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Synaptotagmin-1 Is Required for Fibroblast Growth Factor-1 Release*

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Theresa M. LaVallee‡, Francesca Tarantini§¶, Susan Gamble‖, Carla Mouta Carreira§, Anthony Jackson, and Thomas Maciag§‡‡**

From the Department of Molecular Biology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855 and the §Center for Molecular Medicine, Maine Medical Center Research Institute, South Portland, Maine 04106

By using p65 synaptotagmin-1 and fibroblast growth factor (FGF)-1:β-galactosidase (β-gal) NIH 3T3 cell co-transfectants, we demonstrate that a proteolytic fragment consisting of the extravesicular domain of synaptotagmin-1 is released into the extracellular compartment in response to temperature stress with similar kinetics and pharmacological properties as FGF-1:β-gal. Using a deletion mutant that lacks 95 amino acids from the extravesicular domain of synaptotagmin-1, neither synaptotagmin-1 nor FGF-1:β-gal are able to access the stress-induced release pathway. Furthermore, the p40 extravesicular fragment of synaptotagmin-1 is constitutively released in p40 synaptotagmin-1 NIH 3T3 cell transfectants, and this release is potentiated when the cells are subjected to temperature stress. These data demonstrate that the p40 fragment derived from synaptotagmin-1 is able to utilize the FGF-1 non-classical exocytotic pathway and that the release of FGF-1 is dependent on synaptotagmin-1.

FGF[1]-1 and FGF-2 are the prototype members of a large family of related heparin-binding growth factors that regulate numerous biological processes including mesoderm formation, neurogenesis, and angiogenesis *in vivo* (1). The FGF prototypes are distinguished among the FGF family members by the absence of a classical signal peptide sequence to direct their secretion through the conventional endoplasmic reticulum (ER)-Golgi pathway. Because the FGF prototypes initiate their biological responses through the activation of high affinity receptors on the surface of target cells, it has been suggested that the secretion of both FGF-1 and FGF-2 may be regulated by novel release pathways. Indeed, the ligation of a signal peptide sequence to FGF-1 followed by somatic gene transfer, which results in the constitutive release of FGF-1, yields a functional oncogene *in vitro* and exaggerated vascular hyperplasia *in vivo* (2, 3).

We have reported that FGF-1 is released from stable FGF-1 NIH 3T3 cell transfectants through a non-classical exocytotic pathway as a latent macromolecule in response to temperature stress which requires activation by either $(NH_4)_2SO_4$ (4) or reducing agents (5–7). During the structural characterization of FGF-1 (8), a heparin-binding protein (p40) that co-eluted with FGF-1 was identified by amino-terminal sequence analysis as a proteolytic fragment of the synaptotagmin-1 (Syn-1) translation product (8). Syn-1 is a 65-kDa vesicular protein and has been most extensively studied in neuronal and endocrine cells. The Syn gene family currently consists of 11 members (9, 10) and is characterized by a divergent amino terminus, a transmembrane domain, a large cytoplasmic region, which is comprised of two repeats of sequences homologous to the protein kinase C C2 domain, and a highly conserved carboxyl terminus (11, 12). The p40 proteolytic fragment identified in neural tissue corresponds to the extravesicular portion of Syn-1. Genetic and microinjection studies have suggested that Syn-1 is the Ca^{2+} sensor for neurotransmitter release (13).

Recently, our laboratory has identified the presence of the Syn-1 transcript and translation product in NIH 3T3 cells, as well as in human umbilical vein endothelial cells (14). Moreover, we have detected the p40 extravesicular portion of Syn-1 in the conditioned medium from FGF-1-transfected cells exposed to heat shock and have observed that the release of FGF-1 in response to temperature stress can be significantly attenuated by the expression of an antisense-Syn-1 construct (14). Since these data indicate that Syn-1 may be involved in the non-classical secretion pathway employed by FGF-1, we used an established *in vitro* system (4) to study the properties of Syn-1 release. NIH 3T3 cells stably transfected with a FGF-1:β-galactosidase (gal) chimera construct release the FGF-1:β-gal chimera as a structurally intact protein in response to temperature stress (15). Because the endogenous level of Syn-1 expression is low in NIH 3T3 cells, we used FGF-1:β-gal NIH 3T3 cells co-transfected with either p65 Syn-1 or a construct expressing the p40 extravesicular domain of the Syn-1 protein to study the release pathway used by these polypeptides. We report that the p40 extravesicular portion of Syn-1 is released from p65 Syn-1 and FGF-1:β-gal co-transfectants in response to temperature stress with similar kinetics as FGF-1:β-gal. The release of both Syn-1 and FGF-1:β-gal is an ATP-driven process that requires transcription and translation but does not require

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‡ Supported by National Institutes of Health Postdoctoral Fellow Training Grant T32 HL07698. Present address: EntreMed, 9610 Medical Ctr. Drive, Rockville, MD 20850.

¶ Present address: Dept. of Geriatric Medicine, University of Florence, School of Medicine, Florence, Italy.

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‡‡ To whom correspondence should be addressed: Center for Molecular Medicine, Maine Medical Center Research Institute, 125 John Roberts Rd., S. Portland, ME 04106. Tel.: 207-761-9783; Fax: 207-828-8071; E-mail: maciat@mail.mmc.org.

[1] The abbreviations used are: FGF, fibroblast growth factor; β-gal, β-galactosidase; 2-dOG, 2-deoxyglucose; DTT, dithiothreitol; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IL, interleukin; BCS, bovine calf serum; Syn-1, synaptotagmin-1; DMEM, Dulbeccos's modified Eagle's medium; LDH, lactate dehydrogenase; del, deletion.

a structurally intact ER-Golgi apparatus. Deleting 95 amino acids from the extravesicular domain of Syn-1 results in a mutant Syn-1 that is not released in response to temperature stress and also prevents the release of FGF-1:β-gal into the heat shock conditioned medium. Transfecting a construct encoding only the extravesicular domain of Syn-1 into FGF-1:β-gal NIH 3T3 cells results in the constitutive release of the p40 extravesicular fragment of Syn-1, and this release is potentiated when the cells are subjected to temperature stress. Although the constitutive release of p40 Syn-1 from these co-transfectants is not energy-dependent, the potentiated release requires ATP. However, both the constitutive release and the potentiated release are ER-Golgi-independent.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine NIH 3T3 cells were obtained from the American Type Culture Collection, and stable NIH 3T3 FGF-1:β-gal cell transfectants were obtained as described previously (15). Cells were transfected with a eukaryotic expression vector containing a gene encoding resistance to hygromycin and the coding sequence for rat p65 Syn-1 (amino acids 1–421) or rat p65-(del 120–214) or rat p40 Syn-1 (amino acids 119–421) containing a Myc tag. Co-transfectants were maintained in Dulbeccos's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% (v/v) bovine calf serum (BCS, HyClone), 1× antibiotic/antimycotic (Life Technologies, Inc.), and 200 μg/ml G418 (Life Technologies, Inc.) and 250 μg/ml hygromycin (Boehringer Mannheim) on human fibronectin (10 $\mu g/cm^2$)-coated dishes.

