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Cysteine and Glutathione Secretion in Response to Protein Disulfide Bond Formation in the ER

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Protein folding in the endoplasmic reticulum (ER) often involves the formation of disulfide bonds. The oxidizing conditions required within this organelle were shown to be maintained through the release of small thiols, mainly cysteine and glutathione. Thiol secretion was stimulated when proteins rich in disulfide bonds were translocated into the ER, and secretion was prevented by the inhibition of protein synthesis. Endogenously generated cysteine and glutathione counteracted thiol-mediated retention in the ER and altered the extracellular redox. The secretion of thiols might link disulfide bond formation in the ER to intra- and intercellular redox signaling.

The ER is the port of entry and main folding compartment for proteins destined for the central vacuolar system (*1*). Nascent proteins are translocated into the ER in the reduced state and rapidly form disulfides in the suitable redox environment of this organelle (*2*, *3*). Oxidizing conditions are likely to be generated by the import of oxidized glutathione (GSSG) and cystine (*3*) and must be continuously maintained to counteract the vectorial import of reduced cysteine residues that enter the ER as part of the translocated polypeptides. Imported GSSG and cystine can participate in disulfide interchange reactions involving ER-resident as well as newly made proteins (*3*, *4*) and catalyzed by protein disulfide isomerase, ERp72, and other oxidoreductases (*1*, *2*). It is possible that the homeostasis of the redox state in the ER depends on the flux of small disulfides, secreted together with their reduced counterparts, which are generated during the process of protein disulfide bond formation.

To test this model, we monitored the accumulation of thiols in the extracellular space (Fig. 1). As observed for other cell types (*3*), both J558L myeloma cells (Fig. 1, A and C) and *Xenopus laevis* oocytes (Fig. 1E) released thiols. The export of thiols and of constitutively secreted proteins [immunoglobulin (Ig) λ chains] was compared in J558L cells after perturbation of vesicular traffic by treatment at low temperature (20°C) or in the presence of brefeldin A (BFA) (*5*). After the first hour of treatment, the extracellular accumulation of both λ chains and thiols was inhibited (Fig. 1), which suggested that a functional secretory apparatus was required for their release. When cells were kept at 20°C for 3 hours and then transferred to 37°C, the secretion of both thiols and proteins was promptly restored (Fig. 1, A and B). The similar behavior of thiols and λ chains suggests that they did indeed utilize the same transport systems. In agreement with previous observations that J558L cells secrete λ chains that are completely oxidized (*4*), virtually all secreted thiols were soluble in acetone (*6*). Differences in the equilibrium constant (K_{ox}) may explain the coexistence of reduced glutathione and cysteine with oxidized proteins (*7*). Similar findings were also obtained with isolated amphibian oocytes, in which monensin, another inhibitor of protein secretion, inhibited thiol release (Fig. 1E). Monensin was used because BFA is ineffective in *Xenopus* oocytes (*8*).

We also predicted that thiol release should be influenced by the synthesis of secretory proteins containing disulfide bonds. In agreement with this, cyclohexi-

