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Research Article

Familial dysautonomia is a hereditary disorder that affects autonomic and sensory neurons. Nerve growth factor (NGF) is required for the normal development of sympathetic and sensory neurons and it has been postulated that an abnormality involving NGF may be responsible for familial dysautonomia. Previous studies have shown that the beta-NGF gene is not linked to the disease. However, NGF appears to be abnormal by immunochemical assays; the putative altered form of NGF could result from a disturbance in the processing pathway. To study the processing of the 35-kD glycosylated NGF precursor and the secretion of NGF in familial dysautonomia, we have employed a recombinant vaccinia virus vector to express high levels of NGF mRNA in primary fibroblast cultures from patients with the disorder; the processing pathway was then studied directly. Cells from several unrelated patients all produce the same 35-kD NGF precursor, process this normally to NGF within the cell, and release NGF into the medium. There are no differences in the ability of cells from patients and from unaffected relatives to process and secrete NGF. The use of similar recombinant vaccinia virus vectors to express proteins at high level in primary cell lines should facilitate the detection of posttranslational processing defects in a variety of human disorders.

Use of Vaccinia Virus Vectors to Study Protein Processing in Human Disease

Normal Nerve Growth Factor Processing and Secretion in Cultured Fibroblasts from Patients with Familial Dysautonomia

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Abstract

Familial dysautonomia is a hereditary disorder that affects autonomic and sensory neurons. Nerve growth factor (NGF) is required for the normal development of sympathetic and sensory neurons and it has been postulated that an abnormality involving NGF may be responsible for familial dysautonomia. Previous studies have shown that the β-NGF gene is not linked to the disease. However, NGF appears to be abnormal by immunochemical assays; the putative altered form of NGF could result from a disturbance in the processing pathway.

To study the processing of the 35-kD glycosylated NGF precursor and the secretion of NGF in familial dysautonomia, we have employed a recombinant vaccinia virus vector to express high levels of NGF mRNA in primary fibroblast cultures from patients with the disorder; the processing pathway was then studied directly. Cells from several unrelated patients all produce the same 35-kD NGF precursor, process this normally to NGF within the cell, and release NGF into the medium. There are no differences in the ability of cells from patients and from unaffected relatives to process and secrete NGF.

The use of similar recombinant vaccinia virus vectors to express proteins at high level in primary cell lines should facilitate the detection of posttranslational processing defects in a variety of human disorders.

Introduction

Familial dysautonomia (Riley-Day syndrome) is a disorder of autosomal recessive inheritance affecting sympathetic and sensory neurons. Clinically, it is characterized by autonomic instability (involving temperature, blood pressure, and gastrointestinal motility), impairment of pain and temperature sensation, alacrima, and loss of fungiform papillae on the tongue (1). Physiological studies show decreased norepinephrine levels and synthesis, and absence of the cutaneous flare response to histamine (2). Pathological examination shows loss of cells in sympathetic and dorsal root ganglia, with involvement of parasympathetic and central neurons as well (3–5).

Since sympathetic and sensory neurons depend on nerve growth factor (NGF)[1] for survival, it is reasonable to question whether NGF plays a role in the pathogenesis of familial dysautonomia (6, 7). In sera, Siggers et al. (8) found a threefold higher level of immunoreactive NGF than in controls, but the bioassay showed normal levels. Fibroblasts from affected patients have shown reduced NGF by bioassay but normal levels by RIA (9). These results have suggested the accumulation of an abnormal form of NGF, either as a result of mutation within the active protein, or from a defect in prohormone processing. More recent studies have demonstrated that the human beta-NGF gene is not directly linked to familial dysautonomia (10, 11). Thus a defect in NGF itself can not be responsible for the disorder. However, a disturbance of the apparatus involved in the processing and secretion of NGF could produce the same functional defect and explain the abnormal levels of NGF previously detected.

We have shown that two NGF transcripts are produced from the NGF gene as a result of alternative RNA splicing. They predict two distinct forms of the NGF precursor (12). The longer transcript (A) encodes a 34-kD protein with NGF (12.5 kD) at the COOH-terminus; the only significantly hydrophobic domain (putative signal peptide involved in secretion) occurs 70 residues from the predicted NH_2-terminus (Fig. 1). The shorter transcript (B) lacks the initiation codon of A and thus translation is initiated downstream to produce a prohormone of 27 kD. In this precursor, the hydrophobic region is NH_2-terminal, a location more typical for a signal peptide.