Synaptotagmin Constructs—The p65 form of rat Syn-1 (a generous gift of T. C. Sudhof) was cloned into the PCRII vector (Invitrogen, San Diego) after generating an *Nde*I site at the amino terminus and a *Bam*HI site at the carboxyl terminus by polymerase chain reaction. The Myc-tag (5′-GAACAAAAGCTTATTTCTGAAGAAGACTTG-3′) was ligated to the carboxyl terminus and consisted of a 10-amino acid (EQKLISEEDL) epitope recognized by the 9E10 monoclonal antibody (16). The p65-(del 120–214) construct was made by removing the *Afl*II-*Kpn*I fragment from the p65 Syn-1 construct resulting in the loss of amino acids 120–214 and the addition of 3 amino acids (GID). The p40 form of rat Syn-1 was made by deleting the 5′ sequences at the *Afl*II site and thus generated a construct that encoded for amino acids 119–421, which includes the C2A and C2B domains and the conserved carboxyl terminus, as well as the Myc tag. For transfection analysis p65 Syn-1, p65-(del 120–214) and p40 Syn-1 were cloned into the pMEXhygro vector. This vector was generated from the pMEXneo vector (17) by removing the *Stu*I-*Nru*I fragment containing the neomycin resistance gene and inserting a 1774-base pair *Hpa*I-*Eco*3I fragment containing the hygromycin B resistance promoter and gene from the p3′SS vector (Stratagene).

Temperature Stress and Processing of the Condition Medium—For heat shock, the transfected cells were grown to confluence in DMEM containing 10% (v/v) BCS and 1× antibiotic/antimycotic on human fibronectin (10 $\mu g/cm^2$)-coated dishes; the cells were washed with DMEM containing 4 units/ml heparin (The Upjohn Co.) and subjected to heat shock (42 °C for 100 min) in DMEM containing 4 units/ml heparin and 0.5% BCS as described previously (4). Three independent clones were tested for each transfection. In order to study the biochemical properties of secretion, prior to heat shock the transfectants were incubated at 37°C with either 10 μg/ml actinomycin D (2 h, added from a 1 mg/ml stock solution in ethanol; Sigma), 10 μg/ml cycloheximide (2 h, added from a 10 mg/ml stock solution in Me_2SO; Sigma), 5 μg/ml brefeldin A (0.5 h, added from a 1 mg/ml stock solution in ethanol; Epicenter Technologies), 50 mM 2-deoxyglucose (1 h; Sigma), or 5 μM A23187 (Sigma); the transfectants were washed with DMEM containing 4 units/ml heparin and then subjected to heat shock (42 °C for 100 min) in DMEM containing 4 units/ml heparin and 0.5% BCS in the presence of the drug, except for A23187, which was performed as described under "Results." Conditioned media were collected after temperature stress, processed by filtration, and activated with 0.1% (w/v) dithiothreitol (DTT, Sigma) by incubating at 37 °C for 2 h (5). The conditioned medium was adsorbed to a 1-ml heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris, pH 7.4, containing 10 mM EDTA (TEB). The column was washed with TEB and then eluted with TEB containing 1.5 M NaCl. The eluant was concentrated using a Centricon 10 concentrator (Amicon, Inc.) and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (5). To examine the cell lysate, cells were collected, pelleted, and lysed in sample buffer; a fraction of the cell lysate was analyzed by 10% SDS-PAGE.

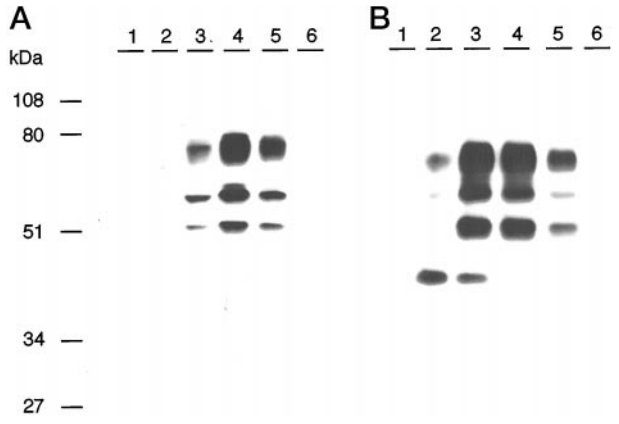

FIG. 1. **A comparison of the endogenous Syn-1 immunoreactive polypeptides with the transfected rat p65 Syn-1 polypeptides.** FGF-1 NIH 3T3 cell transfectants (*A*) or p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants (*B*) were collected and lysed in PBS containing 1% Triton. The lysate was adsorbed to heparin-Sepharose and eluted with increasing concentrations of NaCl, resolved by 10% SDS-PAGE and immunoblotted for Syn-1. *Lane 1,* 0.25 M NaCl; *lane 2,* 0.4 M NaCl; *lane 3,* 0.6 M NaCl; *lane 4,* 0.8 M NaCl; *lane 5,* 1.0 M NaCl; and *lane 6,* 1.5 M NaCl. Approximately 9-fold more cell lysate was used in *A* than in *B* and approximately an 8-fold longer exposure of the immunoblot was needed for *A* than *B*.

Cell viability was assessed by measuring lactate dehydrogenase (LDH) activity in the conditioned medium after filtration as described by Bergmeyer (18) (Sigma procedure DG 1340-UV). To determine total LDH activity cells were lysed in PBS containing 1% Triton X-100 and sonicated. The debris was pelleted, a 1:80 dilution of the cell lysate was made, and LDH activity was measured. This value was used to determine the percent of LDH activity in the conditioned medium.

Immunoblot analysis of the samples resolved by SDS-PAGE was performed as described previously (4). Briefly, after transfer to a nitrocellulose filter, the filters were incubated with 24 mM Tris, pH 7.4, containing 136 mM NaCl, 2 mM KCl, and 0.1% (v/v) Tween 20 (TCB) that contained 5% (w/v) milk for 2 h at 42 °C. The filter was probed with either 5 μg/ml polyclonal anti-human FGF-1 antibody (19) or 5 μg/ml polyclonal anti-rat Syn-1 antibody (14) in TCB containing 5% milk for 1 h at room temperature. The filter was washed with TCB and then visualized using the ECL detection system (Amersham Pharmacia Biotech).

