20. H. Thomann *et al.*, *J. Am. Chem. Soc.* **109**, 7913 (1987).
21. A. E. True, P. McLean, M. J. Nelson, W. H. Orme-Johnson, B. M. Hoffman, *J. Am. Chem. Soc.* **112**, 651 (1990).
22. H. I. Lee, K. S. Thrasher, D. R. Dean, W. E. Newton, B. M. Hoffman, *Biochemistry* **37**, 13370 (1998).
23. R. N. F. Thorneley, D. J. Lowe, in *Molybdenum Enzymes*, T. G. Spiro, Ed. (Wiley-Interscience, New York, 1985), vol. 1, pp. 221–230.
24. H. B. Deng, R. Hoffmann, *Angew. Chem. Int. Ed. Engl.* **32**, 1062 (1993).
25. K. K. Stavrev, M. C. Zerner, *Chem. Eur. J.* **2**, 83 (1996).
26. P. E. M. Siegbahn, J. Westerberg, M. Svensson, R. H. Crabtree, *J. Phys. Chem. B* **102**, 1615 (1998).
27. G. Ertl, H. Knözinger, J. Weitkamp, Eds., *Handbook of Heterogeneous Catalysis* (VCH, New York, 1997), vol. 4.
28. A. Logadottir, J. K. Nørskov, *Surf. Sci.* **489**, 135 (2001).
29. This work was supported by NIH. Stanford Synchrotron Radiation Laboratory operations are funded by the U.S. Department of Energy (DOE), Office of Basic Energy Sciences, and NIH. F.A.T. acknowledges the Burroughs Wellcome Fund and the Helen Hay Whitney Foundation for postdoctoral fellowships. We thank J. C. Peters, J. E. Bercaw, and H. B. Gray for enlightening discussions. Coordinates of the refined MoFe-protein structure have been deposited in the Protein Data Bank (accession code 1M1N).

Supporting Online Material
www.sciencemag.org/cgi/content/full/297/5587/1696/DC1
Materials and Methods
References

13 May 2002; accepted 26 June 2002

Cooperation of GGAs and AP-1 in Packaging MPRs at the Trans-Golgi Network

Balraj Doray,[1]*† Pradipta Ghosh,[1]* Janice Griffith,[2] Hans J. Geuze,[2] Stuart Kornfeld[1]‡

The Golgi-localized, γ-ear–containing, adenosine diphosphate ribosylation factor–binding proteins (GGAs) are multidomain proteins that bind mannose 6-phosphate receptors (MPRs) in the Golgi and have an essential role in lysosomal enzyme sorting. Here the GGAs and the coat protein adaptor protein–1 (AP-1) were shown to colocalize in clathrin-coated buds of the trans-Golgi networks of mouse L cells and human HeLa cells. Binding studies revealed a direct interaction between the hinge domains of the GGAs and the γ-ear domain of AP-1. Further, AP-1 contained bound casein kinase–2 that phosphorylated GGA1 and GGA3, thereby causing autoinhibition. This could induce the directed transfer of the MPRs from GGAs to AP-1. MPRs that are defective in binding to GGAs are poorly incorporated into AP-1–containing clathrin-coated vesicles. Thus, the GGAs and AP-1 interact to package MPRs into AP-1–containing coated vesicles.

