Regulation of Protein Secretion Through Controlled Aggregation in the Endoplasmic Reticulum

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A system for direct pharmacologic control of protein secretion was developed to allow rapid and pulsatile delivery of therapeutic proteins. A protein was engineered so that it accumulated as aggregates in the endoplasmic reticulum. Secretion was then stimulated by a synthetic small-molecule drug that induces protein disaggregation. Rapid and transient secretion of growth hormone and insulin was achieved in vitro and in vivo. A regulated pulse of insulin secretion resulted in a transient correction of serum glucose concentrations in a mouse model of hyperglycemia. This approach may make gene therapy a viable method for delivery of polypeptides that require rapid and regulated delivery.

Secreted proteins regulate diverse cellular processes in mammals, ranging from rapid metabolic changes to growth and differentiation events. Because many of these activities are associated with disease, recombinant proteins are an important class of therapeutic agent. However, broad use of secreted proteins as drugs is hampered by the need for parenteral administration, usually by direct injection—a regime that often cannot maintain protein concentrations within a therapeutic window or provide appropriate delivery kinetics.

Gene therapy is a promising alternative approach for delivering such proteins. Sustained therapeutic concentrations of several proteins have been obtained after stable introduction of their genes into somatic cells (*1*). However, in most cases, safe and effective therapy will require that genes be equipped with a regulatory system that permits natural concentrations and kinetics of protein expression to be reproduced.

Several systems have been developed for regulating transgene expression pharmacologically at the level of transcription (*2*). Typically a small-molecule drug such as tetracycline or rapamycin controls the activity of an engineered transcription factor that in turn regulates the expression of a therapeutic gene. These systems have been successfully used to control the expression of hormones such as erythropoietin (Epo) after gene transfer into mice and primates (*3*). One common feature of these systems is that induction and decay of protein expression occur on a time scale of days: Concentrations in the blood typically peak after about 24 hours and then decrease to background levels over 4 to 14 days. This reflects the early point of regulation and the many potentially rate-limiting steps between transcription and protein secretion, including decay of the inducing drug, transcription factors, mRNA, and therapeutic protein. Such prolonged kinetics are appropriate for proteins such as Epo, and for many other secreted proteins (such as immune modulators and neurotrophins) that regulate similarly paced growth, differentiation, and survival processes.

Recombinant proteins that regulate much faster processes must instead be produced on a time scale of minutes or hours. For example, insulin needs to be delivered postprandially in a brief pulse that lasts ~4 hours (*4*). Production of endogenous insulin and other proteins of this class is regulated at the level of secretion; proteins are stored in secretory granules in specialized cells until their release is signaled. For such proteins to be delivered effectively by a gene therapy approach, direct control of secretion will likely be required.

We set out to develop a system that allows protein secretion to be controlled directly with a cell-permeant, small-molecule drug. Important features of a suitable system include (i) low basal secretion; (ii) rapid, high-level secretion in response to an orally bioavailable drug; (iii) rapid cessation of secretion upon drug withdrawal; and (iv) exclusive use of human proteins to minimize the potential for immunogenicity in clinical applications. In addition, the system should be indifferent to the protein being secreted and should employ the cellular machinery generally used to secrete proteins constitutively so that it can function in a variety of cell types.

A scheme for regulated protein secretion. Our system uses the endoplasmic reticulum (ER) as a storage depot for proteins (Fig. 1A). A therapeutic protein of interest is reversibly retained in the ER by expressing it as a fusion protein that includes a conditional aggregation domain (CAD)—a domain that interacts with itself in a ligand-reversible manner (Fig. 1A). Secreted fusion proteins containing multiple copies of a CAD should form extensive aggregates by virtue of their multivalency. Furthermore, these aggregates would be expected to be too large for transport within vesicles that depart the ER, resulting in retention of the fusion protein within this organelle. Addition of a cell-permeant CAD ligand should dissolve the aggregates, permitting the fusion protein to exit the ER and progress through the constitutive secretory pathway. To produce biologically active therapeutic protein, the CAD moiety must then be removed. A furin cleavage sequence is therefore interposed between the CAD and the therapeutic protein. Furin is a ubiquitously expressed protease that resides in the trans-Golgi and processes protein precursors before their secretion (*5*). Because furin cleaves at the COOH-terminus of its recognition sequence (*5*), an unaltered, natural version of the therapeutic protein should be released and secreted. Thus, in our system, the ER fulfills the function normally performed by secretory granules in specialized secretory cells, and the physiological stimulus that normally triggers exocytosis of secretory granules is replaced by a ligand that controls access of the secreted protein to the constitutive secretory pathway.