RESULTS

The Kinetics of p40 Syn-1 Release in Response to Temperature Stress—Consistent with the results in cell lysates from NIH 3T3 cells that are not transfected with p65 Syn-1, which have been adsorbed to and eluted from heparin-Sepharose, the lysate from the p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants resolves several heparin-binding Syn-1 immunoreactive polypeptides as visualized by Syn-1 immunoblot analysis (compare Fig. 1, *A* with *B*). Tunicamycin treatment and pulse-chase analysis of the p65 NIH 3T3 cell transfectants (data not shown) demonstrated that these polypeptides are partially due to differential *N*-glycosylation, an observation consistent with reports that Syn-1 is *N*-glycosylated and palmitoylated (20, 21). To determine the kinetics of release of Syn-1, p65 Syn-1 was stably transfected into FGF-1:β-gal NIH 3T3 cell transfectants, and the heat shock-conditioned medium from the co-transfectants was collected and examined after adsorption to and elution from heparin-Sepharose. The p40 extravesicular fragment of Syn-1 was clearly detectable in the conditioned medium after 1.5 h at 42 °C (Fig. 2*A*). Supplementing the medium with protease inhibitors did not influence the generation of p40 Syn-1 (data not shown). Analysis of the heat shock-conditioned medium using FGF-1 immunoblot methods demonstrated that FGF-1:β-gal is also released with similar kinetics (Fig. 2*B*). Lactate dehydrogenase (LDH) activity was

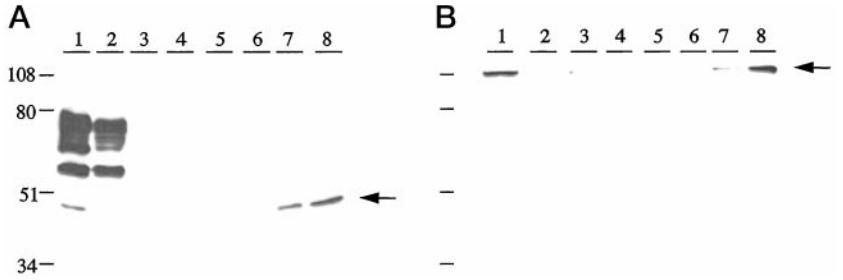

FIG. 2. **The release kinetics of the extravesicular domain of Syn-1 and FGF-1:β-gal from NIH 3T3 cells in response to temperature stress.** Lysate and conditioned medium from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants were collected at various times and immunoblot analysis for Syn-1 (*A*) and FGF-1:β-gal (*B*) were performed as described under "Experimental Procedures." *Lanes 1* and *2* are the lysates from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell transfectants at 37 °C (2 h) and 42 °C (2 h), respectively. *Lanes 3–7* are the conditioned medium from cells treated at 37 °C; *lane 3,* 2 h or at 42 °C; *lane 4,* 0.25 h; *lane 5,* 0.5 h; *lane 6,* 1.0 h; *lane 7,* 1.5 h; *lane 8,* 2.0 h. The *arrow* in *A* denotes the p40 extravesicular fragment of Syn-1, and the *arrow* in *B* denotes FGF-1:β-gal.

measured to demonstrate that the cells did not undergo significant lysis after the cells were subjected to temperature stress. We typically observed 5.6% of the total cell lysate LDH activity in the medium conditioned at 37 °C for 100 min and 8.5% of the total cell lysate LDH activity in the medium conditioned by heat shock (42 °C, 100 min).

Because FGF-1 NIH 3T3 cell transfectants co-transfected with an antisense-Syn-1 construct are impaired in their ability to release FGF-1 in response to temperature stress (14), we evaluated whether Syn-1 release was dependent on FGF-1 expression. The p65 Syn-1 NIH 3T3 cell transfectants, which have very low levels of endogenous FGF-1 and p65 Syn-1 NIH 3T3 cells co-transfected with a Cys-free mutant non-secreted form of FGF-1 (5), demonstrated that the release of p40 Syn-1 was similar to that observed in the p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants (Fig. 3, *A* and *B*). These data suggest that p40 Syn-1 release from the p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants in response to heat shock does not depend on the expression of high levels of cytosolic FGF-1 that is competent to enter the stress-induced release pathway.

The Pharmacology of p40 Syn-1 Release in Response to Temperature Stress—We have previously demonstrated that the release of FGF-1 is dependent on both transcription and protein synthesis because FGF-1 release is blocked by exposure to either actinomycin D or cycloheximide (4). Furthermore, FGF-1 utilizes a non-classical release mechanism since its appearance in the extracellular compartment is not sensitive to treatment with brefeldin A (Fig. 4*B, lane 3–5* (4)). Thus, we analyzed the effects of actinomycin D, cycloheximide, and brefeldin A on the release of Syn-1. Consistent with the results obtained for the release of FGF-1 (4), we observed that the release of p40 Syn-1 is inhibited by actinomycin D and cycloheximide, and its release is insensitive to treatment with brefeldin A (Fig. 4*A, lanes 3–5*). Additionally, FGF-1 is released into the heat shock conditioned medium as a latent low heparin-binding affinity complex that must be activated with either $(NH_4)_2SO_4$ or DTT in order to bind immobilized heparin with high affinity (4, 14). Heparin eluates of conditioned medium from temperature stress-induced p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants that are not activated by DTT contain lower levels of p40 Syn-1 and FGF-1:β-gal (Fig. 4, *A* and *B, lane 7*). These results suggest that the secreted p40 Syn-1 and FGF-1:β-gal exhibit reduced heparin affinity when the conditioned medium is not activated with DTT.