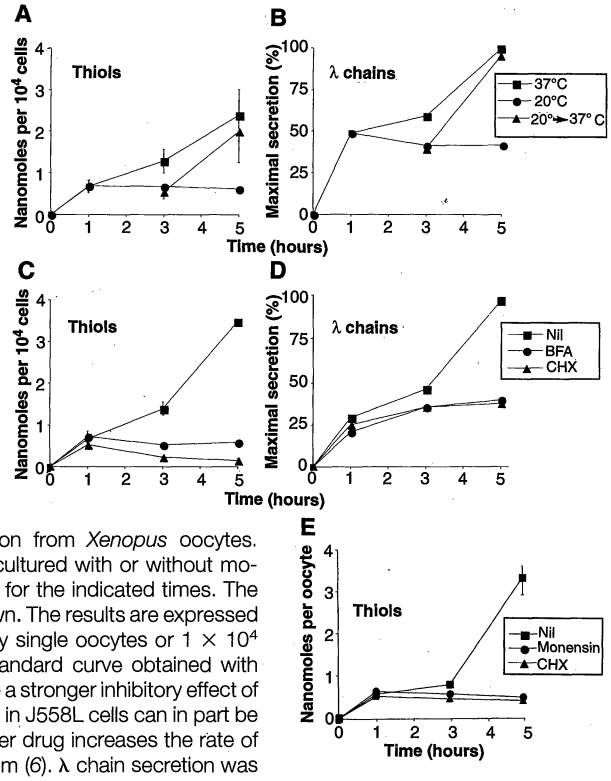

Fig. 1. Thiol release through the secretory pathway requires protein synthesis. (**A** through **D**) J558L cells were cultured in a water bath for the indicated times at 20° or 37°C. After 3 hours, aliquots of cells cultured at 20°C were further cultured for 2 hours at 37°C [(A) and (B)]. J558L cells were cultured in an incubator at 37°C with or without BFA (5 μg/ml) or 500 μM cycloheximide (CHX) [(C) and (D)]. The spent media were harvested by centrifugation and the accumulation of thiols [(A) and (C), mean of three experiments], and λ chains [(B) and (D)] were quantitated by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay (*20*) and immunoblotting (*4*), respectively. (**E**) Thiol secretion from *Xenopus* oocytes. Groups of five oocytes (*21*) were cultured with or without monensin (20 μg/ml) or 500 μM CHX for the indicated times. The average of two experiments is shown. The results are expressed as nanomoles of thiols secreted by single oocytes or 1×10^4 J558L cells, as derived from a standard curve obtained with purified cysteine. What seems to be a stronger inhibitory effect of CHX as compared with that of BFA in J558L cells can in part be explained by the fact that the former drug increases the rate of decay of thiols in the culture medium (*6*). λ chain secretion was normalized assuming as 100% the amount of protein present in control cultures at 5 hours.

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mide (CHX), an inhibitor of protein synthesis, prevented the extracellular accumulation of thiols by both J558L cells (Fig. 1C) and amphibian oocytes (Fig. 1E).

To examine the dependence of thiol secretion on the oxidation of newly synthesized proteins in the ER, we used oocytes that expressed two proteins rich in disulfide bonds, influenza hemagglutinin (HA), or IgM (9). The synthesis of these two polypeptides increased the accumulation of reducing equivalents in the oocyte medium severalfold (Fig. 2). Although small differences were observed at early time points (striped bars), this phenomenon became clearly evident 20 hours after the injection (open bars), possibly reflecting a time-dependent increase in the synthesis of the heterologous proteins (*10*). In contrast, the expression of phaseolin (a protein that translocates into the ER but does not contain cysteine residues) did not significantly induce the accumulation of extracellular thiols. This excludes the possibility that simple protein synthesis or translocation into the ER was responsible for thiol secretion. Dose-response analysis (Fig. 3A) showed that the more IgMs were synthesized, the more thiols were secreted by oocytes; whereas phaseolin was not effective, even when expressed at very high levels (>2 μg per oocyte). A linear relation between IgM synthesis and thiol release was observed when they were plotted on a semilog scale. Thus, the amount of thiols released by cells exceeds the stoichiometric values expected from the formation of disulfide bonds in cargo proteins. It is possible that changes in the pH of the ER lumen or other secondary adaptive responses, including the unfolded protein response (*11*), are elicited when very large amounts of disulfide-rich proteins are synthesized in the ER.

Thus, small thiols were secreted by cells in a quantity that was influenced by the biosynthesis of cysteine-containing proteins in the ER. As determined by reversed-phase high-performance liquid chromatography (rpHPLC) analyses, cysteine and glutathione (GSH) account for most of the secreted thiols. Also, their oxidized counterparts (cystine and GSSG) were detected extracellularly, as were the mixed disulfides between the two species (Cys-SG). Three main phenomena were evident (Fig. 3). First, the expression of IgM and HA, but not of phaseolin, caused a more than 10-fold increase in the thiol/disulfide ratio in the medium (Fig. 3B). Second, the total amount of cysteine (that is, cysteine + cystine + Cys-SG) did not change significantly upon IgM overexpression, whereas there was a shift from the oxidized to the reduced forms (Fig. 3, C and E). Third, the synthesis of cysteine-rich proteins in the ER caused a net increase in the release of glutathione (Fig. 3, D and E). This may suggest the existence of two disulfide transporters, one constitutive and preferential for cystine and the other redox-sensitive and GSSG-specific. In addition, glutathione molecules present as mixed disulfides with the ER protein matrix could be mobilized as a response to the redox changes.

