages is thought to be released primarily from CC-CA complex, as distinct from the proctodeal system in larva. To rule out the possibility that eNSF-IR cells were EH cells, cells stained for α-EH (Fig. 6E) and eNSF-IR (Fig. 6F) were examined both from dorsal and ventral aspects. Axons from eNSF-IR cells project ventrally and run median to VM axons and project to CC through NCC I and II similar to DH-IR in larva (Fig. 6F). This allowed unequivocal assignment of eNSF-IR cells to Type IIa_4 cells. These data rule out most of the known hormones and peptides as candidates for colocalization with eNSR-IR, and thus it constitutes a novel reactivity.

Discussion

Mammalian cell-free systems and genetic studies in yeast have identified a large set of proteins that mediate intracellular transport. Subcellular localization and sequence conservation combined with genetic data have been used increasingly to implicate specific factors in distinct transport steps. Despite the overall conservation of mechanistic aspects between neurotransmitter release and endocrine secretion, far less about hormonal secretion in invertebrates is understood at the molecular level. Our interests are vested in understanding the molecular bases of hormonal secretion in the developmental model *M. sexta*. Here we have taken combined expression and complementation analyses to assign an *in vivo* role for *M. sexta* NSF. Our results clearly indicate that MsNSF is functional in yeast *in vivo*, and its expression levels are critical for this functional complementation. It is also clear that MsNSF is involved in intracellular trafficking in yeast and possibly in *M. sexta*. This is the first demonstration that an animal NSF could genetically complement the *SEC*18 mutation in yeast.

In vivo complementation by MsNSF in yeast occurs without its cofactor SNAP(s) from a homologous source. The higher expression levels required for functional complementation may be due to differences in properties of MsNSF such as its ATPase activity, affinity for Sec17p, or requirement for SNAP (–like) cofactors from an identical source and recognition of SNARE complexes in their right context and from the correct species. Vertebrate p97 (yeast Cdc48p) which, like NSF, has been implicated in fragment assembly of mitotic Golgi (Rabouille et al., 1995) is not functional in a yeast Cdc48p assay for ER membrane fusion; however, crude lysate from *Xenopus* possesses greater Cdc48p activity *in vitro* (Latterich et al., 1995). A reason for lack of complementation by vertebrate NSF and p97 in yeast *SEC*18 and *CDC*48 mutants might be that these proteins require their respective cofactors α-SNAP and p47 to be active in heterologous environments.

Each subunit of NSF hexamer can be divided into distinct domains (for a review, see May et al., 2001). The D1 domain provides the major ATPase activity in disassembling SNARE complexes (Matveeva et al., 1997; Nagiec et al., 1995; Steel and Morgan, 1998). ATP binding to D2 is required for hexamerization of NSF but not for SNARE disassembly (May et al., 2001). The temperature-sensitive mutation of *SEC*18 (^{89}G → D) occurs in the N-domain implicated in interacting with SNAREs (A. Morgan; cited by May et al., 1999). Sequence divergence of this region among cloned NSFs might reflect its structural requirement for interacting with species-specific SNAP-SNAREs. By this criterion, one would expect MsNSF to have lower affinity for yeast SNAREs than Sec18p. Since the N-domain contains a critical pocket proposed to interact with SNAP and, in turn, with SNARE complex, subtle structural differences in this region would underlie the ability to discriminate the substrate from the right species. Another possibility is that MsNSF might be interacting with the mutant form of Sec18p *in vivo* to form a less functional hetero-oligomer, rather than all subunits being contributed by MsNSF to form a fully functional homo-hexamer. *In vitro* experiments suggest that this is an unlikely situation since NSF oligomer containing even a single mutant subunit is completely defective in fusion reactions (Whiteheart et al., 1994).

NSF assembles into a 20 S complex containing SNAREs and SNAP (Wilson et al., 1992). The 20 S complex contains three copies of SNAP bound to one hexameric NSF (Wimmer et al., 2001). Neuronal (Hayashi et al., 1995; Söllner et al., 1993a) and yeast (Rossi et al., 1997) SNAREs also bind three SNAP and Sec17p, respectively, indicating a conserved behaviour in SNARE:SNAP:NSF complex formation. Even though NSF ATPase activity is essential for breaking apart the SNARE complex, the *comatose* equivalent of CHO NSF (^{274}G→E), deficient in ATPase activity, could nonetheless mediate membrane fusion (Müller et al., 1999). These data imply that NSF possibly functions in steps other than the well-studied ATPase-dependent SNARE disassembly-mediated reactions (Schwarz, 1999).

The precise function of NSF in membrane fusion is controversial. NSF-mediated SNARE disassembly might precede fusion (Banerjee et al., 1996) or in a priming step after docking (Kawasaki et al., 1998), or after fusion to initiate another cycle of fusion (Littleton et al., 1998, 2001; Schweizer et al., 1998). Mutations in *Drosophila* NSF1 result in the accumulation of docked vesicles (Kawasaki et al., 1998). This may result from undissociated SNARE complex on vesicles that are recycled, or on the plasma membrane formed after fusion in *comatose* flies. Such tangled SNAREs would be incapable of forming productive v-t-SNAREs in *trans*. NSF-mediated disassembly in wild-type synapses would relieve this constraint after fusion, such that v-SNARE from SNARE bundles formed after fusion are recycled efficiently on endocytosed vesicles, leaving t-SNAREs on plasma membrane free to pair with incoming v-SNARE. Since these events can be viewed as ‘beginning’ and ‘end’ reactions within one round of fusion, prevailing data support the action of NSF not during fusion but in disentangling SNARE bundles residing on the same membrane (*cis*-SNAREs), formed after fusion (Littleton et al., 1998, 2001; Tolar and Pallanck, 1998). This interpretation is also consistent with Sec18p in yeast vacuolar fusion, where it mediates an ATP-dependent priming of vacuoles even before fusion partners come in contact with each other (Ungermann et al., 1998; Wickner and Haas, 2000).

