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Constitutive secretion of serum albumin requires reversible protein tyrosine phosphorylation events in *trans*-Golgi

Rachel J. Webb,¹ Jacob D. Judah,¹ Lee-Chiang Lo,² and Geraint M. H. Thomas¹

¹Department of Physiology, University College London, London, United Kingdom;
and ²Department of Chemistry, National Taiwan University, Taipei, Taiwan

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Webb, Rachel J., Jacob D. Judah, Lee-Chiang Lo, and Geraint M. H. Thomas. Constitutive secretion of serum albumin requires reversible protein tyrosine phosphorylation events in *trans*-Golgi. *Am J Physiol Cell Physiol* 289: C748–C756, 2005. First published April 20, 2005; doi:10.1152/ajpcell.00019.2005.—Serum albumin secretion from rat hepatocytes proceeds via the constitutive pathway. Although much is known about the role of protein tyrosine phosphorylation in regulated secretion, nothing is known about its function in the constitutive process. Here we show that albumin secretion is inhibited by the tyrosine kinase inhibitor genistein but relatively insensitive to subtype-selective inhibitors or treatments. Secretion is also blocked in a physiologically identical manner by the tyrosine phosphatase inhibitors pervanadate and bisperoxo(1,10-phenanthroline)-oxovanadate. Inhibition of either the kinase(s) or phosphatase(s) leads to the accumulation of albumin between the *trans*-Golgi and the plasma membrane, whereas the immediate precursor proalbumin builds up in a proximal compartment. The *trans*-Golgi marker TGN38 is rapidly dispersed under conditions that inhibit tyrosine phosphatase action, whereas the distribution of the *cis*-Golgi marker GM130 is insensitive to genistein or pervanadate. By using a specifically reactive biotinylation probe, we detected protein tyrosine phosphatases in highly purified rat liver Golgi membranes. These membranes also contain both endogenous tyrosine kinases and their substrates, indicating that enzymes and substrates for reversible tyrosine phosphorylation are normal membrane-resident components of this trafficking compartment. In the absence of perturbation of actin filaments and microtubules, we conclude that reversible protein tyrosine phosphorylation in the *trans*-Golgi network is essential for albumin secretion and propose that the constitutive secretion of albumin is in fact a regulated process.

vesicular trafficking; liver; genistein; pervanadate

PROTEIN SECRETION from mammalian cells occurs in either a constitutive or regulated fashion and proceeds through the coordination of a series of complex and highly conserved processes (30, 37, 46). In constitutive secretion, cargo proteins are fed into the pathway by translational events at the endoplasmic reticulum (ER) and they progress through the Golgi and then out of the cell without storage at any stage. On average, the mass of a protein secreted from cells with time is thought to be nearly constant and to depend only on the level of gene transcription. The concentrations of any constitutively secreted protein in the subcompartments of the secretory system are thought to be in near steady state (25, 34). In regulated

secretion, protein is sorted from the Golgi into inert storage granules and released when stimuli trigger their final fusion with the plasma membrane (13).

One of the major physiological functions of the liver is the constitutive secretion of serum albumin, the principal blood plasma protein by one order of magnitude. Blood albumin is replaced at a rate of ~2% per day (40) and so by protein mass this process is the largest internal constitutive secretory process in mammals. The primary amino acid sequences, structure, and function of albumins are highly conserved across species as are the processes of albumin synthesis and secretion (42). In all cases, translation produces the short-lived, primary product prealbumin, which is rapidly co-translationally cleaved in ER lumen, at its NH₂ terminus, to produce the stable precursor proalbumin (17). Proalbumin then passes to the Golgi and is converted to mature albumin by a second NH₂ terminal cleavage (17). This process is easily monitored because proalbumin can be resolved experimentally from albumin. The cleavage occurs in a late Golgi compartment that is indistinguishable from the *trans*-Golgi network (TGN) and depends on Furin (38, 45). Furin and other members of the mammalian subtilisin/Kex2p-like proprotein convertase family are localized largely in the *trans*-Golgi network with small amounts in the plasma membrane and recycling endosomes (28, 35) pinpointing the TGN as the crucial compartment in albumin production from proalbumin. In the absence of efficient proteolysis, e.g., because of mutations in the cleavage signal, proalbumin is not trapped but is itself secreted from the cell (30) indicating that passage through the TGN is not dependent on cleavage but that it is a usual feature.

Some enzymes mediating reversible protein phosphorylation are known to play roles in secretory processes. Protein kinase(s) C (PKCs) were implicated in Golgi vesicle formation (9, 47, 51, 52, 61) and more recently, PKD has been shown to promote the Golgi budding induced by the $\beta\gamma$ -subunits of heterotrimeric G proteins (15, 22, 57). Tyrosine kinases have prominent roles in some forms of regulated secretion acting in signal transduction pathways that can modulate the final exocytotic step (36, 41, 48). Protein tyrosine kinases and phosphatases were not thought to influence the mechanisms of storage/secretory granule formation preceding exocytosis but studies with inhibitors (2) revealed the action of these enzymes in trafficking between the Golgi and the plasma membrane during

Address for reprint requests and other correspondence: G. M. H. Thomas, Dept. of Physiology, Univ. College London, Rockefeller Bldg., 21 Univ. St., London WC1E 6JJ, UK (e-mail: g.thomas@ucl.ac.uk).