A thiol-dependent retention system prevents the ER-to-Golgi transport of incompletely folded or assembled molecules with exposed unpaired cysteines (*4*, *12*). This mechanism, which can be counteracted by exogenous reducing agents (*13*), is also active in amphibian oocytes: The reducing agent 2-mercaptoethanol (2-ME) induces the secretion of free λ chains that are otherwise retained by these cells (*14*). Thus, if indeed thiols were generated in the ER, they should alter the local redox, favoring λ chain secretion. Indeed, the coinjection of concentrated HA mRNA induced a fourfold increase in the amount of λ chains secreted (Fig. 4, A and B). Because HA itself is not retained in the ER by thiol-dependent mechanisms (*15*), saturation of the retention machinery does not explain the increased secretion of free λ chains. Rather, these findings suggest that the total thiol concentration increased in the ER when cysteine-containing proteins were translocated, weakening re-

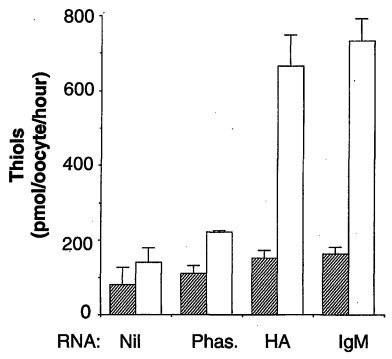

Fig. 2. The synthesis of cysteine-rich ER proteins increases thiol secretion. Groups of oocytes were microinjected (*22*) with water as control (Nil) or with synthetic mRNAs encoding phaseolin (Phas.), influenza HA, or Ig μ and λ chains (IgM) (*23*). The thiols accumulated in the spent medium from 2 to 6 hours (striped bars) or from 22 to 27 hours (open bars) after injection were quantitated by the DTNB assay (*20*).