To study the processing of NGF, we have constructed two recombinant vaccinia viruses corresponding to the two major NGF transcripts A and B, here termed VV:NGF-A and VV:NGF-B. In previous experiments, infection with these viruses has yielded high levels of NGF precursor and NGF in all of the mammalian cell lines tested (13). We have now used the viruses to study NGF processing and secretion in primary fibroblast cultures from patients with familial dysautonomia.

Methods

Cell culture. Human skin fibroblast cell lines were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cells were grown at 37°C in minimal essential medium with Earle's buffered salt solution and 20% FCS.

Virus infection. Construction of the recombinant vaccinia viruses has been previously described (13). Purified viral stocks were prepared for infection by brief sonication in PBS with 1 mM $MgSO_4$ and 0.01% BSA (PBS-M/BSA). The cells were washed once in PBS-M/BSA and the sonicated virus was allowed to adsorb for 30–60 min at room temperature.

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1. *Abbreviations used in this paper:* NGF, nerve growth factor.

Metabolic labeling. Confluent cultures in 16-mm wells were infected at a multiplicity of 10–20 with VV:NGF-A, VV:NGF-B, or wild type virus. The inocula were replaced with 250 μl of 0.5 mCi/ml [^{35}S]methionine and cysteine (translabel; ICN Radiochemicals, Irvine, CA) in methionine- and cysteine-free medium plus 1% dialyzed FCS, and then incubated for 4 h at 37°C.

Immunoprecipitation and gel analysis. The media to be analyzed were centrifuged to remove debris. The cells were harvested, washed once in PBS, and disrupted by repeated freeze-thawing and sonication. The samples were diluted in RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.2% SDS, 0.15 M NaCl, 50 mM Tris, pH 7.4) plus 20 μg/ml PMSF (14). Antibody raised against NGF was added for a final dilution of 1:1,000. After incubation at 4°C for 12 h, protein A-Sepharose was added and the mixture rocked at 4°C for an additional 4–8 h. Precipitates were washed three times in RIPA and disrupted by boiling for 5 min in Laemmli gel buffer. The samples were analyzed by electrophoresis on a 12.5% discontinuous polyacrylamide gel. The gels were fixed in 10% acetic acid, 50% ethanol, and 40% water, enhanced by incubating in 1 M salicylate (each for 20 min), dried, and exposed to Kodak XAR-5 film overnight with an enhancing screen.

Results

Previous studies have shown that many mammalian cell types have the ability to process the NGF precursor and secrete NGF (13). However, it is not clear whether the same or a different set of proteins perform this function in different cells. If a defect in NGF synthesis is responsible for the disturbance of both sympathetic and sensory neurons in familial dysautonomia this defect must occur in the many cell types that these neurons innervate. We have therefore assumed that if a post-translational disturbance of NGF processing occurs in familial dysautonomia, it should be evident in a variety of cells, including the accessible skin fibroblast. NGF has been detected in primary human skin fibroblasts (9) and we have detected NGF mRNA in fibroblast cell lines.

The low levels of NGF expressed by most cells preclude a direct analysis of endogenous NGF synthesis and processing. To make the experiments feasible, we have artificially increased NGF levels by using recombinant vaccinia viruses that can independently express the two major NGF transcripts in a variety of cell types (13) (Fig. 1).

As with all other mammalian cell lines previously tested, infection of primary fibroblast cells with VV:NGF-A or VV:NGF-B (for transcripts A and B, respectively) produced easily detectable amounts of intracellular NGF precursor as well as secreted NGF. Fig. 2 shows the results of infection in cells from an unaffected relative of a patient with familial dysautonomia (GM4632). Immunoprecipitation of medium and extracts from infected cells labeled metabolically for 4 h reveals the appearance within the cell of a 35-kD precursor as well as smaller amounts of NGF, and the secretion of NGF into the medium. There is no apparent difference between the proteins synthesized from transcripts A and B because both precursors are cleaved after the common signal peptide, to yield identical molecules (13). We have observed a very similar pattern in many immortalized cell lines (13). The wild type virus that does not contain NGF gene sequences, does not give rise to the 35-kD NGF precursor or to NGF itself.