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regulated secretion. More recently, protein tyrosine phosphatase-MEG2 has been shown to be the protein tyrosine phosphatase associated with secretory storage granules, which allows maturation and fusion by regulating *N*-ethylmaleimide-sensitive fusion protein tyrosine phosphorylation (59, 63). In addition, studies (1, 18) of stimulated melanosome aggregation in cells from lower vertebrates indicate that protein tyrosine phosphorylation has been shown to be essential for nonsecretory vesicular trafficking.

Here we demonstrate that reversible protein tyrosine phosphorylation is an essential feature of the constitutive albumin secretory pathway in rat hepatocytes, the major natural protein transport system in these cells.

MATERIALS AND METHODS

Materials. Rabbit anti-TGN38 antiserum was the generous gift of Prof. George Banting (University of Bristol). Mouse anti-GM130 was from BD Biosciences. Both mouse anti-rabbit IgG Alexa 488 and goat anti-mouse IgG Alexa 568 were from Molecular Probes Europe (Leiden, The Netherlands). Genistein, staurosporine, PP2, and BpV-(phen) were from Calbiochem, and wortmannin, SU-6656, rapamycin, and PP1 were from Sigma. Fresh pervanadate (peroxovanadate) was prepared by treating a 10 mM stock of neutralized sodium vanadate with equimolar hydrogen peroxide. Occasionally, 0.1 mg/ml catalase (Sigma-Aldrich, Poole, UK) was added to remove any excess peroxide and the enzyme was in turn denatured by heating. Mouse monoclonal anti-phosphotyrosine antibody P-Tyr-100 (Cell Signaling Technologies) was from NEB (Hitchin, UK). Both goat-anti-rabbit IgG-horseradish peroxidase (HRP) and goat-anti-mouse IgG-HRP were from Bio-Rad Laboratories (Hemel Hempstead, UK). Streptavidin-HRP conjugate and [³⁵S]Promix ([³⁵S]-labeled cysteine and methionine) were from Amersham Pharmacia Biotech. Super Signal West chemiluminescent substrate was from Perbio Science (Tattenhall, UK). Disodium ATP from Sigma-Aldrich was converted to the magnesium salt by sequential neutralization with Tris base (2 Mol per Mol ATP) and treatment with equimolar MgCl₂.

Hepatocyte primary cell culture. Cells were prepared from the livers of male rats (200–300 g body wt) by perfusion of the liver with Ringer's solution containing EDTA (2 mM). The cells were washed twice with HEPES-buffered Ringer's solution (HBR) containing 1 mM MgCl₂, 1 mM CaCl₂, 4 mg/ml bovine serum albumin, and 25 mM HEPES (pH 7.4), and then purified on a discontinuous Percoll gradient. The viable cells were suspended in Williams E medium [supplemented with 5% (vol/vol) FCS, 0.04 mg/ml gentamicin, and 2 mM glutathione] at ~5 × 10⁵ cells/ml. Insulin (1 mg/ml) and hydrocortisone (0.1 mM) were added to the suspension, of which 2 ml was placed in 3.5-cm-diameter collagen-coated culture dishes or 50 μl on similarly treated glass coverslips. The cultures were incubated overnight and replenished with fresh medium each day if maintenance is required or washed into HBR three times and then left in a final volume of 1 ml for experimentation. For studies of albumin secretion, 3.7 MBq (100 μCi) of [³⁵S]-labeled methionine was added to the buffer ("pulse"). The cells were incubated for 10 min, after which they were washed with ice-cold saline containing 15 mM unlabeled methionine and cysteine ("chase") and the cells transferred to similar medium containing 10 μg of rat serum albumin, as a carrier, along with any inhibitors. After 30 min of incubation at 37°C, the medium was removed and kept for the analysis of secreted albumin (proalbumin is not released under these conditions). The secretion of albumin over this period is strictly linear with time. The cells were then washed once with fresh buffer and then lysed with the same solution containing 0.5% (wt/vol) Triton X-100. The sample was centrifuged briefly, and the supernatant containing the cellular albumin and proalbumin was kept for analysis.

Analysis of albumin and proalbumin secretion. The albumin and proalbumin in each sample was precipitated by the addition of 110 μl of sheep anti-rat serum albumin, centrifuged, washed twice with 150 mM NaCl, and then taken up in dilute TCA. Redundant antibody was removed by the addition of ethanol. Albumin and proalbumin were reprecipitated from the acid with diethylether, air dried, redissolved in 20 μl of water, and analyzed by one-dimensional isoelectric focusing [5% polyacrylamide gels containing 6 M urea, 5% (vol/vol) glycerol, and ampholines]. The gels were then air dried and phosphorimaged for quantification.

Immunocytochemistry. Cells on optical glass coverslips were treated with indicated compounds in HBR for 10 min at 37°C. After being washed with phosphate-buffered saline (PBS), the cells were fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.1% (wt/vol) TX-100, and then blocked with 5% (vol/vol) goat serum. The coverslips were then individually soaked in rabbit anti-rat TGN38 serum (1/200 dilution), or mouse anti-GM130. After being washed to remove the primary antibodies, anti-rabbit IgG-Alexa 488 or anti-mouse IgG-Alexa 568 were added as appropriate and incubation continued for up to 1 h. After being washed, the cells were sealed in Mowiol on glass microscope slides. Routine confocal microscopy was carried out on a Bio-Rad μRadiance imaging system fitted with a Zeiss Axiovert 100TV inverted microscope. Alexa 488 was excited with the 488-nm line of an Argon laser and the emission collected with a 520-nm (40 nm bandwidth) band-pass filter. Alexa 546 was excited at 514 nm (shoulder on the excitation spectra) and collected with a 520-nm (40-nm bandwidth) band-pass filter. Identical results could be obtained with excitation at 568 nm on a Zeiss LSM510 microscope with a ×40 (1.3 numerical aperture) oil-immersion lens. For data analysis, images of either small groups of hepatocytes (~10–20 members) or individual cells could be scrutinized using MetaMorph software with qualitatively identical results. Changes in the organized, patterned distributions of Golgi staining were quantified by normalizing the standard deviation of pixel intensity to average pixel intensity. The loss of a discrete staining pattern is revealed as a decrease in this index.