It has been suggested that Syn-1 is the Ca^{2+} sensor during neurotransmitter release (22). Furthermore, ATP and calcium have been demonstrated to play an important role in regulated exocytotic pathways. Thus, we sought to determine whether these cofactors play a role in the release of either Syn-1 or FGF-1:β-gal. To determine the ATP requirement for the release of these polypeptides, the NIH 3T3 cell co-transfectants were subjected to heat shock in the presence of 2-deoxyglucose (dOG), an analog of glucose that inhibits glycolysis and thus the generation of ATP (23). As shown in Fig. 4, *A* and *B* (*lane 6*), the release of both p40 Syn-1 and FGF-1:β-gal in response to temperature stress was severely reduced in the presence of 2-dOG. To assess whether an increase in intracellular Ca^{2+} concentration would have an effect on FGF-1:β-gal and p40 Syn-1 release, the p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants were pretreated with the Ca^{2+} ionophore, A23187, at 37 °C and then subjected to heat shock in the absence of the drug. Surprisingly, A23187 did not affect the release of either p40 Syn-1 (Fig. 5*A*) or FGF-1:β-gal (Fig. 5*B*) at 37 or at 42 °C. Additionally, treating the cells with the ionophore at 37 °C for up to 2 h did not stimulate the release of p40 Syn-1 or FGF-1:β-gal (data not shown). Unfortunately, we were unable to assess whether treating the cells with the ionophore for longer intervals during heat shock has an effect on either p40 Syn-1 or FGF-1:β-gal release since these conditions resulted in cell lysis. Ca^{2+} is generally considered to be a transient stimulant, and pretreating the cells prior to temperature stress may not deliver the increase in intracellular Ca^{2+} at the appropriate time. Therefore, we subjected the p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants to heat shock for 1.5 h, added the ionophore, and continued the heat shock in the presence of A23187 for 15 min. Similar to the pretreatment of the cells with the ionophore, delivering the Ca^{2+} signal 1.5 h into the heat shock had no effect on the release of either p40 Syn-1 or FGF-1:β-gal (data not shown).

The Release of Both Syn-1 and FGF-1:β-gal Is Dependent on Residues 120–214 in p65 Syn-1—To investigate further whether Ca^{2+} might play a role in this release mechanism, a 95-amino acid deletion was made in p65 Syn-1 that deleted the Ca^{2+}-binding site, the phosphatidylserine-binding domain, and the casein kinase II phosphorylation site (39, 42) (Fig. 6*A*). Similar to the p65 Syn-1 lysate, the p65-(del 120–214) lysate contained several immunoreactive Syn-1 polypeptides, including a 35-kDa fragment suggesting that the deletion mutant is competent to generate a soluble extravesicular domain (Fig. 6*B, lane 2*). However, examining the heat shock conditioned medium of the p65-(del 120–214) and FGF-1:β-gal NIH 3T3 co-transfectants showed that neither p65-(del 120–214) (Fig. 6*B*) nor FGF-1:β-gal (Fig. 6*C*) was present in the extracellular compartment. These data suggest that this 95-amino acid region is required for both Syn-1 and FGF-1:β-gal to access the stress-induced release pathway.

The p40 Syn-1 Domain Is Released from NIH 3T3 Cells Independent of Temperature Stress—The detection of only the p40 extravesicular portion of p65 Syn-1 in the extracellular

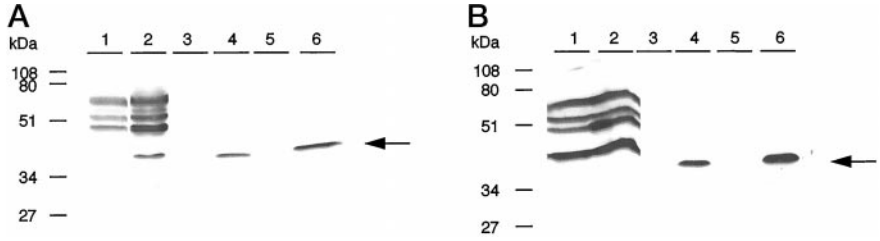

FIG. 3. **The release of the extravesicular domain of p65 Syn-1 is not dependent on high levels of FGF-1 that are able to access the stress-induced exocytotic pathway.** *A,* lysate and conditioned medium were collected, and immunoblot analysis for Syn-1 was performed as described under "Experimental Procedures." *Lane 1* is the lysate from p65 Syn-1 NIH 3T3 cell transfectants at 37 °C. *Lane 2* is the lysate from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants at 37 °C. *Lanes 3* and *4* are the conditioned medium from p65 Syn-1 NIH 3T3 cell transfectants treated at 37 °C (100 min) or at 42 °C (100 min), respectively. *Lanes 5* and *6* are the conditioned medium from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants treated at 37 °C (100 min) or at 42 °C (100 min), respectively. *B,* lysate and conditioned medium were collected, and immunoblot analysis for Syn-1 was performed as described under "Experimental Procedures," except cells were lysed in PBS containing 1% Triton X-100, and lysate was adsorbed to heparin-Sepharose and eluted at 1.5 M NaCl. *Lane 1* is the lysate from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants at 37 °C. *Lane 2* is the lysate from p65 Syn-1 and Cys-free FGF-1 NIH 3T3 cell co-transfectants at 37 °C. *Lanes 3* and *4* are the conditioned medium from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants treated at 37 °C (100 min) or at 42 °C (100 min), respectively. *Lanes 5* and *6* are the conditioned medium from p65 Syn-1 and Cys-free FGF-1 NIH 3T3 cell co-transfectants treated at 37 °C(100 min) or at 42 °C (100 min), respectively.

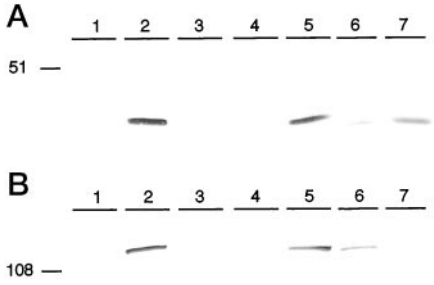

FIG. 4. **The biochemical properties of the release of the extravesicular domain of Syn-1 and FGF-1:β-gal from NIH 3T3 cells.** The p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants were treated, and conditioned medium was collected, and immunoblot analyses for Syn-1 (*A*) and FGF-1:β-gal (*B*) were performed as described under "Experimental Procedures." *Lanes 1* and *2* are conditioned medium collected from untreated cells at 37 and 42 °C, respectively. *Lanes 3–6* are heat shock conditioned medium from cells treated with the following: *lane 3,* actinomycin D; *lane 4,* cycloheximide; *lane 5,* brefeldin A; *lane 6,* 2-dOG; and *lane 7* is heat shock conditioned medium not activated with DTT. Figure is a composite of several experiments. The levels of Syn-1 and FGF-1:β-gal expression in cell lysate were unaffected by treatment with the various drugs (data not shown). LDH activity was measured to demonstrate that the cells did not undergo significant lysis in the presence of the drugs and temperature stress: 37 °C 5.6%, 42 °C 8.5%, actinomycin D 11.0%, cycloheximide 8.9%, brefeldin A 10.4% and 2-dOG 7.8%.