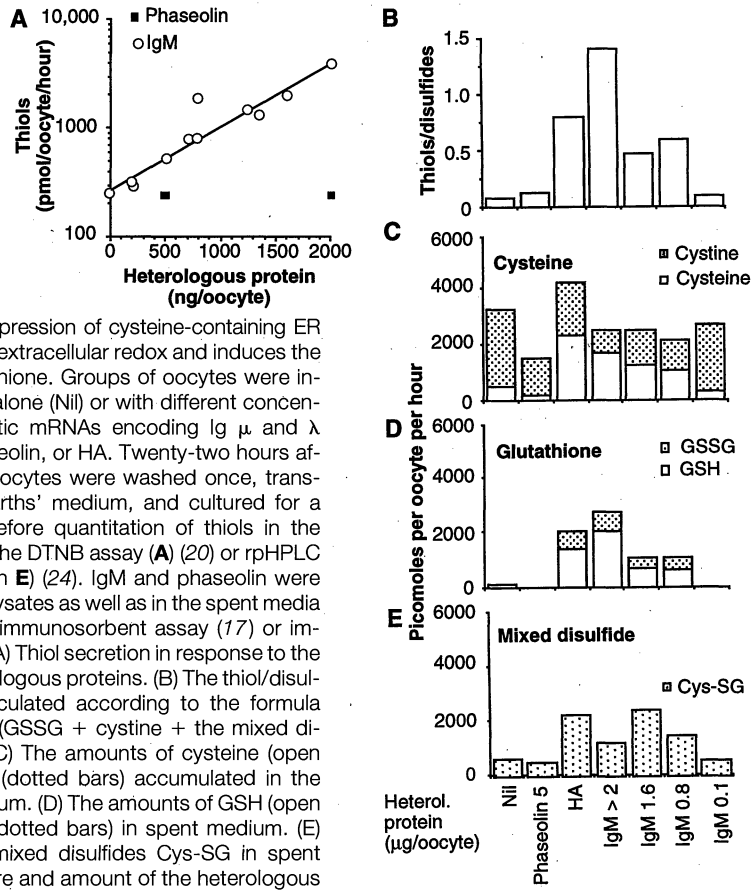

Fig. 3. The overexpression of cysteine-containing ER proteins alters the extracellular redox and induces the secretion of glutathione. Groups of oocytes were injected with water alone (Nil) or with different concentrations of synthetic mRNAs encoding Ig μ and λ chains (IgM), phaseolin, or HA. Twenty-two hours after the injection, oocytes were washed once, transferred to fresh Barths' medium, and cultured for a further 5 hours before quantitation of thiols in the spent medium by the DTNB assay (**A**) (*20*) or rpHPLC analysis (**B** through **E**) (*24*). IgM and phaseolin were quantitated in the lysates as well as in the spent media by enzyme-linked immunosorbent assay (*17*) or immunoblotting (*4*). (A) Thiol secretion in response to the synthesis of heterologous proteins. (B) The thiol/disulfide ratio was calculated according to the formula (GSH + cysteine)/(GSSG + cystine + the mixed disulfide Cys-SG). (C) The amounts of cysteine (open bars) and cystine (dotted bars) accumulated in the oocyte spent medium. (D) The amounts of GSH (open bars) and GSSG (dotted bars) in spent medium. (E) The amounts of mixed disulfides Cys-SG in spent medium. The nature and amount of the heterologous proteins synthesized by oocytes are indicated at the bottom of panel (E). We were unable to quantitate HA because of the lack of a purified standard.

tention as occurs when 2-ME is added (*14*).

As expected, the coinjection of phaseolin mRNA did not induce λ chain secretion (Fig. 4C), excluding the possibility that protein synthesis per se inhibits retention. In contrast, the overexpression of λ chains, which contain five cysteines and rapidly form intrachain disulfide bonds (*16*), influenced thiol-mediated retention. The increased secretion of thiols (and of free λ chains) observed upon the overexpression of cysteine-rich ER proteins did not reflect an acceleration of vesicular transport. Indeed, the secretion rate of lysozyme did not change when oocytes were coinjected with different amounts of phaseolin or λ chain mRNAs (Fig. 4D).

Thus, the formation of disulfide bonds in the ER induced the secretion of cysteine and GSH. This mechanism is likely to be one of the key elements controlling redox homeostasis in the exocytic pathway. Whether the thiol/disulfide ratio and the relative abundance of the single molecular species are the same in the ER and in the pool of secreted molecules remains to be established. However, the increased secretion of cysteine and GSH in response to the synthesis of cysteine-containing proteins is likely to be linked to a change in the redox of the ER. Although the formation of most protein disulfides will not be affected by these redox changes, some crucial thiols like the one of Cys^{214} in λ chains appear to be more sensitive (*4*). Another example of a cysteine residue whose activity is likely to be regulated by the ER redox is Cys^{575} of Ig μ heavy chains. This residue plays a key role in the developmental control of IgM secretion, mediating both the retention and the assembly of unpolymerized subunits (*17*). Oocytes secrete unpolymerized IgM precursors when injected with large amounts of μ and λ chain mRNAs (*14*). The most likely interpretation of this finding is that endogenous reducing agents are generated in the ER that counteract retention. This suggests that under conditions of excessive synthesis, the redox buffering capacity of the ER can be saturated and quality control weakened.

The secretion of thiols through the exocytic pathway in response to disulfide bond formation may provide a direct link between the synthesis of a large class of secretory and membrane proteins and key regulatory molecules that are known to be modulated by the extracellular redox (*18*). Further investigation is required to determine whether ER-generated thiols can be exploited as markers of bulk flow transport through the exocytic pathway.