Preparation of rat liver Golgi membranes. In a variation of the surgical and perfusion procedures for the isolation of hepatocytes, the livers were cleared of blood and then removed and placed in ice-cold 0.5 M sucrose. The tissue was then processed up to the gradient centrifugation step described by Taylor et al. (54). All procedures were conducted on ice and all centrifuge rotors were of the swinging-bucket type unless otherwise stated. Briefly, the tissue was chopped and then minced with a stainless steel press before two successive steps of disruption in a Dounce homogenizer. After low-speed centrifugation (1,000 g_{max}) to remove large debris and nuclei, the microscopic particulate fraction was recovered from the supernatant by further centrifugation at higher speed (5,000 g_{max}). After resuspension of this pellet in 250 mM sucrose buffer, the particulate fraction was floated on a discontinuous density gradient consisting of 2, 1.3, 1.1, and 0.85 M sucrose and then ultracentrifuged (200,000 g_{av}, 2 h). The Golgi-enriched fraction (which was clearly resolved from the ER-rich particles in the 1.3 M fraction) was recovered from the 0.85:1.1 M sucrose interface. This preparation could be snap frozen in liquid nitrogen and stored in aliquots at –80°C if required.

Probing Golgi membranes for tyrosine phosphatases, ATP-binding proteins, and phosphotyrosyl-containing proteins. For these experiments, snap-frozen Golgi preparations were thawed and the sucrose was diluted with an equal volume of water to allow the membranes to be sedimented by centrifugation (100,000 g_{av}, 30 min, fixed angle rotor). Membranes were then resuspended in an assay buffer composed of (in mM) 20 HEPES, pH 7.5, 1 MnCl₂, 1 MgCl₂, and 1 DTT. We have already established that freeze-thaw, followed by centrifugation in fixed-angle rotors, removes the soluble contents from the lumen of rat liver Golgi (45) providing only the membranes for analysis. The Golgi membrane preparations in assay buffer were then treated with various agents. In one set of investigations membranes

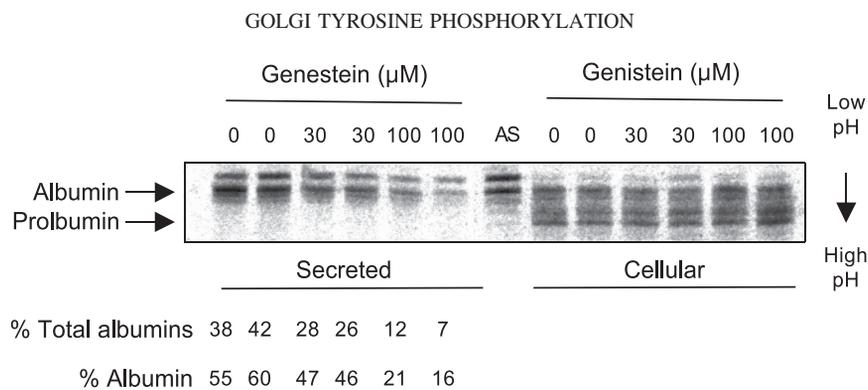


Fig. 1. The inhibition of protein serum albumin secretion from primary rat hepatocytes by genistein. Cell cultures were established and duplicate samples treated with genistein at the concentrations indicated. After 30 min of incubation, both albumin and proalbumin were collected by immunoprecipitation from the culture medium and corresponding cell lysates. Samples were displayed by one-dimensional isoelectric focusing and migration compared with a radiolabeled authentic albumin standard (AS). Data below the figure indicate the impact of genistein on albumin secretion obtained by densitometric analysis of the phosphorimaged gels. The fraction of the total radiolabeled albumin-like protein synthesized by the hepatocytes (proalbumin + albumin) that was secreted from the cells is described as %total albumin. The fraction of the mature albumin alone that was ultimately secreted is shown as %albumin.

were treated with various concentrations of MgATP in the presence or absence of 100 μM pervanadate and a second set of membranes were treated with 1 mM LCL2 (diluted from a 50 mM stock in water). In both cases, after 1 h at 37°C, the membranes were recovered by brief ultracentrifugation (100,000 g_{av} , 10 min), taken up in SDS-PAGE sample buffer, and heated (80°C for 5 min). Golgi membrane proteins were displayed on 10% SDS-PAGE gels and transferred to nitrocellulose. After a blocking step with bovine serum albumin or milk protein, as appropriate, the membranes were probed with either mouse monoclonal anti-phosphotyrosine IgG or streptavidin-HRP as needed. In membranes probed with anti-phosphotyrosine, a secondary goat anti-mouse HRP conjugate antibody was added after being washed. In each experiment, reactive bands were revealed by a chemiluminescent reaction. The apparent molecular weight of any reactive bands were estimated conventionally: we modeled the migration distance in relation to \log_{10} molecular weight using either first-, or occasionally, second-order polynomial functions and regression interpolation.