In higher eukaryotic cells, the sorting of newly synthesized acid hydrolases to lysosomes is dependent on the mannose 6-phosphate (Man-6-P) recognition system (*1*). A key step in this pathway is the binding of the Man-6-P–tagged hydrolases to MPRs in the trans-Golgi network (TGN). The receptors are then packaged into transport vesicles for delivery to endosomal compartments, where the hydrolases are released and transferred to lysosomes. The MPRs are localized to AP-1–containing clathrin-coated vesicles (AP-1–CCVs) at the TGN, implicating AP-1 as the coat protein involved in transport vesicle assembly (*2*). The MPRs also bind to the GGA family (*3–5*). The GGAs are modular proteins with four domains: an NH_2-terminal VPS-27, Hrs, and STAM (VHS) domain, then a GGA and TOM (GAT) domain, a connecting hinge segment, and a COOH-terminal γ-adaptin ear (GAE) domain. The GAT domain binds adenosine diphosphate ribosylation factor–guanosine 5′-triphosphate complexes and mediates recruitment of the protein from the cytosol onto the TGN (*6*, *7*). The VHS domain then interacts specifically with the acidic cluster–dileucine (AC-LL) motif in the cytoplasmic tails of the MPRs (*3–5*, *8*, *9*). Mutations in the AC-LL motif impair acid hydrolase sorting and decrease binding of the MPRs to the GGAs but not to AP-1, indicating that the GGAs have a major role in the sorting process (*4*, *10*, *11*). One explanation for these findings is that the GGAs and AP-1 function in parallel to package MPRs into different vesicular carriers at the TGN, as has been proposed to occur in yeast (*12*). Alternatively, the GGAs could bind the MPRs and facilitate their entry into forming AP-1–CCVs. We sought to distinguish between these two possibilities.

We first examined the distribution of GGA2 and AP-1 in mouse L cells by means of the cryo-immunogold technique. If the two proteins nucleate their own transport vesicles in the TGN, then they should be detected on separate coated buds and vesicles in the TGN, whereas if they cooperate in the packaging of MPRs, they might be found together. GGA2 was associated with tubules, buds, and CCVs at the TGN (Fig. 1A). In double-labeling experiments, GGA2 and AP-1 colocalized on the buds and CCVs (Fig. 1, B to D; Tables 1 and 2). About 50% of GGA2 was found on clathrin-coated TGN membranes, of which half was on identifiable buds. Forty-one percent of the coated TGN buds contained both proteins (Table 2). Similarly, GGA1 and AP-1 colocalized in coated buds at the TGN of HeLa cells. These findings are consistent with an interaction between the two proteins.

To examine this possibility, we expressed the three GGAs in SF9 insect cells and tested them for binding to glutathione *S*-transferase (GST) fusion proteins containing either the γ-ear domain or the hinge segment of AP-1 coupled to glutathione beads in pulldown experiments. All three GGAs bound to the GST–γ ear fusion protein, whereas no binding to the GST-γ hinge was detected (Fig. 2A). The binding was direct because purified GGAs also bound to the GST-γ ear (Fig. 2B). The GGAs interacted poorly with the ear domains of AP-2 and GGA2, showing that the binding was specific for the AP-1–γ ear (Fig. 2C). Binding was lost when the GGA1 hinge was truncated from 429 to 370 residues (Fig. 2D). Thus, the hinge segments of the GGAs bind to the γ-ear domain of AP-1. That truncated GGA1 lacking the hinge traps the MPRs in the TGN (*3*) supports the idea that the GGA–AP-1 interaction is essential for normal MPR trafficking.

The fact that the GGAs interact with AP-1 but are undetectable in isolated CCVs (*13*) raises the possibility that they bind the MPRs in the TGN and present them to AP-1 for packaging into CCVs. In this case there should be a mechanism whereby the GGAs release their cargo molecules upon interacting with AP-1. MPR binding to the VHS domains of GGA1 and GGA3 is regulated by competitive binding of an AC-LL motif in the hinge segment (*14*). This intramolecular binding requires casein kinase–2 (CK-2)–mediated phosphorylation of a serine located three residues upstream of the acidic cluster. Meresse *et al.* have reported that AP-1 isolated from CCVs has an associated CK-2–type activity (*15*). If this kinase were to

[1]Department of Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA. [2]Department of Cell Biology, University Medical Center and Institute for Biomembranes, Utrecht University, 3584 CX Utrecht, Netherlands.