We have found that a single amino acid change (Phe^{36} to Met) converts the monomeric human protein FKBP12 into a CAD (*6*). The mutant protein (hereafter referred to as F_M) forms dimers with micromolar affinity that can be completely dissociated by small, synthetic ligands. Moreover, fusion proteins comprising four copies of F_M joined to green fluorescent protein form large cytoplasmic aggregates in cells that disperse within minutes upon addition of ligand (*6*). Numerous synthetic ligands have been designed that bind tightly to F_M [dissociation constant (K_d) ≈ 1 nM)] but negligibly to endogenous wild-type FKBP12 (Fig. 1B) (*7*).

Secreted F_M fusion proteins are retained in the ER. To determine if F_M and its ligands could control protein secretion, we constructed fusion proteins (Fig. 1C) comprising the signal sequence from human growth hormone, four copies of F_M, a furin cleavage sequence, and the coding sequence for human proinsulin or growth hormone (GH), two proteins whose expression is nat-

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urally regulated at the level of secretion. Because the enzymes that normally process proinsulin to insulin are present only in certain endocrine and neural cells, sequences at the B-C and C-A junctions were altered (*8*) to create additional recognition sequences for furin. Vectors in which these fusion proteins are expressed constitutively were stably transfected into HT1080 cells, a human fibrosarcoma cell line.

Localization of the fusion proteins was determined by fluorescence microscopy. In the absence of ligand, the insulin and GH fusion proteins were retained in the ER and colocalized with resident ER but not Golgi proteins (Fig. 2, A and B). Electron microscopy further revealed that the fusion proteins accumulated in the ER (Fig. 2C). In contrast to parental HT1080 cells, the ER in insulin- and GH-expressing cell lines were dilated and filled with dense flocculent material that was recognized by antibodies to insulin (anti-insulin) or anti-GH. These data indicate that the ER can be coopted as a storage depot for secreted proteins.

Ligand-induced secretion and processing of F_M fusion proteins. Cells expressing the fusion proteins were next treated with vehicle or increasing concentrations of ligand (AP21998) for 2 hours, and secretion of the protein was assessed. Release of insulin and GH was extremely low in the absence of ligand (Fig. 3A). However, addition of ligand induced secretion of both proteins in a concentration-dependent manner. At saturating doses of ligand (1 to 2 μM), the rate of protein secretion was greater than 250 times the basal rate. Thus, accumulated protein was released by ligand, and the rate of release could be controlled by varying the dose over an ~20-fold range.

Correct cleavage of the fusion protein by furin is essential if the released therapeutic protein is to be fully active and nonimmunogenic. The cell lysate and supernatant from GH-producing cells were therefore analyzed by immunoblotting with antibodies to GH and FKBP (Fig. 3B). In the absence of ligand, the 75-kD F_M-GH fusion protein was present only in the cell lysate. Two hours after addition of ligand, the fusion protein had been cleaved appropriately and secreted, as determined by the appearance in the supernatant of a 22-kD protein that was recognized by anti-GH and that comigrated with purified recombinant GH. The remaining 55-kD regulatory portion of the fusion protein (F_M) was also detected in the supernatant with anti-FKBP. Similar analysis of the insulin-producing cell line showed that proinsulin was also released from the fusion protein before secretion and that subsequent processing of proinsulin to insulin occurred with ~50% efficiency (*9*, *10*).