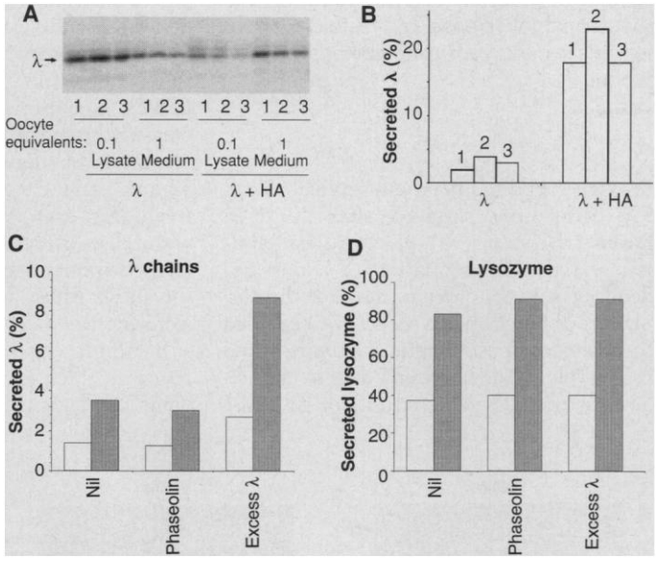

Fig. 4. The overexpression in the ER of cysteine-containing proteins counteracts thiol-mediated retention. (**A** and **B**) Oocytes injected with λ chain mRNA at low concentration were coinjected with or without HA mRNA at high concentration. Twenty hours after injection, oocytes were individually pulse-labeled with [^{35}S]methionine and cysteine for 4 hours (*25*). At the end of a 20-hour chase, lysates and supernatants of single oocytes were immunoprecipitated with purified antibodies to λ (anti-λ) (*26*) and resolved by SDS-PAGE (A) under nonreducing conditions. To allow the detection and the densitometric quantitation (B) of secreted λ chains from individual oocytes, one-tenth of the proteins immunoprecipitated from oocyte lysates and all the proteins immunoprecipitated from incubation media were loaded onto the gel. Three different oocytes per set (1, 2, and 3) were analyzed. Also, the upper band present in the lysates of HA-coinjected oocytes, which is recognized by anti-λ in immunoblotting experiments (*6*, *14*), was included in the densitometric quantitations. (**C** and **D**) Groups of oocytes injected with small amounts of lysozyme and Igλ mRNAs were coinjected with highly concentrated phaseolin, Igλ (excess λ) transcripts, or water as control (Nil). Twenty-four hours after injection, oocytes were pulse-labeled for 4 hours with [^{35}S]-labeled amino acids and chased for 5 hours (open bars) or 20 hours (gray bars). λ chains (C) and lysozyme (D) were immunoprecipitated with specific antibodies and resolved by SDS-PAGE under reducing conditions. The percent of secreted molecules was calculated from densitometric analyses (*4*). Note that the secretion of lysozyme is considerably faster and more efficient than that of λ chains.

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9. To confirm the synthesis of heterologous proteins, oocytes were lysed 27 hours after the injection, and proteins were electrophoresed under reducing conditions and transferred to nitrocellulose. Filters were incubated with antibodies specific for phaseolin, HA, or IgM and were developed by ECL (Amersham, Milano, Italy) to detect the presence of the specific proteins.
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20. Thiols in solution were quantitated with Ellman's reagent DTNB [P. W. Riddles, R. L. Blakeley, B. Zerner, *Anal. Biochem.* **94**, 75 (1979) and references therein]. Briefly, the spent media of lymphoid cells or oocytes were spun at 8000*g* for 2 min at 4°C and diluted in 50 mM tris-HCl (pH 8.2) and 5 mM EDTA. Ten microliters of DTNB (10 mM in methanol) were added to 1 ml of diluted sample, and the absorbance at 412 nm was immediately determined with a Beckman DU-70 spectrophotometer. The concentration of thiols was determined by comparison to a cysteine standard.
21. Oocytes were obtained from large females of *Xenopus laevis* and maintained in modified Barths' saline as described (*19*). Animals were kept and oocytes were obtained in accordance with the institutional guidelines. For the determination of secreted thiols and disulfides, groups of five oocytes were cultured for different times in 50 μl of modified Barths' saline lacking nystatin. The basal levels of thiol secretion varied in batches of oocytes isolated from different frogs, possibly corresponding to different levels of synthesis of endogenous proteins.
22. Oocytes were microinjected with 50 nl of mRNA solution as described (*19*) with the use of a Narishige IM-1 automatic microinjector controlled by an IM-1T timer.
23. The preparation of transcription constructs for the synthesis of Igμ, Igλ, phaseolin, and HA mRNAs has been previously described (*14*, *15*) [A. Ceriotti *et al.*, *Eur. J. Biochem.* **202**, 959 (1991)]. Linearized plasmids were transcribed in vitro by SP6 polymerase (Promega), and synthetic mRNA was prepared for microinjection as previously described (*14*).
24. Samples (25 μl) were diluted 10-fold with 0.5 M