RESULTS

Effects of inhibitors on albumin secretion. A range of inhibitors was applied to primary rat hepatocytes and the constitutive secretion of pulse-chase-labeled albumin monitored along side untreated paired controls. Table 1 shows the impact of these compounds on the total secretion of albumin, which is in the range of 45 to 65% over 30 min in the controls. The

inhibitor concentrations used are in line with those for physiological or pharmacological investigations reflecting variables like cell permeability, cellular catabolism (especially in hepatocytes) and high intracellular ATP concentrations (competition). The established inhibitors of protein secretion, brefeldin A (BFA), and monensin, were tested as positive controls and to provide authentic blockades for comparison with the effects of other inhibitors. Genistein, a broad-spectrum inhibitor of protein tyrosine kinases, caused a strong inhibition of albumin secretion across the concentration range 10 to 100 μM (Fig. 1, Table 1, and Fig. 2). Approximately 60% of constitutive albumin secretion could be inhibited with 50 μM genistein (Table 1 and Fig. 2A). Indeed, genistein was the most potent agent among all of the protein kinase inhibitors tested (see below). Following the study by Austin and Shields (2), we tested the effects of pervanadate (peroxovanadate), an inhibitor of protein tyrosine phosphatases, and also found this compound to be an inhibitor of albumin secretion. More than half of albumin secretion could be eliminated with 50 μM pervanadate (Table 1 and Fig. 2B). Hepatocytes contain large amounts of catalase, therefore, it is unlikely that the effects of pervanadate are due to free hydrogen peroxide; however, to guard against artefacts, we used two strategies. First, pervanadate

Table 1. The effects of inhibitors on secretion of serum albumin

Agent	<i>n</i>	Concentration	Control	Treated	<i>P</i> Value
Genistein	8	100 μM	49.2 \pm 8.5	21.7 \pm 10.3	4.2 $\times 10^{-5}$
Pervanadate	6	1 mM	45.7 \pm 6.3	7.9 \pm 4.0	1.5 $\times 10^{-6}$
BpV(phen)	2	100 μM	56	29–27	
Rapamycin	4	5 μM	58.4 \pm 4.5	59.3 \pm 6.1	0.83
PP2	4	5 μM	56.7 \pm 1.9	54.7 \pm 7.3	0.75
SU-6656	5	50 μM	55.0 \pm 2.1	44.0 \pm 2.5	0.03
No serum	4	N/A	45.3 \pm 2.8	45.8 \pm 4.7	0.86
Staurosporine	4	0.1 μM	55.4 \pm 2.2	46.8 \pm 6.0	0.03
Wortmannin	6	0.1 μM	54.7 \pm 5.8	60.7 \pm 5.3	0.09
BFA	4	10 $\mu\text{g}/\text{ml}$	65.2 \pm 22.8	4.1 \pm 4.5	0.002
Monensin	6	3 μM	51.1 \pm 1.1	5.9 \pm 6.1	6.3 $\times 10^{-9}$
Latrunculin B	6	25 $\mu\text{g}/\text{ml}$	56.8 \pm 3.0	48.2 \pm 8.9	0.059

Values are means \pm SE for control incubations and with drug treatment are shown for the percent secretion of albumin hepatocytes; *n*, number of replicates of each experiment. BpV(phen), bisperoxo(1,10-phenanthroline)-oxovanadate; NA, not applicable; BFA, brefeldin A. Cell cultures were established and the secretion of albumin estimated, as described in MATERIALS AND METHODS. Drugs, at the various concentrations indicated, were added to the hepatocytes at the start of a 30-min albumin secretion assays without preincubation. The statistical significance of differences between paired control and experimental incubations are shown as *P* values from paired *t*-tests.

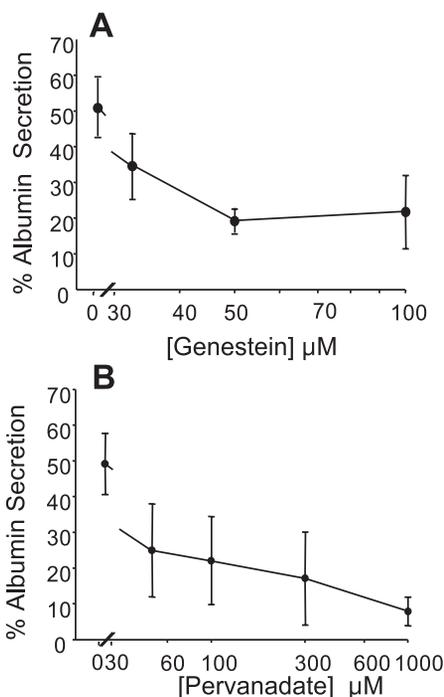


Fig. 2. The concentration-dependent inhibition of protein serum albumin secretion from primary rat hepatocytes by genistein and pervanadate. *A*: effects of increasing genistein concentrations. *B*: effects of increasing pervanadate concentrations. Cell cultures were established and the secretion of albumin estimated exactly, as described in MATERIALS AND METHODS. Without treatment, albumin secretion is normally $\sim 50\%$ within 30 min. The data shown are the mean of 3 individual experiments within which determinations were made in duplicate. Bars indicate means \pm SE.

preparations were pretreated with catalase to consume residual hydrogen peroxide, and second, we examined the effects of bisperoxo(1,10-phenanthroline)-oxovanadate [BpV(phen)], another inhibitor of protein tyrosine phosphatases. In both cases, albumin secretion was inhibited.