The low basal rate of secretion of insulin and GH results from the high degree of aggregation, and therefore retention, conferred by the incorporation of four F_M domains into the fusion proteins. Incorporation of fewer F_M domains should reduce the extent of aggregation (*6*) and thereby increase the rate of secretion in the absence of ligand. Comparison of basal and ligand-induced rates of secretion from cell lines expressing insulin (Fig. 4) or GH (*9*) fused to four or fewer F_M domains revealed that as the number of F_M domains decreased, the rate of basal secretion increased. Therefore, to maximize the difference between basal and induced levels, fusion proteins with four copies of the F_M domain were further analyzed.

Rapid and transient kinetics of ligand-induced secretion. To assess the kinetics of protein release, cells were treated with a saturating dose of ligand, and samples of medium were collected periodically and assayed for the presence of secreted insulin and GH (Fig. 5A). Little or no protein was secreted in the first 20 min, consistent with the time required for proteins to progress from the ER to the Golgi-secretory vesicle compartment (*11*). The highest rate of secretion was observed over the next 30 min, presumably reflecting bulk release of the stored protein. One hour after exposure to ligand, the rate of secretion declined, and after 2 to 3 hours, when all of the stored protein had apparently been released, the rate of secretion of newly synthesized protein was 10 to 20% of peak levels. This suggests that in the absence of ligand, cells store an amount of protein equivalent to that synthesized over a 5- to 10-hour period. Consistent with this, after the stores are fully depleted, cells must be incubated in the absence of ligand for at least 10 hours before secretion of an equivalent amount of protein can be reinduced (*9*).

To determine the kinetics with which protein secretion shuts down after withdrawal of ligand, insulin-producing cells were incubated with a subsaturating (750 nM) dose of ligand for 1 hour and then in the absence of ligand for four subsequent 1-hour intervals. Between each