borate buffer (pH 7.7) and placed in an autosampler HPLC system (Merck-Hitachi, Darmstad, Germany) that automatically performs the fluorenyl methoxycarbonyl (FMOC) derivatization by adding 250 μl of a 15 mM solution of 9-fluoromethoxycarbonylchloride (FMOC-Cl; Sigma-Aldrich, Milano, Italy) in acetone and, after 45 s, removing the excess reagent by two pentane extractions (900 μl). HPLC-grade reagents were purchased from Romil (Cambridge, UK). rpHPLC analyses of the FMOC-derivatized compounds were performed under the following conditions: A column RP-8 Superspher 60 LiChroCart was used; eluents were H_2O/CH_3CN (80:20) and 40 mM CH_3COONa (pH 4.6) (A); H_2O/CH_3CN (20:80) and 10 mM CH_3COONa (pH 7.0) (B); and CH_3CN (C). Gradient: 0 min, 100% A; 30 min, 50% A, 50% B; 40 min, 100% C; 45 min, 100% C; 50 min, 100% A. Flow was at 1.25 ml/min at a temperature of 45°C; ultraviolet monitoring was used (wavelength, 263 nm). The concentration of thiols in the samples was determined by comparison with calibrated amounts of standard solutions of cysteine, GSH, GSSG, and the mixed disulfide between cysteine and glutathione (Cys-SG). The identity of individual peaks in the chromatograms was further confirmed by time-of-flight matrix-assisted laser desorption ionization mass spectrometry (*4*) [T. Tanaka *et al.*, *Rapid Comm. Mass Spectrom.* **2**,151 (1988)].

25. For pulse and chase analysis, injected oocytes were cultured overnight at 20°C in modified Barths' saline, washed, and resuspended in modified Barths' saline (5 μl per oocyte) containing [^{35}S]methionine-cysteine (1 mCi/ml) (PRO-MIX, Amersham). After the pulse, the medium was removed, and the oocytes were washed thoroughly with modified Barths' saline and incubated in the same medium supplemented with excess cold methionine and cysteine. After the medium was harvested, each oocyte was homogenized in 40 μl of homogenization buffer (*19*), supplemented with 50 mM iodoacetamide. Media and homogenates were frozen in liquid nitrogen and stored at −80°C.
26. Homogenates and incubation media were diluted to 1 ml with Net-gel buffer (*14*) and incubated with specific antibodies for 4 hours on ice before addition of 50 μl of a 10% suspension of protein A–Sepharose CL 4B (Pharmacia, Uppsala, Sweden). After overnight incubation at 4°C, the beads were washed three times with NET-gel buffer and eluted by heating at 95°C in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer [0.062 M tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.001% bromophenol blue].
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Synaptic Efficacy Enhanced by Glial Cells in Vitro

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In the developing nervous system, glial cells guide axons to their target areas, but it is unknown whether they help neurons to establish functional synaptic connections. The role of glial cells in synapse formation and function was studied in cultures of purified neurons from the rat central nervous system. In glia-free cultures, retinal ganglion cells formed synapses with normal ultrastructure but displayed little spontaneous synaptic activity and high failure rates in evoked synaptic transmission. In cocultures with neuroglia, the frequency and amplitude of spontaneous postsynaptic currents were potentiated by 70-fold and 5-fold, respectively, and fewer transmission failures occurred. Glial cells increased the action potential–independent quantal release by 12-fold without affecting neuronal survival. Thus, developing neurons in culture form inefficient synapses that require glial signals to become fully functional.