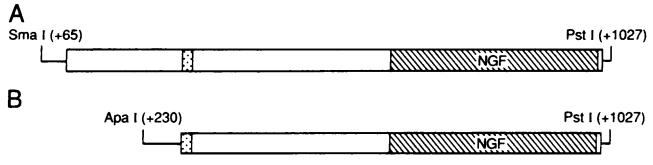


Figure 1. Structure of the predicted NGF precursors (12). A single line represents the untranslated and a box represents the translated sequences of the NGF cDNA. The hatched region denotes the NGF moiety and the stippled area is the hydrophobic domain. The restriction enzyme sites used for cloning into the vaccinia virus vector are shown with their nucleotide position relative to transcript A.

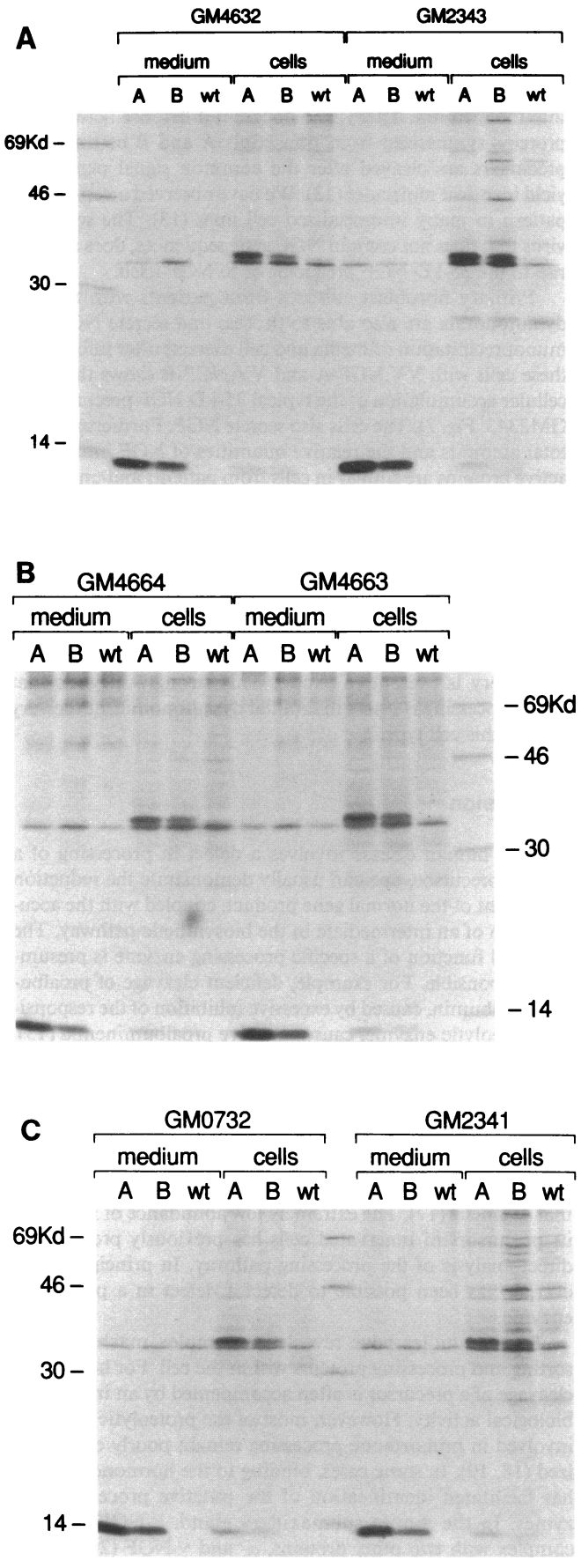
Primary fibroblast cultures from patients with familial dysautonomia are also able to process and secrete NGF. Immunoprecipitation of media and cell extracts after infection of these cells with VV:NGF-A and VV:NGF-B shows the intracellular accumulation of the typical 35-kD NGF precursor (see GM2343, Fig. 2). The cells also secrete NGF. Furthermore, the total amounts and the relative quantities of NGF immunoreactive proteins are similar in cells from patients and unaffected relatives. A similar analysis of four other unrelated patients showed a similar profile of processing and secretion (see GM2343, GM4663, GM0732, and GM2341, Fig. 2).

The levels of NGF obtained using the vaccinia expression vector vastly exceed those normally present in vivo. Because the cells from patients with familial dysautonomia can still process these large amounts, we conclude that the biosynthetic machinery is present in excess. This strongly suggests that NGF processing is intact in familial dysautonomia, at the very least in this cell type.