compartment suggests the function of a protease that may be important to relieve p65 Syn-1 from its transmembrane constraint. The activity of this protease may be induced by temperature stress and thus may be a regulated step in the Syn-1 exocytotic pathway. To determine whether the generation of the p40 proteolytic fragment of Syn-1 is regulated, we stably co-transfected a construct expressing the p40 extravesicular fragment of Syn-1 into the FGF-1:β-gal NIH 3T3 transfectants and examined the conditioned medium for the presence of p40 Syn-1 and FGF-1:β-gal prior to and after heat shock. Interestingly, we observed the constitutive release of the p40 Syn-1 fragment at 37 °C (Fig. 7*A*). In addition, we did not observe an increase in the release of FGF-1:β-gal at 37 °C from the p40 Syn-1 and FGF-1:β-gal co-transfectants (Fig. 7*B*). Furthermore, following temperature stress, the release of p40 Syn-1 was potentiated in the p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants (Fig. 7*A, lanes 4* and *5*). Consistent with the results observed with the p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants, the release of p40 Syn-1 (Fig. 7*A*, compare *lanes 4* and *5* with *lanes 6* and *7*) and FGF-1:β-gal (Fig. 7*B*) from the p40 Syn-1 and FGF-1:β-gal co-transfectants was unaffected by treatment with brefeldin A. In contrast, however, the release of p40 Syn-1 from the p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants was not attenuated at 37 °C in the presence of 2-dOG (Fig. 7*C* compare *lanes 4* and *6*), but the potentiation of p40 Syn-1 release and the release of FGF-1:β-gal upon heat shock was inhibited in the presence of 2-dOG (Fig. 7*C* compare *lanes 5* and *7* and Fig. 7*D* compare *lanes 4* and *6*). These data suggest that the release of p40 Syn-1 may not be regulated by the conditions of temperature stress, rather it may be regulated by the function of a protease whose activity is induced by temperature stress.

DISCUSSION

Syn-1 has been characterized as a constituent of a multiprotein complex involved in the regulation of neurotransmitter release. Although Syn-1 is thought to be the Ca^{2+} sensor for neurotransmitter release, Syn-1 Ca^{2+}-independent interactions have been identified, and several of the Syn family members have been reported to function in a Ca^{2+}-independent manner (9, 22). Our attempts to increase the intracellular concentration of Ca^{2+} by treating the cells with the ionophore A23187 did not stimulate the release of either Syn-1 or FGF-1:β-gal *in vitro*, suggesting that this release mechanism may utilize a component of the Ca^{2+}-independent function of Syn-1. However, we cannot conclude that Ca^{2+} does not play a role in this non-classical release mechanism, since it is possible that the ionophore was not delivered either at the appropriate time or in the appropriate cellular context, or perhaps it may need additional co-factors to function in this pathway. Moreover, deleting residues 120–214 from Syn-1 blocks the release of both Syn-1 and FGF-1 in response to temperature stress. This region contains the Ca^{2+}-binding site, the phosphatidylserine-binding site, and the casein kinase II phosphorylation site (39, 42) and thus suggests that one of these activities may be required in this release mechanism. Likewise, 2-dOG affects the generation of ATP and thus a number of cellular processes including glycosylation. Whereas the decrease in the temperature stress-induced release of both Syn-1 and FGF-1:βgal in the presence of 2-dOG suggests that ATP may play a role in this pathway, we cannot conclude what is the exact mechanism by which ATP is utilized.

Even though the FGF-1 non-classical release pathway is utilizing components of the classical exocytotic pathway, the mechanism of FGF-1 release appears to be novel. It has been proposed that during neurotransmitter release, Syn-1 facilitates the docking of the vesicle at the plasma membrane and then, in an ATP-dependent step, the vesicle fuses with the plasma membrane and subsequently releases the vesicular

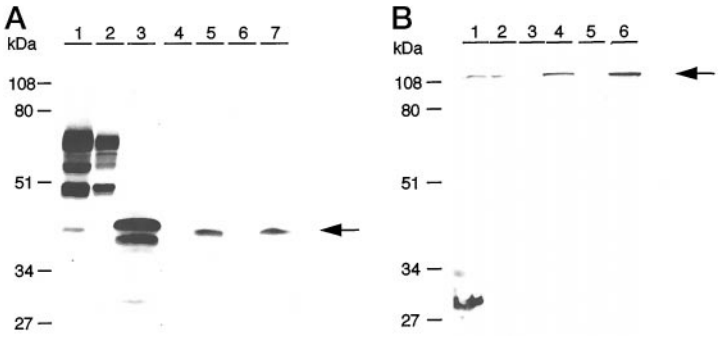

FIG. 5. **Raising the intracellular concentration of calcium does not stimulate the release of Syn-1 or FGF-1:β-gal.** The p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants were pre-treated with A23187 for 15 min, washed, and incubated in the absence of drug at 37 or 42 °C for 100 min; conditioned medium was collected, and immunoblot analyses for Syn-1 (*A*) and FGF-1:β-gal (*B*) were performed as described under "Experimental Procedures." *A,* Syn-1 immunoblot. *Lanes 1* and *2* are lysate collected from untreated cells at 37 and 42 °C, respectively. *Lane 3* is 50 ng of recombinant rat Syn-1 extravesicular domain. *Lanes 4* and *5* are conditioned medium collected from untreated cells at 37 °C and 42 °C, respectively. *Lanes 6* and *7* are conditioned medium collected from cells pretreated with the ionophore at 37 and 42 °C, respectively. *B,* FGF-1 immunoblot. *Lanes 1* and *2* are cell lysates collected from untreated cells at 37 and 42 °C, respectively. *Lanes 3* and *4* are conditioned medium collected from untreated cells at 37 and 42 °C, respectively. *Lanes 5* and *6* are conditioned medium collected from cells pretreated with the ionophore at 37 and 42 °C, respectively. LDH values in the presence of A23187 were 5.8% at 37 °C and 11.7% at 42 °C