To examine the possibility that members of the Src family of nonreceptor tyrosine kinases are the site of genistein's action, hepatocytes were treated with either PP2 or SU-6656 (4). PP2 used at $5 \mu\text{M}$ was without effect, but $50 \mu\text{M}$ SU-6656 decreased albumin secretion by up to 20%.

We also studied the effects of serum starvation to determine if surface receptor kinases or phosphatases or any of the many coupled downstream kinases might be loci for genistein's action. We found that serum can be withdrawn for up to 20 h (the longest time tested) without any measurable effect on albumin secretion. The subsequent readdition of serum did not produce any rebound effect. Many serum factors stimulate the synthesis of 3-phosphorylated polyphosphoinositides, but there was no effect of wortmannin on albumin secretion even at high, nonspecific, concentrations ($1\text{--}10 \mu\text{M}$). Similar tests with LY-294002, a complimentary phosphatidylinositol 3-kinase inhibitor (4), also failed to disrupt albumin secretion from hepatocytes (data not shown).

Once we obtained inhibition of secretion with broad-spectrum inhibitors of tyrosine kinases and phosphatases, we contrasted the effects of genistein and pervanadate with broad-spectrum inhibitors of other classes of protein kinases. Staurosporine caused a relatively small inhibition of albumin secretion from hepatocytes ($\sim 20\%$ of total). Staurosporine is

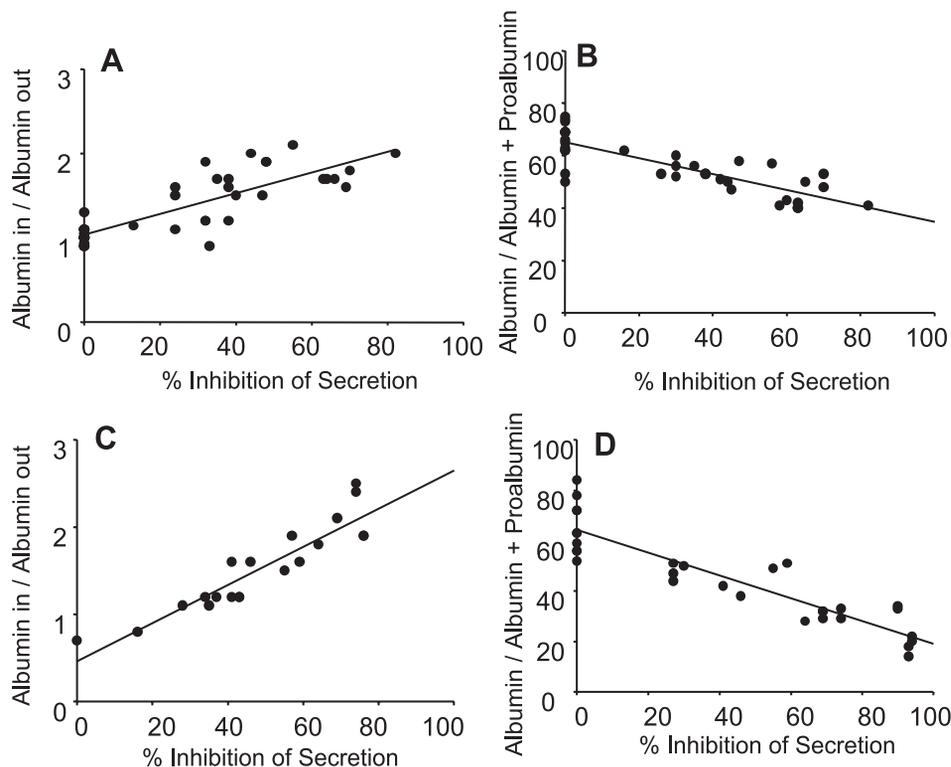
known to inhibit various serine/threonine kinases, including isoforms of protein kinases C (PKCs) (62), cAMP-dependent protein kinase (PKA) (44), calmodulin kinase II (65), and myosin light chain kinase (32).

Finally to exclude the possibility that we have characterized a novel, upregulated de novo radioprotein synthesis rather than the normal constitutive albumin production, we examined the effects of rapamycin and found no significant inhibition.

Both genistein and pervanadate inhibit constitutive secretory pathway at the level of trans-Golgi. Because both genistein and pervanadate have a similar effect on the secretion of albumin, we can hypothesize that a cycle of protein phosphorylation and dephosphorylation might be important in the function of the constitutive secretory pathway. By analyzing the relationship between the observed inhibition of albumin secretion (%inhibition) and the distribution of albumin and proalbumin between the inside and the outside of the cells, we were able to establish important features of the inhibitory processes using two complimentary indexes. The first index reports the ratio of mature, cleaved albumin found inside and outside the hepatocytes (albumin in/albumin out, Fig. 3, A and C). The second index is the ratio of mature, cleaved albumin inside the cells to the total albumin-like protein inside the cells [the albumin-like protein is taken as the sum of albumin and proalbumin (Fig. 3, B and D)]. We also confirmed that albumin trapped inside the hepatocytes is not degraded by intracellular proteases. With the use of either index it is immediately apparent that both genistein and pervanadate cause similar changes in the distribution of the proteins between the inside and outside of the cells (compare Fig. 3A with 3C and 3B with 3D). Genistein and pervanadate increase the mass of unsecreted albumin with increasing inhibition of secretion indicating a blockade after the cleavage of proalbumin to albumin, a TGN-mediated process. Proalbumin also accumulates inside the cells showing that there is no alternative pathway that bypasses the blocked TGN. This indicates that once the export of albumin is blocked a compartment proximal to the TGN accommodates the stalled precursor pool.

