Genetic construction and properties of a diphtheria toxin-related substance P fusion protein: In vitro destruction of cells bearing substance P receptors

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Contributed by Susan E. Leeman, March 25, 1996

ABSTRACT We have genetically replaced the native receptor binding domain of diphtheria toxin with an extended form of substance P (SP); SP-glycine (SP-Gly). The resulting fusion protein, DAB389SP-Gly, is composed of the catalytic and transmembrane domains of diphtheria toxin genetically coupled to SP-Gly. Because native SP requires a C-terminal amidation for high affinity to the SP receptor, the precursor form of the fusion toxin, DAB389SP-Gly, was converted to DAB389SP by treatment with peptidylglycine-α-amidating monoxygenase. We demonstrate that following conversion, DAB389SP is selectively cytotoxic for cell lines that express either the rat or the human SP receptor. We also demonstrate that the cytotoxic action of DAB389SP is mediated via the SP receptor and dependent upon passage through an acidic compartment. To our knowledge, this is the first reported use of a neuropeptide as the targeting ligand for a fusion toxin; and the first instance in which an inactive precursor form of a fusion toxin is converted to the active form by a posttranslational modification.

Substance P (SP) is an 11-amino acid peptide. In the biosynthetic pathway of SP, a precursor form of SP, SP-glycine (SP-Gly), is processed by peptidylglycine-α-amidating monoxygenase (PAM) to yield an amidate moiety at its carboxyl terminus (1,2) which is important for high affinity binding to its receptor. SP is mainly released from neurons and acts upon target cells evoking various cellular responses throughout the central and peripheral nervous systems [reviewed by Otsuka and Yoshioka (3)]. SP receptors have been demonstrated in neurons in discrete areas of the central nervous system, glial cells, endothelial tissue, and selected endocrine glands (4–6). SP receptors are also expressed in various types of tumors, including glioblastomas and astrocytomas, as well as in peritumoral vasculature associated with several types of solid tumors (7).

Diphtheria toxin (DT)-related fusion toxins are composed of the catalytic and transmembrane domains of DT, to which various eukaryotic cell surface targeting ligands [e.g., α-melanocyte stimulating hormone, epidermal growth factor, interleukin (IL)-2, IL-4, IL-6, and CD4] have been genetically linked. Genetic substitution of the native DT receptor binding domain with these ligands has resulted in the formation of a family of DT-based fusion proteins that are selectively cytotoxic for eukaryotic cells that express the appropriate targeted cell surface receptor (8). In general, the cytotoxic action of the DT-related fusion proteins is dependent upon binding of the toxin to the targeted cell surface receptor, receptor-mediated endocytosis of the bound toxin into endocytic vesicles, and upon acidification, the facilitated delivery of the catalytic domain through the endocytic vesicle membrane into the cell cytosol. Once delivered to the cytosol, the catalytic domain catalyzes the ADP-ribosylation of elongation factor 2 (EF2), thereby inhibiting protein synthesis, resulting in cell death. Since the SP/SP-receptor complex has been shown to be internalized into endosomes, we reasoned that an SP-receptor targeted fusion toxin would be selectively cytotoxic (9,10). To permit bacterial expression of the protein, we constructed a plasmid encoding the inactive precursor fusion protein, DAB389SP-Gly, which was converted to the active form, DAB389SP, by treatment with PAM in vitro. DAB389SP was found to be highly potent and selectively cytotoxic to a number of cell lines expressing either the rat or human SP receptor. In contrast, HUT102/6TG cells, which are devoid of the SP receptor, are resistant to the action of this fusion toxin. We also demonstrate that the cytotoxic action of this fusion protein is mediated via the SP receptor and dependent upon endosomal acidification. We anticipate that DAB389SP will prove useful for studies of SP-receptor distribution and SP function, and may have future clinical applications.

PROCEDURES

Oligonucleotides. Oligonucleotides were synthesized using an Applied Biosystems model 391 PCR Mate DNA synthesizer. Two complementary oligonucleotides were designed that, when annealed, encoded SP-Gly followed by a translational stop signal. Following synthesis, the oligonucleotides were removed from the columns and deprotected in NH₄OH as described by the manufacturer. Oligonucleotides were then vacuum dried, resuspended in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0), phenol/chloroform extracted, NaAc/ethanol precipitated, and annealed. The resulting double stranded DNA fragment possessed a ½ SphI site on the 5' end and a ½ HindIII site on the 3' end.