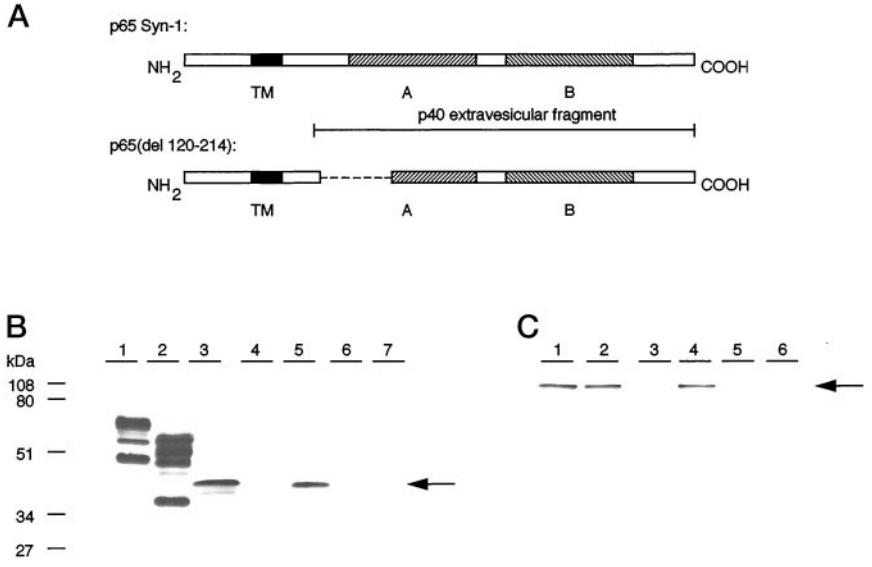

FIG. 6. **Deleting residues 120–214 results in a mutant Syn-1 that inhibits the release of both Syn-1 and FGF-1:β-gal.** *A,* a schematic illustration of the domain structure of p65 Syn-1 (*top*) and the site of the deletion in p65-(del 120–214) (*bottom*). *TM* represents the transmembrane region; *A*, the first C2 domain; *B*, the second C2 domain. The p40 extravesicular portion of Syn-1 that is released into the heat shock conditioned medium is denoted. *B,* lysate and conditioned medium were collected, and immunoblot analysis for Syn-1 was performed as described under "Experimental Procedures." *Lane 1* is the lysate from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants at 37 °C. *Lane 2* is the lysate from p65-(del 120–214) and FGF-1:β-gal NIH 3T3 cell co-transfectants at 37 °C. *Lane 3* is 50 ng of recombinant rat Syn-1 extravesicular domain. *Lanes 4* and *5* are the conditioned medium from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell transfectants treated at 37 °C (100 min) or at 42 °C (100 min), respectively. *Lanes 6* and *7* are the conditioned medium from p65-(del 120–214) and FGF-1:β-gal NIH 3T3 cell co-transfectants treated at 37 °C (100 min) or at 42 °C (100 min.), respectively. *Arrow* indicates p40 Syn-1. *C,* lysate and conditioned medium were collected, and immunoblot analysis for FGF-1 was performed as described under "Experimental Procedures." *Lane 1* is the lysate from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants at 37 °C. *Lane 2* is the lysate from p65-(del 120–214) and FGF-1:β-gal NIH 3T3 cell co-transfectants at 37 °C. *Lanes 3* and *4* are the conditioned medium from p65 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants treated at 37 °C (100 min) or at 42 °C (100 min), respectively. *Lanes 5* and *6* are the conditioned medium from p65-(del 120–214) and FGF-1:β-gal NIH 3T3 cell co-transfectants treated at 37 °C (100 min) or at 42 °C (100 min), respectively. *Arrow* denotes FGF-1:β-gal.

contents into the extracellular compartment (23). In contrast, we have observed the release of the extravesicular (cytoplasmic) domain of Syn-1 (p40) into the medium conditioned by heat shock, and our data suggest that the release of p40 Syn-1 in response to stress occurs by an ER-Golgi-independent mechanism that requires time, energy, transcription, and translation. These features are also conserved within the non-classical FGF-1 release pathway, and the release of both polypeptides requires a 95-amino acid domain contained within the extravesicular portion of Syn-1.

The data presented here demonstrate that Syn-1 is required for the release of FGF-1 and, more specifically, that it is the sequences within the extravesicular domain of Syn-1 that are involved in the regulation of this release. Moreover, these data confirm our prior observation that antisense-Syn-1 NIH 3T3 cell transfectants are attenuated in their ability to release FGF-1 in response to temperature stress (14). Syn-1 has been demonstrated to bind a large number of proteins and phospholipids, and the majority of these interactions are mediated by the C2 domains in the extravesicular portion of Syn-1 (9, 24, 25). Although deleting residues 120–214 from Syn-1 results in a dominant-negative mutant Syn-1 that blocks FGF-1:β-gal release, when either p40 Syn-1 or p65 Syn-1 was co-transfected into FGF-1:β-gal NIH 3T3 cells, we did not observe an increase in FGF-1:β-gal release in response to temperature stress when compared with singly transfected FGF-1:β-gal NIH 3T3 cell