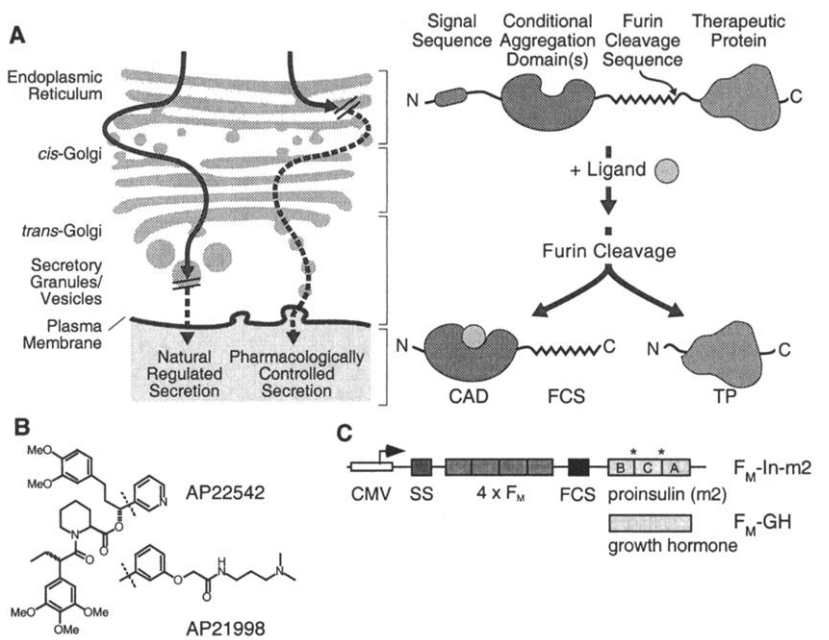

Fig. 1. Scheme for the pharmacologic control of protein secretion. (**A**) (Left) Natural control of protein secretion (protein is stored in the secretory granules) is contrasted with the scheme for pharmacological control (protein is stored in the ER). (Right) The therapeutic protein of interest (TP) is expressed as part of a fusion protein that contains, at its NH_2-terminus, a signal sequence, a conditional aggregation domain (CAD), and a furin cleavage sequence (FCS). Processing and secretion of the TP is induced by ligand (see text). (**B**) Chemical structures of ligands used (*6*). (**C**) Schematic diagrams of plasmids used. Chimeric proteins were generated from the following domains and expressed under control of the human cytomegalovirus (CMV) immediate early enhancer-promoter from a pCGNN-derived expression vector (*19*): SS [signal sequence; amino acids −26 to −1 of human growth hormone (*20*)]; 4 × F_M [four tandem repeats of FKBP12 in which the Phe^{36} was mutated to Met (*6*)]; FCS [furin cleavage signal (Ser-Ala-Arg-Asn-Arg-Gln-Lys-Arg) present in human stromelysin-3]; proinsulin-m2 [entire human proinsulin sequence (*21*)] containing mutations (*) at the B-C and C-A peptide junctions that create recognition sequences for the protease furin (*8*); human growth hormone (entire mature coding sequence). Not shown are two related vectors used in these studies. In F_M-In-m3, a third mutation is introduced into the proinsulin gene [His at amino acid B10 is mutated to Asp (*8*)]. In EGFP-F_M-GH, the entire coding sequence for enhanced green fluorescent protein [EGFP (*22*)] was inserted immediately downstream of the signal sequence in F_M-GH. Vectors were stably transfected into HT1080 cells (ATCC CCL-121) with Superfect (Qiagen) to generate the following cell lines: F_M-In-m2 (HT101-4B); F_M-In-m3 (HT101-10P); F_M-GH (HT90-4D); and EGFP-F_M-GH (HT92H).

interval, medium was replaced and the amount of insulin released into the medium was determined. Secretion was largely terminated within 1 hour of ligand withdrawal (Fig. 5B). Because secretion shut off rapidly and administration of a subsaturating dose of ligand ensured that the internal insulin store was not depleted, it was possible to induce secretion multiple times in a 12-hour period (Fig. 5B). In this experiment, cells were exposed to ligand for 1-hour periods at times selected to assess the ability to induce brief pulses of insulin secretion at meal times. Short-lived pulses of GH secretion could similarly be induced, and for both insulin and GH, the magnitude of induction of individual pulses could be controlled by altering the concentration of ligand (*9*).

Control of therapeutic protein secretion in vivo. Regulated secretion in experimental animals was tested by implanting GH-expressing cells intramuscularly into mice. Intravenous administration of the F_M ligand AP22542 (*12*) induced rapid and dose-dependent secretion of GH from these cells, as determined by the appearance of GH in the serum 2 hours later (Fig. 6).

To induce secretion of biologically active insulin in vivo, engineered cells (*13*) were implanted into mice that were made diabetic by treatment with streptozotocin (STZ). STZ-treated mice have low numbers of insulin-producing beta cells, and serum glucose concentrations are elevated to ~350 mg/dl over normal concentrations of 100 mg/dl. Administration of vehicle or a low dose of ligand failed to induce insulin secretion or normalize serum glucose concen-