Plasmids and Bacterial Strains. The parental plasmid for construction of the gene encoding DAB389SP-Gly was pET-JV127 (11). pET-JV127 was digested with SphI and HindIII, the large DNA fragment was purified by agarose gel electrophoresis, and the DNA encoding SP-Gly was ligated into the SphI and HindIII sites. The resulting plasmid, pETDAB389SPG, encoded the catalytic and transmembrane domains of DT linked to SP-Gly. Expression of the gene fusion was under control of a T7 polymerase promoter. Plasmid DNA was prepared using a QIAprep spin plasmid kit (Qiagen, Chatsworth, CA) and sequencing was performed using a Sequenase.

Abbreviations: SP, substance P; PAM, peptidylglycine α-amidating monoxygenase; SP-Gly, substance P-glycine; DT, diphtheria toxin; EF, elongation factor; IPTG, isopropyl β-D-thiogalactopyranoside; H1A, radiolabo-inoassay; KNA, neurokinin A; NKB, neurokinin B.

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Expression and Purification of DAB_{389}-Gly. E. coli HMS174(DE3) pETDAB389SGP were grown in 1.0 liter of Luria–Bertani broth containing 100 μg of ampicillin per ml at 37°C with shaking to an A_{600} between 0.8 and 1.0. Expression of the chimeric tox gene was then induced by the addition of isopropyl β-d-thiogalactopyranoside (IPTG) to 1 mM. Under these conditions, the fusion protein accumulated in the E. coli cytosol as inclusion bodies. Following the addition of IPTG, the culture was incubated for 3 hr at 37°C with shaking. The bacteria were harvested by centrifugation at 6000 × g and the inclusion bodies purified as described (11). The final inclusion body pellet was resuspended in 5.0 ml of denaturing buffer (7 M guanidine hydrochloride/100 mM Tris·HCl, pH 8.0/10 mM EDTA) and dithiothreitol was added to a final concentration of 6 mM. The sample was resuspended by sonication and dialyzed overnight, at 4°C, against 2 liters of 30 mM (pH 6.0) 2-(N-morpholino)ethanesulfonic acid (Mes; Sigma). After dialysis, the protein concentration was determined using Pierce protein assay reagent. The protein purity was assessed by electrophoresis in 12% SDS/polyacrylamide gels and immunoblots by using polyclonal anti-DT antibodies. The partially purified DAB_{389}-Gly was stored at −70°C until the amidation reaction was performed.

Amidation. DAB_{389}-Gly was converted to its active form in 30 mM Mes (pH 6.0), 0.001% Triton X-100, 1.0% ethanol, 5 mM potassium iodide, 59 μg of catalase per ml, 0.5 μM CuSO_{4}, 1.5 mM sodium ascorbate, 1.0 × 10^{-6} M fusion protein, and 20,000 units of PAM per ml (Unigene, Fairfield, NJ). The reaction mixture was incubated for 2 hr at 37°C. The potassium iodide and sodium ascorbate solutions were prepared immediately before use. Following incubation, the reaction mixture was centrifuged at 2500 × g for 10 min. The pellet containing the amidated DAB_{389}SP was solubilized in 10 ml denaturing solution and refolded into an active conformation by dialysis overnight at 4°C, against two 1 liter changes of refolding buffer (50 mM Tris·HCl, pH 8.0/50 mM NaCl/5 mM reduced glutathione/1 mM oxidized glutathione). The final concentration of DAB_{389}SP was determined by radioimmunoassay (RIA).

RIA. The concentration of DAB_{389}SP was determined by comparison to the standard curve for displacement of radio-labeled SP in the SP RIA (12). The control was DAB_{389}SP-Gly that was subjected to the same amidating conditions used to prepare DAB_{389}SP, without the addition of PAM.

Cytotoxicity Assay. The stably transfected CHO cell lines HNK-1, RNK-1, HNK AR-2, and HNK-3 were maintained in α-minimal essential medium (GIBCO) supplemented with 10% fetal bovine serum (HyClone) and 800 μg of genetin per ml (GIBCO) at 37°C in a 5% CO_{2}/95% air atmosphere. IM9 and HUT102/6TG cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μg of streptomycin per ml, and 50 units of penicillin per ml at 37°C in a 5% CO_{2}/95% air atmosphere. AR4 cells were also maintained in RPMI 1640 medium, except 20% fetal bovine serum was used. For cytotoxicity assays, 1 × 10^{5} of the transfected CHO cells were seeded in 100 μl of complete media into 96-well flat bottomed plates (Costar). AR4 cells were seeded at a concentration of 5 × 10^{5} cells in 100 μl volumes into flat-bottomed plates in complete media, and IM9 and HUT 102/6TG cells in 5 × 10^{4} cells in 100 μl volumes were seeded in complete media into 96-well V-bottomed plates (Linbro).