Structural studies on NSF (Lenzen et al., 1998; May et al., 1999; Yu et al., 1998, 1999), Sec18p (Babor and Fass, 1999) and p97 (Rouiller et al., 2000; Zhang et al., 2000) are providing new avenues for improving our understanding of how conformational changes associated with nucleotide binding and hydrolysis might possibly disassemble SNAREs (see Dalal and Hanson, 2001; Hanson et al., 1997; May et al., 2001). Similar to neuronal activity-dependent phosphorylation of synaptic proteins (Greengard et al., 1993), vertebrate NSF could be phosphorylated by protein kinase C (PKC) in synaptosomes (Matveeva et al., 2001), which is consistent with our previous proposal (Pullikuth and Gill, 1999). It remains to be seen how such modifications can modulate the several membrane fusion reactions that are catalyzed by NSF.

NSF is critical for synaptic transmission, since vesicles accumulate (Kawasaki et al., 1998) and lead to paralysis when its function is impaired (Pallanck et al., 1995a; Siddiqi and Benzer, 1976). Apart from its better-understood role in neurotransmitter release, NSF mediates trafficking or proper insertion of glutamate receptor subunits at postsynaptic sites (Osten et al., 1998; Song et al., 1998). Further, NSF interacts with β_2-adrenergic receptor (Cong et al., 2001) and β-arrestin 1 (McDonald et al., 1999), probably to facilitate receptor internalization and recycling. This indicates that NSF might have varied functions depending on the local cellular context. Despite its established role in neurotransmission, little is known about NSF distribution and function in insect endocrine cells. Our histochemical analysis of MsNSF expression suggests that, in *M. sexta*, NSF expression is under developmental control. The specific enrichment of MsNSF in Type IIa_4 cells suggests that NSF plays a physiologically important role in later development. We did not detect significant colocalization of neuropeptides and hormones with MsNSF enrichment. Thus, MsNSF expression in Type IIa_4 cells is a novel phenotype. It has been speculated that TypeIIa_4 cells, which produce diuretic hormone (DH) in larvae, are atrophied during pupal development since DH production shifts to a cluster of smaller cells derived from a common neuroblast (Veenstra and Hagedorn, 1991). Incidentally, DH immunoreactivity was the only means of readily identifying these cells. Our observation that NSF-IR localizes to Type IIa_4 cells in late pupal and adult stages suggests that these cells are not atrophied but in fact might govern crucial behavior in development, probably through an NSF-dependent mechanism that regulates secretion from CC.

Another important aspect of NSF expression is at the level of sites known to release hormones and neuropeptides that are synthesized in the brain. If NSF were a constitutive member of the release machinery then one would expect its expression to be more or less uniform in all identified sites of hormone release. The fact that eNSF-IR is found only in CC and not in CA strongly indicates that its preferential role is mediating the release of a subset of hormones that are synthesized in the brain and released from CC. Hormones secreted from CA might thus use machinery distinct from NSF that is characterized here, or might be NSF-independent. Of the sequenced genomes, *Drosophila* is the only organism so far to have two distinct NSF homologues, dNSF1 and 2. dNSF1 is required for early adult development whereas dNSF2 is required for early larval development. The expression patterns of neither of them have been adequately examined in endocrine cells, even though one can genetically rescue the defect in the other (Golby et al., 2001). The D1 domain, towards which our antibody was raised, is highly conserved (>80 %) in both dNSF homologues and would be expected to react with such homologues if present in *M. sexta*. MsNSF expression in a subset of enteric endocrine cells indicates that MsNSF might play a role in the regulation of processes emanating from factors produced in the midgut. The strategically located pediculate cells reactive for MsNSF are exposed to the hemolymph and thus could receive information from hemolymph-borne factors governing several aspects of development and homeostasis.

In summary, we have provided *in vivo* evidence for the functional role of MsNSF. Our results experimentally verify that MsNSF could functionally replace Sec18p *in vivo*. This supports the findings of Steel et al. (1999), who showed that purified Sec18p could participate in Ca^{2+}-triggered exocytosis in chromaffin cells. The developmental regulation of NSF expression in neurosecretory cells points to a novel role of NSF in peptidergic pathways dictating developmental aspects of *M. sexta*. Enrichment of MsNSF in enteric endocrine cells suggests that NSF might play a preferential role in hormonal secretion in a subset of cells, whereas the remainder might use either NSF-independent pathways or other NSF-like molecules distinct from the one characterized here. We hope that this study will encourage the use of yeast as a tractable genetic system to understand key roles for proteins in secretion from organisms where cell-free assays are not currently available or are difficult to develop.

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