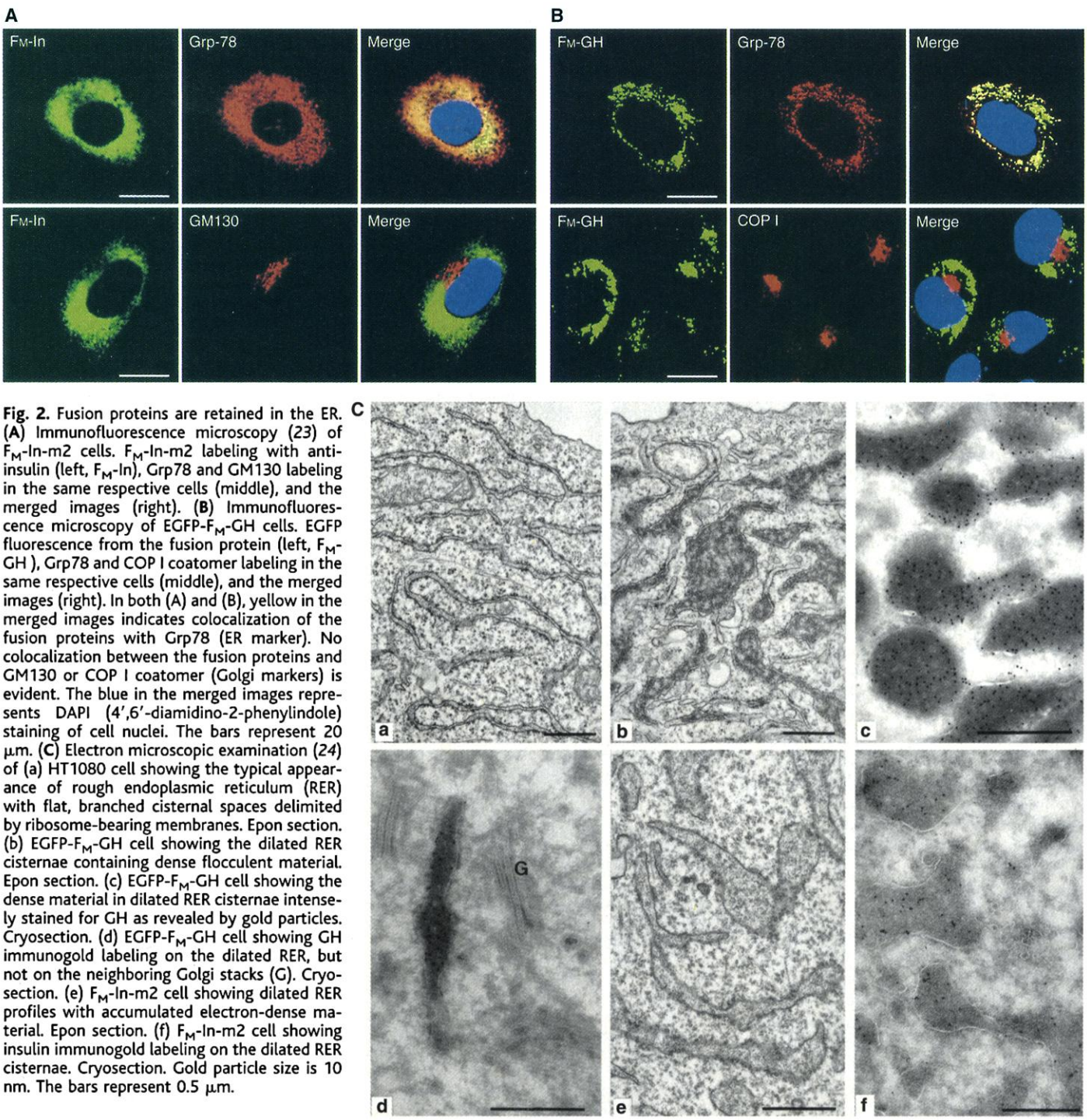

Fig. 2. Fusion proteins are retained in the ER. (**A**) Immunofluorescence microscopy (*23*) of F_M-In-m2 cells. F_M-In-m2 labeling with anti-insulin (left, F_M-In), Grp78 and GM130 labeling in the same respective cells (middle), and the merged images (right). (**B**) Immunofluorescence microscopy of EGFP-F_M-GH cells. EGFP fluorescence from the fusion protein (left, F_M-GH), Grp78 and COP I coatomer labeling in the same respective cells (middle), and the merged images (right). In both (A) and (B), yellow in the merged images indicates colocalization of the fusion proteins with Grp78 (ER marker). No colocalization between the fusion proteins and GM130 or COP I coatomer (Golgi markers) is evident. The blue in the merged images represents DAPI (4′,6′-diamidino-2-phenylindole) staining of cell nuclei. The bars represent 20 μm. (**C**) Electron microscopic examination (*24*) of (a) HT1080 cell showing the typical appearance of rough endoplasmic reticulum (RER) with flat, branched cisternal spaces delimited by ribosome-bearing membranes. Epon section. (b) EGFP-F_M-GH cell showing the dilated RER cisternae containing dense flocculent material. Epon section. (c) EGFP-F_M-GH cell showing the dense material in dilated RER cisternae intensely stained for GH as revealed by gold particles. Cryosection. (d) EGFP-F_M-GH cell showing GH immunogold labeling on the dilated RER, but not on the neighboring Golgi stacks (G). Cryosection. (e) F_M-In-m2 cell showing dilated RER profiles with accumulated electron-dense material. Epon section. (f) F_M-In-m2 cell showing insulin immunogold labeling on the dilated RER cisternae. Cryosection. Gold particle size is 10 nm. The bars represent 0.5 μm.