Brain development and function depends on glial cells, as they guide the migration of neuronal somata and axons (*1*), promote the survival and differentiation of neurons (*2*), and insulate and nourish neurons (*3*, *4*). It is not known, however, whether glial cells also promote the formation and function of synapses, although glial processes ensheath most synapses in the brain (*4*, *5*). The recent development of methods to purify (6) and culture a specific type of neuron from the central nervous system (CNS) (*7*) has allowed us to investigate whether CNS neurons can form functional synapses in the absence of glial cells.

We cultured purified postnatal rat retinal ganglion cells (RGCs) without glial cells under serum-free conditions (Fig. 1A) (*8*) that supported neuronal survival (57 ± 5% of neurons survived after 20 days; mean ± SEM; $n = 3$), electrical excitability, and the differentiation of axons and dendrites (*7*). In order to monitor the formation of functional synapses, we recorded spontaneous postsynaptic currents from RGCs (Fig. 1B) (9). After 5 days in culture, 50% of the RGCs tested (15 of 31 cells) showed low levels of synaptic activity with excitatory postsynaptic currents (EPSCs) occurring at a mean frequency of 3 ± 1 min^{-1} and with a mean peak amplitude of −11 ± 1 pA (Fig. 2A). After 20 days of culture, 63% of the RGCs tested ($n = 24$) displayed spontaneous synaptic activity. The frequency and amplitude of the EPSCs had increased to mean values of 18 ± 7 min^{-1} and −16 ± 1 pA ($n = 15$; Fig. 2A), respectively.

To study the effect of neuroglia on synapse formation, we cultured RGCs with glial cells from their target region, the superior colliculus (*10*). After 5 days of serum-free culture with glial cells, 90% of the RGCs tested ($n = 34$) showed spontaneous EPSCs. Coculture with collicular glia increased the mean frequency and the mean amplitude of spontaneous EPSCs to 41 ± 12 min^{-1} and −29 ± 3 pA ($n = 30$), respectively (Fig. 2A). After 20 days of coculture with glia, every RGC tested ($n = 20$) showed spontaneous synaptic activity, and the mean EPSC frequency and amplitude were increased to 1265 ± 212 min^{-1} and −78 ± 8 pA, respectively ($n = 20$; Fig. 2A). A nearly identical glia-induced enhancement of synaptic activity was observed

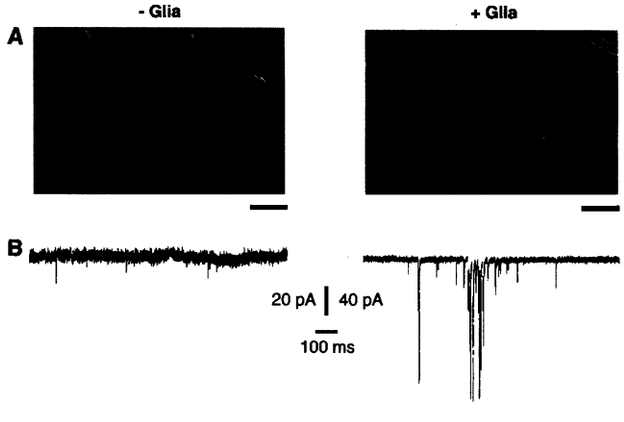

Fig. 1. Spontaneous synaptic activity in purified RGCs that were cultured for 5 days in defined, serum-free medium in the absence (left) or presence (right) of collicular glia. (**A**) Hoffmann-modulation contrast micrographs of RGCs. Scale bar, 50 μm. The density of neurons was similar in both cultures. (**B**) Whole-cell patch-clamp recordings of spontaneous EPSCs from RGCs at a holding potential of −70 mV.

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