Pervanadate causes redistribution of trans-Golgi marker proteins. Because the analysis of secretion indicated a site of action for genistein and pervanadate at the level of the trans-Golgi, we investigated the effects of these compounds on the morphology of the Golgi. TGN-38 was usually found in relatively large, discrete "bodies" but this distributed to a much more highly dispersed punctate pattern in the presence of pervanadate (Fig. 4). For comparison, BFA had no significant effect on this marker and digital analysis of the change in the staining patterns with pervanadate and BFA showed a statistically significantly change in both cases ($P < 0.05$, not shown). Genistein did not affect TGN38 in six separate experiments (Fig. 4). We find GM130, a component of the Golgi scaffold always enriched in the cis-Golgi, to be distributed as discrete ribbon-like structures, reminiscent of the forms described by others (33, 55). Characteristically, treatment with BFA rapidly dissolved this pattern (Fig. 3) but no similarly gross rearrangement could be seen with either genistein or pervanadate. We evaluated the possibility that gross disruptions of either actin filaments or microtubule networks explain the effects of genistein or tyrosine phosphatase inhibitors on albumin secretion from rat hepatocytes. We find that nocodazole abolishes the extensive cytoplasmic microtubule network within our

Fig. 3. The effects of inhibition of protein serum albumin secretion by either genistein or pervanadate are biochemically indistinguishable. Two different indexes were used to assess the nature of the effects of genistein (A and B) and pervanadate (C and D) on serum albumin from primary rat hepatocyte cultures. A and C: fraction of total synthesized albumin that is released from the cells after its formation by proteolysis in the *trans*-Golgi network (TGN). B and D: efficiency with which albumin is formed from proalbumin as the ratio of albumin to the total albumin-like protein. In all cases, each point represents one individual duplicate experiment and data from up to 29 different experiments are included. In some cases data points closely overlap and this redundancy has been eliminated for clarity.



hepatocytes but there was no quantifiable disruption with either 100 μM genistein or pervanadate (not shown). Latrunculin B eliminated the cytosolic actin filaments (mostly found immediately below the cell surface) and that either 100 μM genistein or pervanadate had any effect on these structures in side-by-side incubations (not shown). Despite the impact of latrunculin B on the hepatocellular actin distribution, this compound was without effect on albumin secretion (Table 1).

Isolated rat liver Golgi membranes contain protein tyrosine kinases, phosphatases, and corresponding substrates. To substantiate the hypothesis that the machinery for a cycle of reversible protein tyrosine phosphorylation plays a role in the trafficking of albumin through the Golgi, we probed this organelle for elements of this machinery. For a cycle to be localized in the Golgi membranes, it is obligatory for at least one member of the group of cycle elements, either a tyrosine kinase, tyrosine phosphatase, or a shared protein substrate to be a resident Golgi component. Treatment of Golgi membranes individually with either ATP or pervanadate did not reveal any detectable phosphotyrosyl proteins. However, the addition of ATP in the presence of pervanadate produced a number of these species (Fig. 5A). Figure 5A also shows that by increasing the concentration of ATP over the range of 0 to 100 μM in the presence of pervanadate increases the amounts of detectable phosphotyrosyl proteins. Further analysis reveals apparent saturation kinetics for the tyrosine phosphorylation of at least 13 proteins (not shown).

To investigate further the presence of protein tyrosine phosphatases in Golgi we used the probe LCL2 (26). This compound contains both a phosphotyrosyl recognition head and a trapping group (a transiently generated quinone methide) that efficiently biotinylates only proteins that hydrolyze the tyrosine phosphate group rather than merely bind to it. As a result,

LCL2 is a selective probe for active protein tyrosine phosphatases and Fig. 5B clearly shows that Golgi membranes contain several LCL2-reactive proteins and that interaction is resistant to the blockade of nonspecific interactions with phosphate. Estimates of the apparent molecular weights range from ~ 34 to 68 kDa, a span known to contain many species of non-receptor phosphotyrosine and dual-specificity phosphatases (21).

DISCUSSION

The observation that both pervanadate and genistein inhibit albumin secretion from primary rat hepatocytes is surprising given the predominance of evidence for the role of tyrosine phosphorylation and dephosphorylation in regulated secretion but the complete absence of any corresponding evidence in the constitutive pathway. The postulation of a cycle of protein tyrosine phosphorylation during constitutive secretion follows immediately from these results but the precise kinases and phosphatases are not identifiable with these broad-spectrum inhibitors. The comparatively poor effects of the complementary agents PP2 and SU-6656 (and PP1, data not shown) probably exclude as genistein's targets the well-described Src family tyrosine kinases, and all coupled downstream actors (4). Because cell-surface receptors often rely on tyrosine kinase or phosphatase activity and continue to signal when endocytosed into the vesicular trafficking pathways (7), we investigated the withdrawal and readdition of serum but neither intervention affected albumin processing or secretion. With the possible exception of hepatocyte surface ligands, our cell-washing protocol, combined with the strict linearity of albumin secretion mitigates against paracrine or autocrine agents unless they are secreted rapidly to response-saturating concentrations by ice-cold hepatocytes. Unlike bile production and volume regula-