*These authors contributed equally to this work.
†Present address: Genome Institute of Singapore, 1 Science Park Road, The Capricorn #05-01 Singapore 117528.
‡To whom correspondence should be addressed. E-mail: skornfel@im.wustl.edu

phosphorylate GGA1 or GGA3 to induce autoinhibition, then the MPR cargo molecule would be released for binding to AP-1 and the GGA might return to the cytoplasm. To test this possibility, we purified AP-1 from tris extracts of bovine liver CCVs by antibody affinity chromatography followed by gel filtration. Immunoreactive CK-2 eluted in the same fractions as AP-1 (Fig. 3A). This CK-2 phosphorylated GGA1 but not GGA2 (Fig. 3B). It also phosphorylated a GST-GGA1 peptide (residues 342 to 367), which encoded the region of the hinge containing the AC-LL motif and the critical serine, to the same extent as a GST–cation-independent (CI)–MPR peptide, which encoded a consensus CK-2 site (Ser^{159}-Asp-Glu-Asp-Leu-Leu^{164}) (Fig. 3C). No phosphorylation was observed when the serine or the downstream aspartate was mutated to alanine, as would be expected for a CK-2–type kinase.

If the GGAs and AP-1 cooperate in the packaging of the MPRs into CCVs, we would predict that a mutant MPR that is defective in binding to the GGAs would be poorly incorporated into AP-1–CCVs. To test this prediction, we performed double-label immunogold studies on mouse L cells expressing either wild-type bovine CI-MPR (CC2) or a mutant [Asp^{158} → Ala (D158A)] that fails to bind GGAs (*4*). A number of CCVs in the TGN that are double-labeled for wild-type MPR and AP-1 are shown in Fig. 4A, whereas Fig. 4B illustrates that the mutant CI-MPR was present in the TGN tubules but only occasionally found in the AP-1–positive CCVs. The quantitation of these data (Table 3) indicates that 59% of the CCVs were positive for AP-1 and the wild-type CI-MPR, whereas only 15% were positive for AP-1 and the mutant CI-MPR. Furthermore, the gold signal for the mutant CI-MPR per CCV is only 25% of that of the wild-type CI-MPR. This result indicates that the failure of the mutant CI-MPR to bind to the GGAs impairs its incorporation into AP-1–CCVs.

These data provide evidence that the GGAs function as adaptor proteins that select

Table 1. Quantitative distribution of GGA2 and AP-1. Immunogold was quantified on GGA2 and AP-1 double-labeled cryosections from L cells expressing GGA2-HA. To count gold labeling for GGA2 and AP-1, we randomly photographed Golgi areas at 20,000× and assigned gold particles to noncoated membranes of Golgi stacks and TGN and to membranes with a visible clathrin coat such as those on TGN buds and CCVs.

Immunogold	GGA2	AP-1
Noncoated membranes	52%	18%
Coated membranes	48%	82%
Identifiable coated buds	24%	25%
Membranes with clathrin lattices; CCVs	24%	57%
Total gold	2070	528