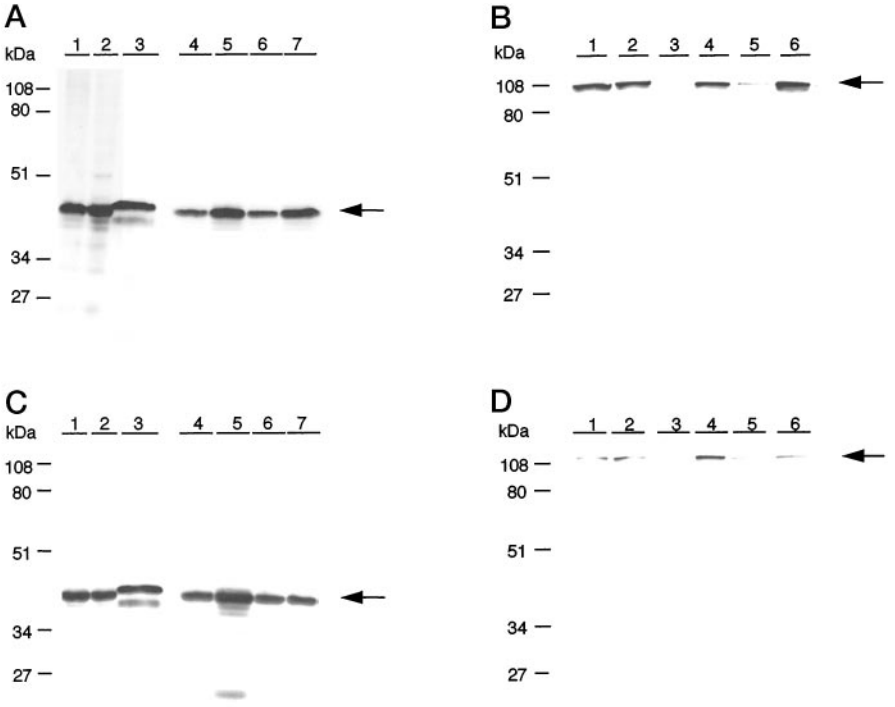

FIG. 7. **The release of the extravesicular domain of Syn-1 from p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants.** The extravesicular domain of Syn-1 is constitutively secreted from p40 Syn-1 and FGF-1:β-gal co-transfected NIH 3T3 cells, and its secretion is insensitive to brefeldin A. The p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants were treated, lysate and conditioned medium were collected, and immunoblot analyses for Syn-1 (*A*) and FGF-1:β-gal (*B*) were performed as described under "Experimental Procedures." *A,* Syn-1 immunoblot. *Lanes 1* and *2* are the lysates from p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell transfectants at 37 °C untreated or treated with brefeldin A, respectively. *Lane 3* is 50 ng of recombinant rat Syn-1 extravesicular domain. *Lanes 4* and *5* are conditioned medium collected from untreated cells at 37 and 42 °C, respectively. *Lanes 6* and *7* are conditioned medium collected from cells treated with brefeldin A at 37 and 42 °C, respectively. *Arrow* indicates p40 Syn-1. *B,* FGF-1 immunoblot. *Lanes 1* and *2* are the lysates from p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell transfectants at 37 °C untreated or treated with brefeldin A, respectively. *Lanes 3* and *4* are conditioned medium collected from untreated cells at 37 and 42 °C, respectively. *Lanes 5* and *6* are conditioned medium collected from cells treated with brefeldin A at 37 and 42 °C, respectively. *Arrow* indicates FGF-1:β-gal. *C* and *D* demonstrate the constitutive release of p40 Syn-1 at 37 °C is unaffected by treatment with 2-dOG; however, the potentiation of p40 Syn-1 release in response to temperature stress is sensitive to treatment with 2-dOG. The p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants were treated, lysate and conditioned medium were collected, and immunoblot analyses for Syn-1 (*C*) and FGF-1:β-gal (*D*) were performed as described under "Experimental Procedures." *C,* Syn-1 immunoblot. *Lanes 1* and *2* are the lysates from p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell transfectants at 37 °C untreated or treated with 2-dOG, respectively. *Lane 3* is 50 ng of recombinant rat Syn-1 extravesicular domain. *Lanes 4* and *5* are conditioned medium collected from untreated cells at 37 and 42 °C, respectively. *Lanes 6* and *7* are conditioned medium collected from cells treated with 2-dOG at 37 and 42 °C, respectively. *Arrow* indicates p40 Syn-1. *D,* FGF-1 immunoblot. *Lanes 1* and *2* are the lysates from p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell transfectants at 37 °C untreated or treated with 2-dOG, respectively. *Lanes 3* and *4* are conditioned medium collected from untreated cells at 37 and 42 °C, respectively. *Lanes 5* and *6* are conditioned medium collected from cells treated with 2-dOG at 37 and 42 °C, respectively. *Arrow* indicates FGF-1:β-gal. LDH activity was measured to demonstrate that the cells did not undergo significant lysis in the presence of the drugs and temperature stress: 37 °C, 4.0% and 42 °C, 7.2%; brefeldin A: 37 °C, 8.5% and 42 °C, 9.1%; and 2-dOG: 37 °C; 5.1% and 42 °C, 5.1%.

transfectants. Moreover, p40 Syn-1 was released following transfection into NIH 3T3 cells that do not overexpress FGF-1 or that express the cysteine-free mutant FGF-1 that is not competent to enter the release pathway (Fig. 3). FGF-1:β-gal release may not be increased when either p40 Syn-1 or p65 Syn-1 is overexpressed because, similar to the regulation of the conventional secretion pathway, this non-classical pathway may be a multiprotein complex and another factor(s) may be limiting. The identification of a S100 gene family member as a component of a multiprotein Syn-1 and FGF-1 complex in ovine brain (26) and the inhibition of the temperature stress-induced release of FGF-1 and Syn-1 from cells treated with amlexanox (26), an anti-inflammatory agent and S100A13-binding compound (see Ref. 27 and GenBank™ accession number AB001567), support this premise. The observation that p40 Syn-1 release is not dependent on the expression of FGF-1 suggests that Syn-1 or perhaps Syn gene family members may also be involved in the regulation of other proteins that utilize non-classical release pathways, such as FGF-2 and the interleukin (IL)-1 prototypes IL-1α and IL-1β.

The p40 Syn-1 and FGF-1:β-gal NIH 3T3 cell co-transfectants exhibited the constitutive release of p40 Syn-1 at 37 °C. Since treatment with 2-dOG only inhibits the potentiation of p40 Syn-1 release after temperature stress, the 2-dOG data suggest that only a small percentage of the p40 Syn-1 may be able to access the non-classical FGF-1 release pathway (Fig. 7*C*). It is also possible that the soluble non-vesicular p40 Syn-1 may be able to enter the regulated non-classical release pathway(s) through protein-protein interactions. This may occur by potential interactions with endogenous p65 Syn-1, since the C2B domain of Syn-1 has been demonstrated to self-associate (28–30).

The constitutive release of p40 Syn-1 from p40-transfected NIH 3T3 cells and the regulated release of p40 Syn-1 from p65-transfected NIH 3T3 cells suggests that a protease may be involved in the regulation of the FGF-1 and Syn-1 release pathways. However, the generation of a soluble form of Syn-1 is not the only requirement for release, since the p65-(del 120–214) construct produces a p35 proteolytic fragment that is not competent to enter the stress-induced release pathway. p65 Syn-1 has been characterized to contain a single hypersensitive proteolytic site (between amino acids 111 and 112) *in vitro,* and cleavage at this site results in the liberation of the extravesicular Syn-1 fragment from vesicles into the soluble fraction (12,

31). Increasingly, intracellular proteases are proving to play pivotal roles in the regulation of biological systems, such as the cell cycle proteases in the regulation of cell growth (32), the caspase family of proteases in the regulation of apoptosis (33), and the chaperone-regulated proteases in the regulation of the stress response (34). Indeed, proteases have been demonstrated to play a role in the release pathways of IL-1β (35, 36) and annexin-1 (37), two proteins that lack a signal sequence.

Whereas the components of the classical secretion pathway are currently under characterization, non-classical secretion pathways are not well defined. Recently, Cleves *et al.* (38) expressed a mammalian protein, galectin-1, in yeast and identified three proteins that were involved in the non-classical exocytosis of this protein, suggesting that non-classical secretion pathways may also be evolutionarily conserved. However, FGF-1 and FGF-2 utilize divergent exocytotic pathways, since FGF-2 is not secreted in response to temperature stress (15) and FGF-1 may utilize a nonvesicular exocytotic mechanism, since the soluble p40 extravesicular portion of Syn-1 is secreted with FGF-1. Although we do not know the mechanism that FGF-1 and p40 Syn-1 use for release, both polypeptides bind phosphatidylserine (7, 29, 39), which may enable them to interact with the cytosolic face of membranes and, possibly, each other. Deleting a region of the Syn-1 protein that contains the phosphatidylserine-binding site, p65-(del 120–214), blocks the release of both Syn-1 and FGF-1:β-gal and further suggests that phosphatidylserine may play a role in this non-classical release mechanism. Additionally, FGF-1 exhibits molten globule character at 42 °C which may allow FGF-1 to disrupt/interrupt membrane structural integrity (40). Whether p40 Syn-1 exhibits a similar feature is not known; however, it had recently been shown that the C2A domain of Syn-1 is able to directly penetrate the lipid bilayer (41).