The fusion proteins DAB_{389}SP-Gly and DAB_{389}SP were serially diluted such that the addition of 100 μl volumes to assay plates resulted in final concentrations ranging from 1.0 × 10^{-8} to 1.0 × 10^{-13} M. Assay plates were incubated for 18 hr

![Diagram](https://example.com/diagram.png)

**FIG. 1.** DNA and deduced amino acid sequence of the C-terminal extended SP-Gly, and the scheme used for the genetic construction of the fusion gene encoding DAB_{389}SP-Gly.
E. coli was removed. IPTG; 3, the cell construction of the sequence before induction with IPTG; 4, purified inclusion bodies from E. coli HMS174(DE3)PETDAB389SPG; 5, purified DAB389SP after amidation reaction (the M, ~ 56,500 protein is catalase, which is used in the amidation reaction). (B) Immunoblot analysis of DAB389SP-Gly and DAB389SP fusion proteins using antibody to DT. Lanes: 1, molecular weight standards (M, × 10^{-3}); 2, E. coli HMS174(DE3)PETDAB389SPG extracts before induction with IPTG; 3, E. coli HMS174(DE3)PETDAB389SPG extracts after induction with IPTG; 4, purified inclusion bodies from E. coli HMS174(DE3)PETDAB389SPG; 5, purified DAB389SP after amidation reaction. The cells and cultures, the cell construction of the sequence before induction with IPTG; 4, purified inclusion bodies from E. coli HMS174(DE3)PETDAB389SPG; 5, purified DAB389SP after amidation reaction. The DAB389SP-Gly encoding fragment described (11).}

at 37°C in a 5% CO_{2}/95% air atmosphere. The medium was carefully aspirated and replaced with 200 μl of leucine-free minimal essential medium (GIBCO) containing 1.0 μCi/ml [14C]leucine (0.250 mCi/mmol; 1 Ci = 37 GBq; DuPont/ NEN), 2 mM L-glutamine, 50 μg of streptomycin per ml, and 50 units of penicillin per ml. In the case of suspension cell cultures, the assay plates were centrifuged at 170 × g for 5 min to pellet the cells before replacement of the culture medium. The cell cultures were incubated for 90 min, and the medium was removed. Cells were lysed and total protein was precipitated, collected, and counted in a liquid scintillation counter as described (11). Medium alone served as the control, and all assays were performed in quadruplicate.

RESULTS

The sequence of the oligonucleotides encoding the C-terminal glycine extended form of SP and the scheme used for the construction of the plasmid encoding the fusion protein DAB389SP-Gly are shown in Fig. 1. Following ligation of the SP-Gly encoding fragment into the vector and transformation of E. coli JM101, transformants were selected on Luria-Bertani ampicillin agar plates. Several recombinant clones were purified, and the DNA sequence of their respective chimeric tox genes was determined to ensure that the SP-Gly insert was cloned in single copy in the correct orientation and that the correct translational reading frame was maintained through the fusion junction. A single clone was selected, and plasmid DNA was prepared and used to transform E. coli HMS174(DE3) in order to produce DAB389SP-Gly in high yield. E. coli HMS174(DE3)PETDAB389SPG was grown to an A_{590nm} of 0.8 and chimeric tox gene expression was induced by the addition of IPTG. As shown in Fig. 2, there is leaky expression of DAB389SP-Gly in the absence of IPTG. Following the addition of IPTG, there is a marked increase in the yield of the fusion protein which accumulates in inclusion bodies. As analyzed by SDS/PAGE, DAB389SP-Gly has an M, of 43,000. This value is in excellent agreement with the molecular weight of 43,800 as deduced from the nucleic acid base sequence of the fusion protein gene.

To convert the precursor DAB389SP-Gly to the mature amidated DAB389SP form, we treated partially purified preparations of DAB389SP-Gly with PAM in vitro. As shown in Fig. 2, the addition of the PAM reaction mixture, which contains catalase (subunit M, = 56,500; Fig. 2A), and incubation at 37°C for 2 hr does not result in the degradation of the fusion toxin.