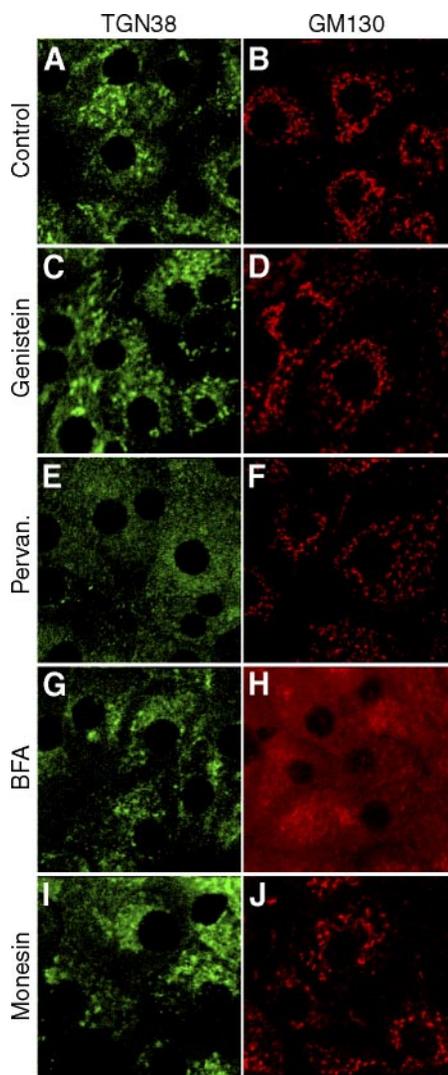


Fig. 4. The effects of genistein and pervanadate and other drugs on the distribution of the Golgi marker (GM) proteins TGN38 and GM130. Primary rat hepatocyte cultures on glass coverslips were treated for up to 30 min with the following drugs: control (A and B, no treatment), genistein (C and D, 100 μ M), pervanadate (E and F, 100 μ M), brefeldin A (BFA; G and H, 10 min, 5 μ g/ml), monensin (I and J, 10 μ M). Cultures were fixed, permeabilized, stained either with antibodies to TGN38 or GM130, and analyzed, as described in MATERIALS AND METHODS. The effects of the drug vehicles (ethanol 0.01% vol/vol final and DMSO 0.02% vol/vol final) had previously been found to have no effect on the distribution of the target proteins.

tion in hepatocytes (19, 31), there is no role for class-1 phosphatidylinositol 3-kinases in the trafficking, processing, or secretion of albumin. Because the activity of these enzymes is often linked to receptor activation this also argues against some forms of receptor tyrosine kinase signaling. Because it is established that $PI(3,4,5)P_3$ and $PI(3,4)P_2$ have major roles in the regulated plasma membrane fusion events required for hepatocyte bile secretion and volume regulation, then this lack of effect establishes a clear difference between regulated and constitutive pathways. Among the other broad-spectrum inhibitors tested, only staurosporine, which inhibits many serine/threonine kinases, had any effect, and this accords with earlier investigations of Golgi function (51). This might indicate a role for some permutation or combination of PKCs, PKA, calmod-

ulin kinase II, myosin light chain kinase, or even PKD. PKD is recruited to diacylglycerol containing membranes [including the TGN (27)] along with PKC- α , - ϵ , or - η kinases, which phosphorylate the PKD activation loop (6, 57, 58), and which are also recruited by diacylglycerol. The activities of PKC- α , - ϵ , - η , and PKD are inhibited by staurosporine (12, 16, 50) and alternative routes to PKD activation by tyrosine phosphorylation (53) can be expected to be staurosporine sensitive (Src-PKC δ path) (29) or alternatively PP2 and SU-6656 sensitive (Src-Abl pathway). Hence, the effects of staurosporine may be due to multiplicative action on several or all of these targets, but significantly, the effect of staurosporine and therefore the importance of its targets are small compared with the targets of genistein and pervanadate. Finally, because rapamycin, a relatively specific compound (8), had no effect then we can probably exclude changes in ribosome function mediated through ribosomal S6 kinase and other members of the rapamycin sensitive pathways, i.e., mitogen receptor kinases, phosphatidylinositol 3-kinase, PDK1, and mTOR (43) from consideration. We also discount roles for ERKs 1/2, p38-SAPK, and JNKs 1/2. Strong activation of these kinases in primary rat hepatocytes induced with either IL-1 or IL-6 (20, 23, 24, 64) downregulating albumin expression after 24 h, but notably is without short-term effect (3, 11). Furthermore, MAP kinases require serine/threonine phosphorylation and their activation in hepatocytes can be sensitive to inhibitors of PKCs (39).

We examined the effects of genistein and pervanadate on the cleavage of proalbumin to albumin. Because cleavage occurs in the hepatocyte TGN, the sensitivity of this step can then discriminate between blockade upstream or downstream of this compartment. We find that both agents prevent the transfer of