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REFERENCES

1. Burgess, W. H., and Maciag, T. (1989) *Annu. Rev. Biochem.* **58,** 575–606
2. Forough, R., Xi, Z., MacPhee, M., Friedman, S., Engleka, K. A., Sayers, T., Wiltrout, R. H., and Maciag, T. (1993) *J. Biol. Chem.* **268,** 2960–2968
3. Nabel, E. G., Yang, Z.-Y., Plautz, G., Forough, R., Zhan, X., Haudenschild, C. C., Maciag, T., and Nabel, G. J. (1993) *Nature* **362,** 844–846
4. Jackson, A., Friedman, S., Zhan, X., Engleka, K. A., Forough, R., and Maciag, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10691–10695
5. Jackson, A., Tarantini, F., Gamble, S., Friedman, S., and Maciag, T. (1995) *J. Biol. Chem.* **270,** 33–36
6. Engleka, K. A., and Maciag, T. (1992) *J. Biol. Chem.* **267,** 11307–11315
7. Tarantini, F., Gamble, S., Jackson, A., and Maciag, T. (1995) *J. Biol. Chem.* **270,** 29039–29042
8. Burgess, W. H., Mehlman, T., Friesel, R., Johnson, W., and Maciag, T. (1985) *J. Biol. Chem.* **260,** 11389–11392
9. Sudhoff, T. C., and Rizo, J. (1996) *Neuron* **17,** 379–388
10. Kwon, O. J., Gainer, H., Wray, S., and Chin, H. (1996) *FEBS Lett.* **378,** 135–139
11. Perin, M. S., Johnston, P. A., Ozcelik, T., Jahn, R., Francke, U., and Sudhof, T. C. (1991) *J. Biol. Chem.* **266,** 615–622
12. Perin, M. S., Brose, N., Jahn, R., and Sudhof, T. C. (1991) *J. Biol. Chem.* **266,** 623–629
13. Kelly, R. B. (1995) *Curr. Biol.* **5,** 257–259
14. Tarantini, F., LaVallee, T., Jackson, A., Gamble, S., Garfinkel, S., Mouta Carreira, C., Burgess, W. H., and Maciag, T. (1998) *J. Biol. Chem.* **273,** 22209–22216
15. Shi, J., Friedman, S., and Maciag, T. (1997) *J. Biol. Chem.* **272,** 1142–1147
16. Kolodziej, P. A., and Young, R. A. (1991) *Methods Enzymol.* **194,** 508–519
17. Martin, G. M., Sprague, C. A., and Epstein, C. J. (1970) *Lab. Invest.* **23,** 86–92
18. Bergmeyer, H.-U. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed) pp. 736–743, Academic Press, New York
19. Sano, H., Forough, R., Maier, J. A., Case, J. P., Jackson, A., Engleka, K., Maciag, T., and Wilder, R. L. (1990) *J. Cell Biol.* **110,** 1417–1426
20. Bennett, M. K., Miller, K. G., and Scheller, R. H. (1993) *J. Neurosci.* **13,** 1701–1707
21. Chapman, E. R., Blasi, J., An, S., Brose, N., Johnston, P. A., Sudhof, T. C., and Jahn, R. (1996) *Biochem. Biophys. Res. Commun.* **225,** 326–332
22. Li, C., Ullrich, B., Zhang, J. Z., Anderson, G. W., Brose, N., and Sudhof, T. C. (1995) *Nature* **375,** 594–599
23. Bennett, M. K., and Scheller, R. H. (1994) *Annu. Rev. Biochem.* **63,** 63–100
24. Sutton, R. B. (1995) *Cell* **80,** 929–938
25. Brose, N., Hofmann, K., Hata, Y., and Sudhof, T. C. (1995) *J. Biol. Chem.* **270,** 25273–25280
26. Mouta Carreira, C., LaVallee, T., Tarantini, F., Jackson, A., Tait-Lathrop J., Hampton B., Burgess W. H., and Maciag, T. (1998) *J. Biol. Chem.* **273,** 22224–22231
27. Oyama, Y., Shishibori, T., Yamashita, K., Naya, T., Nakagiri, S., Maeta, H., and Kobayashi, R. (1997) *Biochem. Biophys. Res. Commun.* **240,** 341–347
28. Sugita, S., Hata, Y., and Sudhof, T. C. (1996) *J. Biol. Chem.* **271,** 1262–1265
29. Chapman, E. R., An, S., Edwarson, J. M., and Jahn, R. (1996) *J. Biol. Chem.* **271,** 5844–5849
30. Damer, C. K., and Creutz, C. E. (1996) *J. Neurochem.* **67,** 1661–1668
31. Tugal, H. B., Leeuwen, F. V., Apps, D. K., Haywood, J., and Phillips, J. H. (1991) *Biochem. J.* **279,** 699–703
32. King, R. W., Deshaies, R. J., Peters, J.-M., and Kirschner, M. W. (1997) *Science* **274,** 1652–1659
33. Miller, D. K. (1997) *Semin. Immunol.* **9,** 35–49
34. Goldberg, A. L., Akopian, T. N., Kisselev, A. F., Lee, D. H., and Rohrwild, M. (1997) *Biol. Chem. Hoppe-Seyler* **378,** 131–140
35. Kostura, M. J., Tocci, M. J., Limjuco, G., Chin, J., Cameron, P., Hillman, A. G., Chartrain, N. A., and Schmidt, J. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5227–5231
36. Wilson, K. P., Black, J. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) *Nature* **370,** 270–275
37. Christmas, P., Callaway, J., Fallon, J., Jones, J., and Haigler, H. T. (1991) *J. Biol. Chem.* **266,** 2499–2507
38. Cleves, A. E., Cooper, D. N. W., Barondes, S. H., and Kelly, R. B. (1996) *J. Cell Biol.* **133,** 1017–1026
39. Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R., and Sudhof, T. C. (1990) *Nature* **345,** 260–263
40. Mach, H., and Middaugh, C. R. (1995) *Biochemistry* **34,** 9913–9920
41. Chapman, E. R., and Davis, A. F. (1998) *J. Biol. Chem.* **273,** 13995–14001
42. Davktov, B., Sontag, J. M., Haya, Y., Petrenko, A. G., Fyske, E. M., Jahn, R., and Sudhof, T. C. (1993) *J. Biol. Chem.* **268,** 6816–6822