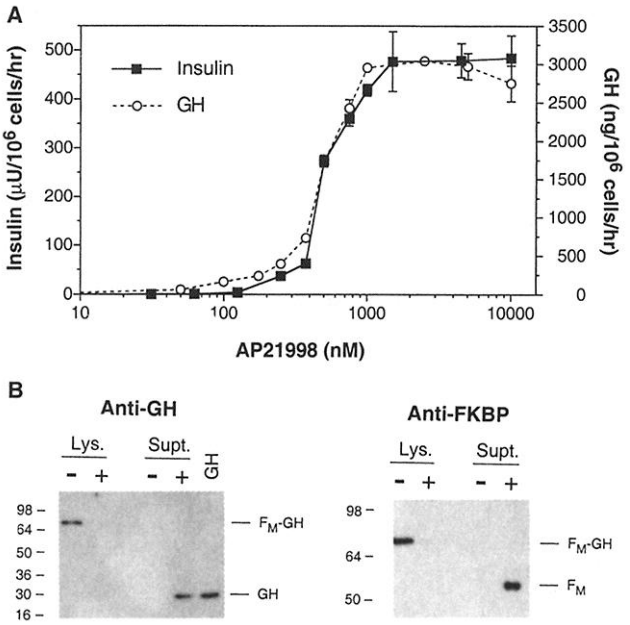

Fig. 3. Ligand-induced secretion and processing of insulin and GH. (**A**) Concentration-dependent secretion of insulin and GH. F_M-In-m2 and F_M-GH cells were incubated in the absence or presence of increasing concentrations of AP21998 for 2 hours. Insulin [human insulin–specific radioimmunoassay (RIA, Linco)] and GH (Nichols Diagnostic) secreted into the medium was measured. The insulin RIA measures mature insulin and does not cross-react with proinsulin. Mean values ± SD are plotted. In the absence of ligand, basal rates of secretion were 0.82 μU of insulin per 10^6 cells per hour and 11.6 ng of GH per 10^6 cells per hour. (**B**) Secretion of mature GH. Immunoblot analysis of the lysate and supernatant of F_M-GH cells treated with vehicle (−) or 2 μM AP21998 (+) for 2 hours. One percent of the cell lysate and supernatant, and 10 ng of recombinant GH (Sigma) were probed with anti-GH (1:1000, Dako) and anti-FKBP12 (1:1000, Affinity Bioreagents) as indicated.

trations (Fig. 7A). However, 2 hours after administration of a 10 mg/kg dose of ligand, circulating insulin concentrations increased to greater than 200 pM, and serum glucose decreased concomitantly to normal concentrations. Similar results were obtained upon oral administration of higher doses of AP22542 (*14*).

To examine the kinetics of secretion, serum insulin concentrations were measured at various times between 5 min and 10 hours after intravenous administration of ligand (Fig. 7B). Insulin was detected in serum within 15 min and peaked by 2 hours at a circulating concentration of 800 pM. Coincident with the induction of insulin secretion, serum glucose declined rapidly from preinduction concentrations of >450 mg/dl and were reduced by 40% within 30 min and by 90% within 2 hours. Two hours after the administration of ligand, circulating insulin decreased and serum glucose increased toward hyperglycemic concentrations. These kinetics are similar to those observed for natural insulin secretion.

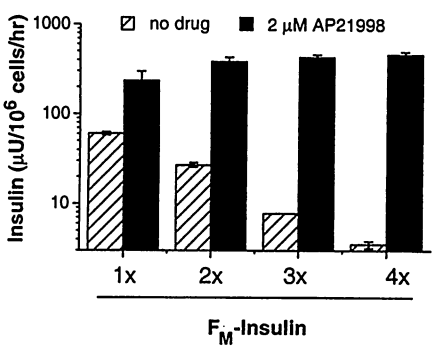

Fig. 4. Control of basal secretion by manipulation of the number of F_M domains. Pools of HT1080 cells stably transfected with vectors expressing F_M-In-m2 fusion proteins containing one, two, three, or four copies of F_M were incubated in the presence or absence of 2 μM AP21998 for 2 hours. Insulin secreted into the medium was measured (Iso-insulin EIA, ALPCO). Mean values ± SD are plotted.