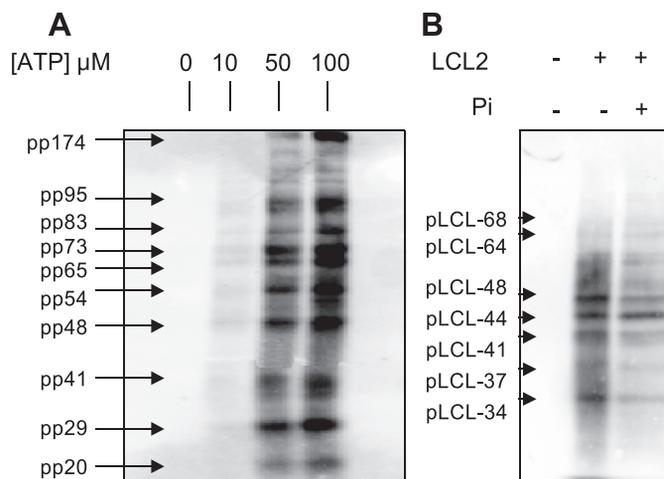


Fig. 5. Components of cyclic protein tyrosine phosphorylation and dephosphorylation are resident in purified Golgi membranes. Golgi membranes were isolated, as described in MATERIALS AND METHODS. A: membranes were treated 100 μ M pervanadate and with increasing final concentrations of ATP from 0 to 100 μ M as shown. B: membranes were left as controls or treated with combinations of 1 mM LCL2 and 50 mM phosphate, as indicated. Phosphate was included to reduce any nonspecific reactivity. In each experiment after incubation at 37°C for 60 min, the samples were treated with hot SDS-PAGE sample buffer to stop the reactions. Samples were then displayed by SDS-PAGE (10% gels) and analyzed by Western blotting with (A) antiphosphotyrosine monoclonal antibody or (B) for biotin-containing proteins using streptavidin. The apparent molecular mass of reactive proteins was estimated by reference to standards included in the same gel. Prefixes pp and pLCL2 indicate phosphoprotein and LCL2-binding protein, respectively.

the newly formed mature albumin from the TGN to the outside of the cell and that proalbumin is trapped in a proximal compartment (for examples, see Figs. 1 and 3). The common factor with both genistein and pervanadate is failure of the secretory pathway at the level of the *trans*-Golgi.

Hepatocytes account for ~93% of liver volume, and Golgi represents ~2% of hepatocellular membrane (5), making the rat liver one the best-described tissues for the biochemical study of Golgi function. We isolated Golgi membranes free of luminal contents (including albumin and presumably any soluble protein kinases, phosphatases, or substrates that might be in transit) from Golgi preparations proven to authentically catalyze the cleavage of proalbumin (45). To test our case in general (before proceeding to the specific) and to avoid bias in searches for specific proteins, we screened these membranes with nonspecific probes, particularly broad specificity anti-phosphotyrosine antibodies to detect tyrosine kinases and endogenous substrates and with an activity probe for tyrosine phosphatases. In principle, at least one component of a cycle for protein tyrosine phosphorylation (one of either the family of tyrosine kinases, phosphatases, or suitable substrates) must be resident in the Golgi to anchor a cycle in this compartment. The absence of all three elements would allow the hypothetical cycle to be immediately dismissed. We found that isolated membranes do not contain a pool of phosphotyrosyl proteins that survives postmortem subcellular fractionation. However, because we could cause phosphotyrosyl proteins to be generated by ATP *in vitro* but only in the presence of pervanadate then Golgi membranes must contain active systems for both the rapid generation and removal of resident phosphotyrosyl proteins. Phosphoprotein synthesis showed apparent saturation kinetics with ATP over the 0–100 μ M range (not shown) which is significant because virtually all well-described protein tyrosine kinases have micromolar K_m s for ATP. The apparent molecular weights of some phosphoproteins are roughly in accord with elements already described by Austin and Shields (2) in regulated secretion. A probe of these same membranes for active tyrosine phosphatases revealed several candidate Golgi tyrosine phosphatases and consequently it is established that all of the machinery for reversible protein tyrosine phosphorylation is present in rat liver Golgi membranes.

Correct Golgi function is associated with the structural and organisational integrity of this dynamic compartment. We found that pervanadate but not genistein caused major changes in the distribution of the *trans*-Golgi marker TGN38 and that neither compound induced any change in the localization of the *cis*-Golgi marker GM130. A simple hypothesis is that the accumulation of one or more unphosphorylated proteins stabilizes the TGN structure and blocks cargo exit (and also import from distal compartments), whereas the accumulation of the corresponding phosphotyrosyl protein(s) permits outward traffic but prevents final exit from the cell.

Gross disruptions to either actin filaments or microtubule networks might explain the effects of genistein or tyrosine phosphatase inhibitors. However, we discount genistein and pervanadate sensitivity in the actin cytoskeleton because this can be entirely dispersed with latrunculin B without significant effect on albumin secretion. This is surprising given the proposed role of myosins and actin in the organization and function of Golgi during protein secretion from cultured cell lines (56, 60). Microtubules are essential for the operation of

the secretory pathway providing tracks for migrating transport intermediates. Colchicine and nocodazole disrupt hepatocellular microtubules and block albumin secretion (10, 14, 49), but we find no gross perturbation of microtubules with either genistein or pervanadate. This is not surprising because there is little evidence for an obligatory role for any tyrosine kinase or phosphatase in the organization of microtubules except possibly effects mediated through MAP (serine/threonine) kinases, which are discussed above.

In conclusion, rat liver Golgi membranes contain all of the elements for the endogenous cyclic generation of resident phosphotyrosyl proteins. Inhibition of this cycle at either one of two different points (phosphorylation or dephosphorylation) causes an identical trafficking lesion at the level of the *trans*-Golgi and in the case of phosphatase blockade can cause the redistribution of an established TGN marker. Despite the absence for precise targets for the effective kinase and phosphatase inhibitors our results indicate a previously unacknowledged, permissive role for protein tyrosine phosphorylation at the level of the TGN during the constitutive secretion of serum albumin from the liver. Because this new role for reversible tyrosine phosphorylation is established for a large-scale, constitutive secretory process in primary cells then this mechanism is highly likely to be universal.

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