![Fig. 2. SDS/PAGE of DAB389SP-Gly and DAB389SP fusion proteins used in this study. (A) SDS/12% polyacrylamide gel following electrophoresis under reducing conditions and staining with Coomassie Brilliant blue. Lanes: 1, molecular weight standards (M, × 10^{-3}); 2, E. coli HMS174(DE3)pETDAB389SPG extracts before induction with IPTG; 3, E. coli HMS174(DE3)pETDAB389SPG extracts after induction with IPTG; 4, purified inclusion bodies from E. coli HMS174(DE3)pETDAB389SPG; 5, purified DAB389SP after amidation reaction.](image1)

To develop an assay to quantitate the conversion of DAB389SP-Gly to DAB389SP, we compared the reactivity of DAB389SP-Gly and DAB389SP in the RIA for SP. Fig. 3 shows

![Fig. 3. Displacement of ^{125}I-labeled SP in the SP radioimmunoassay. •, SP; □, DAB389SP; and ●, DAB389SP-Gly.](image2)

![Fig. 4. Effect of DAB389SP-Gly, DAB389SP, and DA(E149S) B_{389}SP on [^{14}C]leucine incorporation into SP-receptor negative IM-9 cells. •, DAB389SP-Gly; □, DAB389SP; and ●, DA(E149S)B_{389}SP.](image3)

![Fig. 5. Effect of DAB389SP-Gly and DAB389SP on [^{14}C]leucine incorporation into SP-receptor negative HUT102/6TG cells. □, DAB389SP; and ●, DAB389SP-Gly.](image4)
Table 1. Sensitivity of various eukaryotic cell lines to DAB389SP

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Receptor</th>
<th>Receptor number</th>
<th>IC50, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM-9</td>
<td>Human CML</td>
<td>hSP</td>
<td>4,000</td>
<td>1.8 x 10^-11</td>
</tr>
<tr>
<td>HNK-1</td>
<td>Transfected CHO</td>
<td>hSP</td>
<td>490,000</td>
<td>5.1 x 10^-12</td>
</tr>
<tr>
<td>RNK-1</td>
<td>Transfected</td>
<td>rSP</td>
<td>10,000</td>
<td>1.6 x 10^-11</td>
</tr>
<tr>
<td>AR4</td>
<td>Rat pancreatic Ascinar</td>
<td>rSP</td>
<td>20,000</td>
<td>5.4 x 10^-11</td>
</tr>
<tr>
<td>HUT102/6TG</td>
<td>Human T cell</td>
<td>hSP</td>
<td>ND</td>
<td>1.0 x 10^-8</td>
</tr>
<tr>
<td>HNK AR-2</td>
<td>Transfected CHO</td>
<td>hNKKA</td>
<td>630,000</td>
<td>4.2 x 10^-9</td>
</tr>
<tr>
<td>HNK-3</td>
<td>Transfected CHO</td>
<td>hNKB</td>
<td>190,000</td>
<td>2.4 x 10^-8</td>
</tr>
</tbody>
</table>

NKA, neurokinin A; NKB, neurokinin B; ND, not detected; h, human; r, rat.

that DAB389SP-Gly does not displace radiolabeled SP in this assay system. In marked contrast, following treatment of DAB389SP-Gly with PAM and conversion to the amidated form, DAB389SP displaces radiolabeled SP in the assay. Importantly, the displacement of labeled SP by DAB389SP is indistinguishable from that of unlabeled SP.

We next examined the inhibition of protein synthesis in a variety of eukaryotic cell lines after incubation with DAB389SP-Gly and DAB389SP. In the case of IM9 cells, a chronic myelogenous leukemia cell line which is known to express ~4000 SP receptors/cell, the IC50 for DAB389SP was found to be 1.8 x 10^-11 M. In contrast, the IC50 for the precursor DAB389SP-Gly form of the fusion protein was greater than 1 x 10^-8 M (Fig. 4 and Table 1).

To determine whether the inhibition of protein synthesis in IM9 cells was directly due to the effect of ADP ribosylation of EF2 by the catalytic domain, we constructed by site-directed mutation an ADP ribosyltransferase defective mutant, DA(E149S)B389SP-Gly. Following expression, purification, and in vitro treatment with PAM, DA(E149S)B389SP was found to be devoid of cytotoxic activity toward IM9 cells (Fig. 4).

Several lines of evidence demonstrate that the action of the fusion toxin is mediated through the SP receptor (Table 2). The addition of excess SP, SP antibody, or DA(E149S)B389SP to the incubation mixture inhibits the action of DAB389SP on IM9 cells in vitro. The addition of chloroquine, which prevents endosomal acidification, also inhibits the action of the fusion toxin.

We also examined the effect of the fusion toxin on a variety of cell lines in vitro. As shown in Table 1, the IC50 for DAB389SP on a variety of cell lines that express either the human or rat SP receptor is <10^-10 M. In contrast, cell lines that express either the human neurokinin A (NKA) or neurokinin B (NKB) receptor are 100 to 1000-fold less sensitive to DAB389SP; whereas, the HUT102/6TG cell line which is devoid of the SP receptor was found to be more than 1000-fold less sensitive to the fusion toxin.