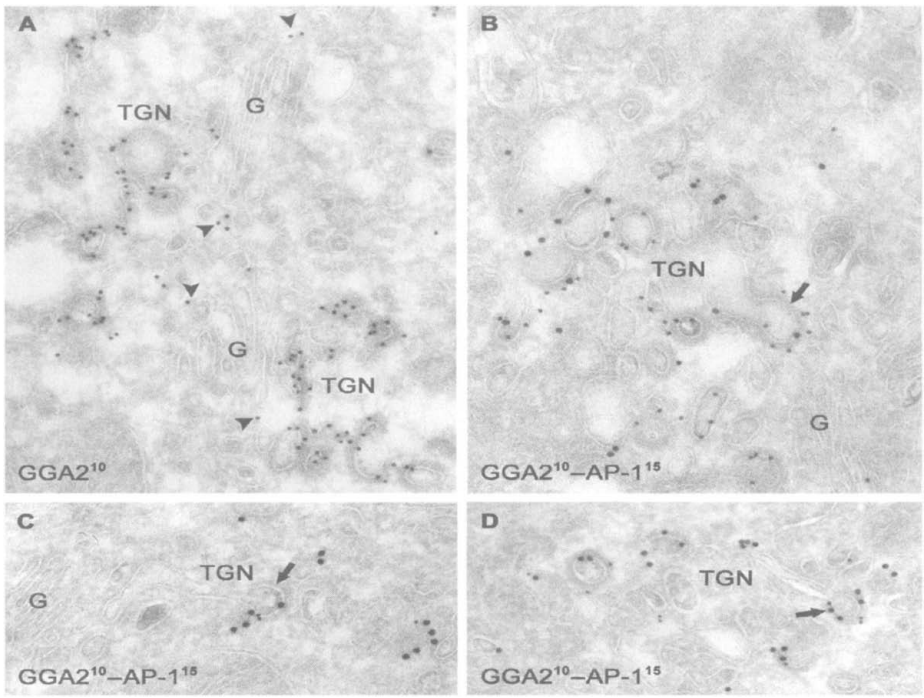

Fig. 1. GGA2 and AP-1 colocalize at the TGN. Ultrathin cryosections of L cells expressing GGA2-HA were single-labeled for GGA2 (**A**) or double-labeled for GGA2 and AP-1 [(**B**) to (**D**)] with 10-nm and 15-nm gold particles as indicated in superscript (*24*). GGA2 was immunolabeled with antibodies to HA (Upstate, Biotechnology, Lake Placid, New York) and γ-subunit of AP-1 (Transduction Laboratories, Lexington, Kentucky). Cryosectioning and immunogold labeling procedures were as described (*25*, *26*). In (A), Golgi stacks (G) are associated with two fields of TGN containing numerous GGA2-positive, clathrin-coated tubular and vesicular profiles. Some gold particles are located on noncoated membranes (arrowheads). The TGN in (B) contains several profiles labeled for both GGA2 and AP-1. The arrows in (B) to (D) point to double-labeled buds at TGN profiles. Magnification in (A) and (B) is 66,000×; in (C) and (D), 57,000×.

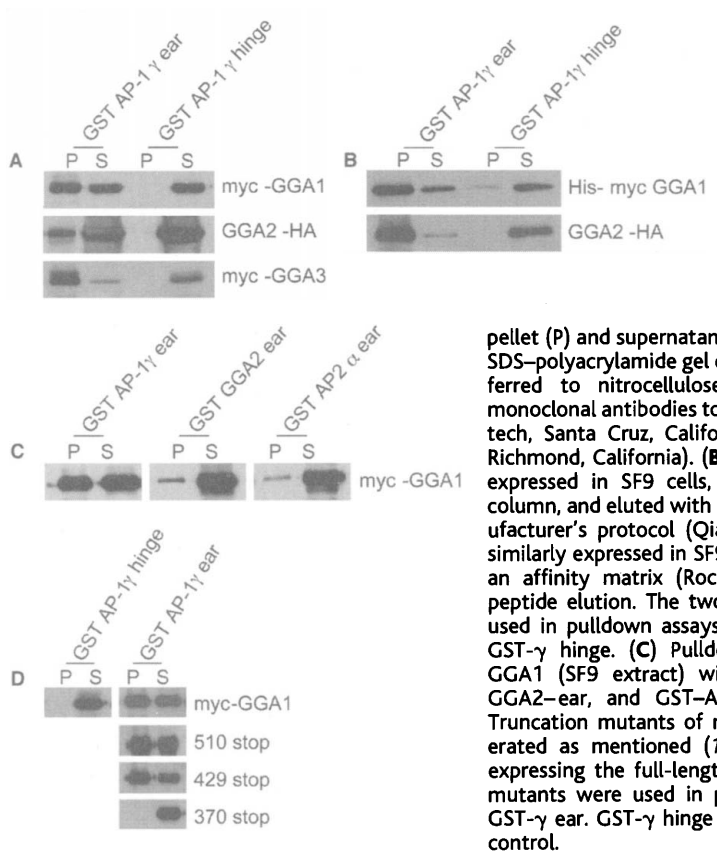

Fig. 2. Binding of GGAs to AP-1–γ ear. (**A**) Pulldown assays were done with SF9 cell extracts containing GGAs 1 through 3, using GST-γ ear and GST-γ hinge of AP-1 as described (*14*, *27*). An aliquot of each GST-fusion protein pellet (P) and supernatant (S) was subjected to SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with monoclonal antibodies to myc (Santa Cruz Biotech, Santa Cruz, California) or HA (BabCO, Richmond, California). (**B**) His-myc-GGA1 was expressed in SF9 cells, purified on a nickel column, and eluted with imidazole as per manufacturer's protocol (Qiagen). GGA2-HA was similarly expressed in SF9 cells and purified on an affinity matrix (Roche), followed by HA peptide elution. The two purified GGAs were used in pulldown assays with GST-γ ear and GST-γ hinge. (**C**) Pulldown assays of myc-GGA1 (SF9 extract) with GST-γ ear, GST-GGA2–ear, and GST–AP-2–α ear (*27*). (**D**) Truncation mutants of myc-GGA1 were generated as mentioned (*14*). SF9 cell extracts expressing the full-length wild type (wt) and mutants were used in pulldown assays with GST-γ ear. GST-γ hinge was used as negative control.