Discussion

When a human disease involves a defect in processing of a protein precursor, one can usually demonstrate the reduction in amount of the normal gene product, coupled with the accumulation of an intermediate in the biosynthetic pathway. The impaired function of a specific processing enzyme is presumably responsible. For example, deficient cleavage of proalbumin to albumin, caused by excessive inhibition of the responsible proteolytic enzyme, causes massive proalbuminemia (15). In addition, alterations in protein modification produce a variety of disorders, including the defective targeting of molecules to cellular organelles. In Zellweger's cerebrohepatorenal syndrome, there is a deficiency of all peroxisomal enzymes, presumably as a result of a sorting defect (16). A number of lysosomal storage disorders are caused by disturbed targeting into that organelle (17). The extremely low abundance of NGF and its precursors in innervated cells has previously precluded a direct analysis of the processing pathway. In principle, however, it has been possible to detect a defect in a processing enzyme.

Recent studies have revealed a complex machinery for sorting and processing proteins within the cell. For hormones, cleavage of a precursor is often accompanied by an increase in biological activity. However, most of the proteolytic enzymes involved in prohormone processing remain poorly characterized (18, 19). In some cases, binding to the hormone product has facilitated identification of the putative processing enzymes. In the mouse submaxillary gland, β-NGF forms a complex with two other proteins, α- and γ-NGF (20). These two NGF-binding proteins belong to the kallikrein family of



closely related serine proteases; γ-NGF exhibits proteolytic activity on artificial peptide substrates, whereas α-NGF does not (21, 22). The specific binding of the γ subunit to β-NGF suggests that γ-NGF plays a role in NGF processing (20). We have recently demonstrated that the γ subunit can cleave the prohormone to NGF in vitro (23); however, trypsin can also cleave the NGF precursor in a similar fashion. Further, all mammalian cell types tested appear naturally able to process and secrete NGF, yet several do not contain α- or γ-NGF (24, 25). It thus appears that more than one processing pathway for NGF may exist. Since the putative processing proteins have not been identified, it has not been possible to test directly whether they are defective in familial dysautonomia.

We have studied the processing function by artifically increasing the levels of the NGF precursor formed within cells using a facile vaccinia virus expression vector competent to infect most cell lines, including primary skin fibroblasts. Previous studies suggest that this viral vector does not itself provide a processing function for preproenkephalin (26). In addition, the expression of sorted proteins in Madin-Darby canine kidney cells indicates that vaccinia does not disturb the native secretory pathways (27). Our analysis shows that cells from patients with familial dysautonomia cleave the NGF precursor and secrete NGF normally. The possibility of a cell-specific processing pathway exists but seems remote since other studies using this vector have shown that the processing pathway for NGF in fibroblasts is similar to that in many target cells. Our results provide no explanation for the higher levels of NGF detected by immunoreactivity as compared with bioassay (8, 9). The vaccinia system of course does not address pretranslational events (a defect in the splicing machinery is however considered unlikely), or postsecretory events involving a binding or degrading protein. However, the one-site RIA that was used (8, 9) to quantitate NGF can give falsely elevated determinations as a result of binding proteins also present in the sample. It would be useful to employ a two-site RIA for NGF to confirm the existence of an abnormal form of NGF in familial dysautonomia (28).

The vaccinia virus expression system used in these studies provides a direct way to study potential disorders of protein processing. Specific precursor proteins can be produced at higher than normal levels in primary cells from affected patients. The consequent added demand on the processing system should allow a defect to be readily observed. The vaccinia system also provides a means to study the processing and sorting pathways for less abundant proteins, such as neural peptides, growth factors, and their receptors. The ability to perform a simple, direct, functional assay of various steps in a processing pathway on primary human cell lines obtained by biopsy or amniocentesis, will facilitate diagnosis of this group of clinical disorders.

Figure 2. Infection of primary fibroblast cultures with vaccinia. Cells from two normal relatives, GM4632 and GM4664, and from patients with familial dysautonomia, GM2343, GM4663, GM0732, and GM2341 were infected with recombinant viruses A and B, and wild type virus. After metabolic labeling for 4 h, the media and cells were harvested, immunoprecipitated, and the samples were analyzed by electrophoresis on 12.5% SDS polyacrylamide gels, followed by autoradiography.

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