A general platform for the delivery of secreted proteins. We have used controlled aggregation in the ER to develop a system that allows protein secretion to be regulated with a synthetic, orally bioavailable drug. Direct control of secretion provides faster kinetics of regulation than transcriptional control and therefore represents a complementary technology for controlled delivery of proteins. The system appears to be generally applicable in that it does not require specialized secretory cells and is apparently indifferent to the protein being secreted. We have demonstrated that the system functions in two different cell lines (HT1080 and Rat-1 fibroblast cells) and can regulate secretion of three proteins (insulin, GH, and alkaline phosphatase) (*9*). The system should be useful for delivery of many other polypeptides that are naturally secreted in short bursts, such as those associated with pain and weight control. We have already used the approach to regulate the expression of membrane proteins (*9*), and the creation of rapidly inducible alleles in animal models (*15*) should be feasible. The ability to conditionally generate ER-retained aggregates also provides a tool for analyzing basic mechanisms of vesicle trafficking among compartments in the secretory pathway.

The induction properties of the system are influenced by several factors, each of which could be independently tailored for optimal delivery of a given therapeutic protein. Peak levels of protein secretion are dictated by the dose of ligand (Fig. 3A) (*6*). Secretion kinetics should be determined by the pharmacokinetic properties of the ligand, which could be optimized (*6*, *16*). The rate of basal secretion ("leakiness") of the system can also be

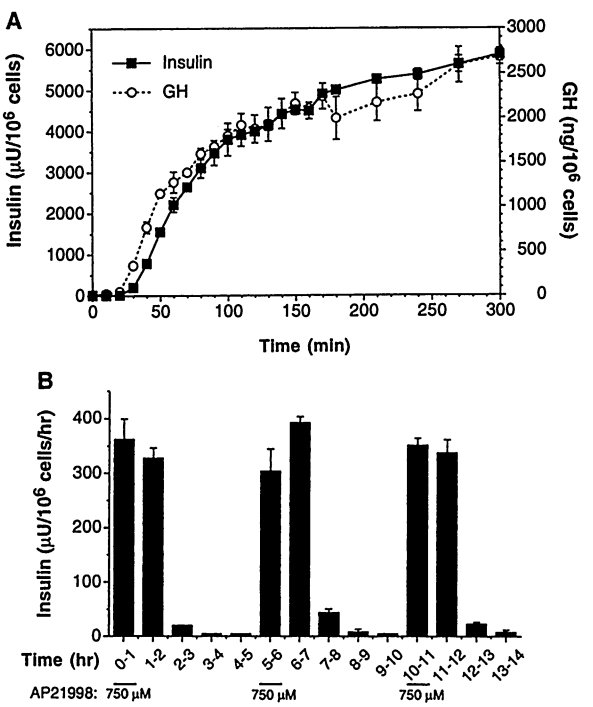

Fig. 5. Rapid kinetics of ligand-induced secretion permit repetitive delivery at short intervals. (**A**) F_M-In-m2 and F_M-GH cells were incubated in the presence of 2 μM AP21998 and samples of medium collected every 10 to 30 min. Accumulated insulin (Iso-insulin EIA) or GH secreted into the medium was measured. Mean values ± SD are plotted. (**B**) F_M-In-m2 cells were incubated in the presence or absence of 750 nM AP21998, as indicated, for 1-hour periods. Between each 1-hour period, medium was saved and the cells were washed extensively to remove any AP21998 or secreted insulin. Samples from each of the 14 intervals were then assayed for the presence of insulin. Mean values ± SD are plotted.

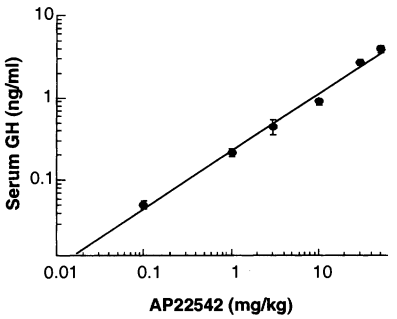

Fig. 6. Dose-dependent secretion of GH in vivo. F_M-GH cells (2×10^6 per animal) were injected intramuscularly into male *nu/nu* mice. One hour later, animals received the indicated dose of intravenous AP22542. Two hours later, serum samples were collected and assayed for GH concentration (Boehringer Mannheim). GH concentrations in mice treated with vehicle were undetectable (<0.0125 ng/ml).