**DISCUSSION**

Many biologically active neuropeptides, including SP, require a C-terminal α-amide moiety to bind to their receptors. In general, the α-amide moiety arises from a posttranslational oxidative cleavage of a glycine-extended precursor by PAM (13, 14). PAM has been used to amitate a number of peptides in vitro, including recombinant glycine-extended salmon calcitonin (15, 16). In this report, we report the genetic construction, expression, and purification of a DT-based fusion protein, DAB389SP-Gly, which can be converted to a mature active form by treatment with PAM in vitro.

Following expression, purification, and activation with PAM, DAB389SP-Gly was shown to be highly potent and selectively cytotoxic for eukaryotic cells that express either the human or rat SP receptor. In addition, we have demonstrated that the action of this fusion toxin is dependent upon binding to the SP receptor through its C-terminal SP receptor binding domain, and that once bound DAB389SP is internalized by receptor mediated endocytosis. Following acidification of the endosome, a required step in the intoxication process of all DT-related proteins (8), the catalytic domain of DAB389SP is delivered to the target cell cytosol where it specifically ADP ribosylates EF2.

Garland et al. (10) demonstrated that the SP/SP-receptor complex is rapidly internalized into endosomes. The acido-tropic agent chloroquine does not affect the equilibrium binding of SP to its receptor or the rate of internalization of 125I-labeled SP into the cell. It is well known that chloroquine and other lysosomotrophic agents block the cytotoxic action of DT and the DT-related fusion toxins by preventing the acidification of the endocytic vesicle (8, 17). These toxins must pass through an acidic compartment in order for the transmembrane domain to insert into the endocytic vesicle membrane and form a portal of entry for the catalytic domain. In this communication, we have shown that the cytotoxic action of DAB389SP is also blocked by chloroquine (Table 2). These results suggest that the SP/SP-receptor complex is internalized by receptor-mediated endocytosis and that the complex rapidly enters an acidic compartment following entry into the cell.

CHO cells, transfected with either rat or human SP receptors, were found to be very sensitive to DAB389SP. In addition, cell lines expressing naturally occurring SP receptors, AR4 and IM9, were also sensitive to the cytotoxic action of DAB389SP. The sensitivity of these cell lines were within the same order of magnitude, except for the human SP receptor transfected CHO cells, which are somewhat more sensitive. This increased sensitivity may be due to the increased number of cell surface SP receptors expressed by these cells. CHO cells transfected with human NKA and NKB receptors were much less sensitive to DAB389SP. It is known that SP is able to bind to both the NKA and NKB receptor, albeit with lesser affinity than to the SP receptor. The order of potency of the neurokinins, SP, NKA, and NKB is, in fact, considered a valid criterion for receptor classification (6, 18). In contrast, HUT102/6TG cells which do not express SP receptors were resistant to DAB389SP (Table 1).

We anticipate that this first neuropeptide receptor directed fusion toxin, DAB389SP, will be useful for studies of both SP-receptor location and function. For example, the ability to selectively lesion cells bearing SP receptors in vivo may prove useful for studies of tumor angiogenesis, and perhaps lead to new clinical treatments. Additionally, the success of the PAM catalyzed conversion of the inactive precursor DAB389SP-Gly

Table 2. Sensitivity of IM9 cells to DAB389SP in the presence and absence of inhibitors

<table>
<thead>
<tr>
<th>DAB389SP, M</th>
<th>Inhibitor</th>
<th>% control incorporation 14C leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 x 10^-10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.0 x 10^-10</td>
<td>Chloroquine, 10 µM</td>
<td>76</td>
</tr>
<tr>
<td>1.0 x 10^-10</td>
<td>SP, 1 x 10^-7 M</td>
<td>83</td>
</tr>
<tr>
<td>1.0 x 10^-10</td>
<td>Anti-SP, 1 µg</td>
<td>100</td>
</tr>
<tr>
<td>1.0 x 10^-10</td>
<td>DA(E149S)B389SP, 1 x 10^-7 M</td>
<td>88</td>
</tr>
</tbody>
</table>
to the active DAB389SP establishes this paradigm as a useful method for the production of other neuropeptide fusion proteins.

We wish to thank David Merkler for advice on the amidation reaction. This study was supported in part by Public Health Service Grant CA-60934 from the National Cancer Institute (J.R.M.). J.C.v.d.S. is a special fellow of the Leukemia Society of America. C.E.F. is supported by a predoctoral fellowship from the National Institute of Mental Health.