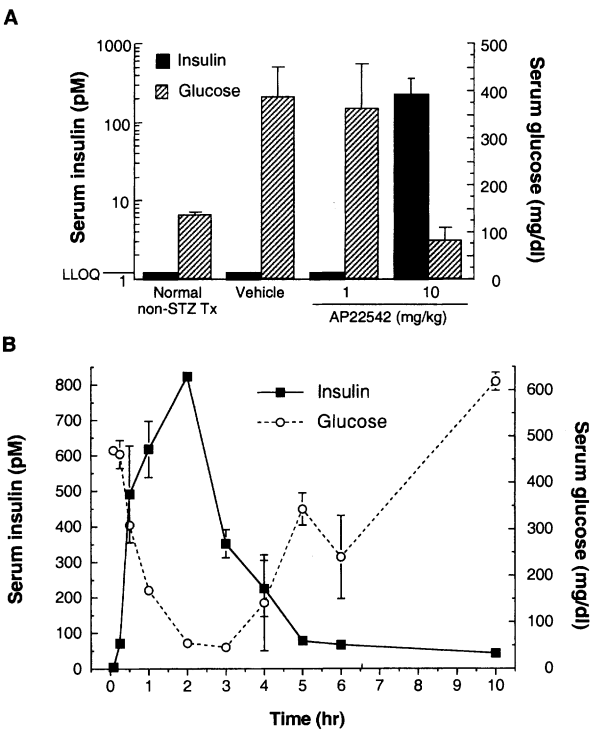

Fig. 7. Rapid and transient regulation of insulin and glucose concentrations in hyperglycemic mice. F_M-In-m3 cells (2×10^7) were implanted intramuscularly into male *nu/nu* mice made hyperglycemic by treatment 2 days earlier with streptozotocin (STZ, 300 mg/kg). About 1 hour after cells were implanted, animals received vehicle or the indicated dose of intravenous AP22542. (**A**) Two hours later, serum samples were collected and assayed for glucose (Sigma) and insulin (Ultrasensitive human insulin-specific RIA, Linco). In STZ-treated mice, predose glucose concentrations were ~350 mg/dl, and predose insulin concentrations were below the lower limit of quantitation of the assay. Also shown are glucose and insulin concentrations in control, non-STZ–treated mice that did not receive implanted cells. (**B**) At the indicated time after administration of AP22542 (30 mg/kg), serum samples were collected and assayed for glucose and insulin. In animals treated with vehicle, glucose concentrations between 400 to 550 mg/dl and insulin concentrations below 7 pM were maintained throughout the duration of the experiments. Mean values ± SEM are plotted.

"tuned" by altering the number of F_M modules incorporated into the fusion protein (Fig. 4); for example, to provide low-level constitutive production of insulin between meals.

Clinical exploitation of regulated secretion in gene therapy applications will require demonstration that the system can function when delivered by established vector systems, such as adeno-associated virus (AAV) or adenovirus vectors, to candidate target tissues such as muscle and liver. In some respects, this may be easier to achieve than for transcriptional control systems, because both therapeutic and regulatory elements are carried on a single gene, and can be accommodated in a single AAV vector. During these studies it will be important to determine whether protein retention has any long-term deleterious influence on the transduced tissue. To date, we have seen no effect in cultured cells (*17*); moreover, natural examples of ER-stored proteins are known (*18*).

The system may be particularly suitable for regulation of insulin secretion in vivo. A single dose of ligand was able to mimic the natural postprandial kinetics of insulin secretion and could transiently correct hyperglycemia in a mouse model (Fig. 7). Repeated injections of recombinant insulin as a therapy for insulin-dependent diabetes might therefore be replaced by a one-time or infrequent delivery of viral vectors containing the regulatory system, followed by the sublingual or oral administration of ligand at meal times.

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