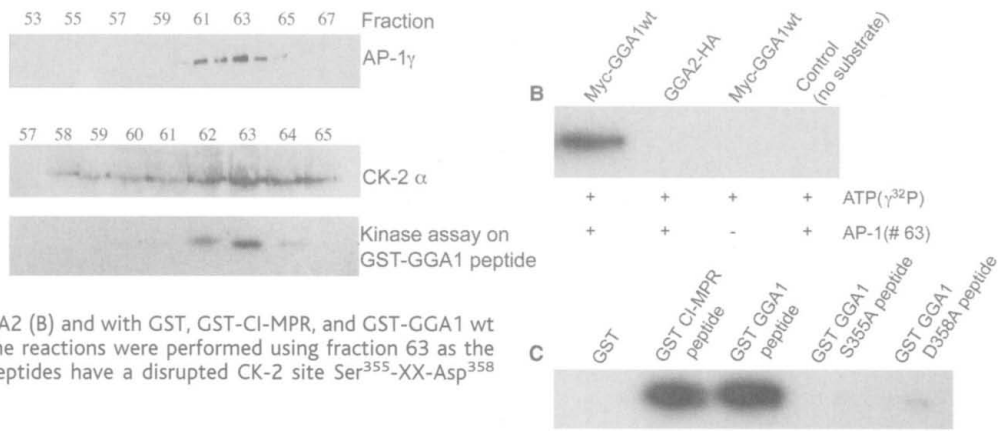

Fig. 3. AP-1 has an associated CK-2 that phosphorylates GGA1. (**A**) Bovine liver CCVs were prepared and extracted with tris as described (*15*). The AP-1 was immunoisolated from the tris extract (*28*), and the purified AP-1 was subjected to gel filtration on a superose 6 column. The fractions were analyzed for AP-1 and CK-2 content by Western blots probed with antibodies to AP-1 γ and CK-2 α and probed for kinase activity (*14*). (**B** and **C**) In vitro kinase reactions with GGA1 and GGA2 (B) and with GST, GST-CI-MPR, and GST-GGA1 wt and mutant peptides (C) as acceptors. The reactions were performed using fraction 63 as the source of enzyme. GST-GGA1 mutant peptides have a disrupted CK-2 site Ser^{355}-XX-Asp^{358} (X = any amino acid) (*27*).

Table 2. Colocalization of GGA2 and AP-1. To establish the degree of colocalization between GGA2 and AP-1, we categorized labeled membrane profiles in the same micrographs as positive for either GGA2 or AP-1 or for both. The membrane profiles were subdivided into noncoated and coated membranes, the latter including membranes with stretches of clathrin lattices, clathrin-coated buds, and CCVs.

Labeled profiles	GGA2 only	AP-1 only	GGA2 and AP-1
Noncoated membranes	68%	30%	2%
Coated membranes	25%	45%	30%
Identifiable coated buds	31%	28%	41%
Total profiles	349	349	117

Table 3. Distribution of wild-type (CC2) and mutant (D158A) CI-MPR and AP-1. CI-MPR gold was quantified at TGN CCVs in cryosections of L cells expressing wild-type (CC2) or mutant (D158A) CI-MPR. In CC2 and in D158A cells, 24 and 23 Golgis were photographed at 20,000×, respectively (*29*). Numbers in parentheses indicate portion of the type of CCV as a percentage of the total.

	CC2	D158A
Total CCVs	277	218
CI-MPR–positive CCVs	191 (69%)	37 (17%)
CI-MPR gold per CCV	1.2	0.3
CI-MPR– and AP-1–positive CCVs	163 (59%)	32 (14.7%)

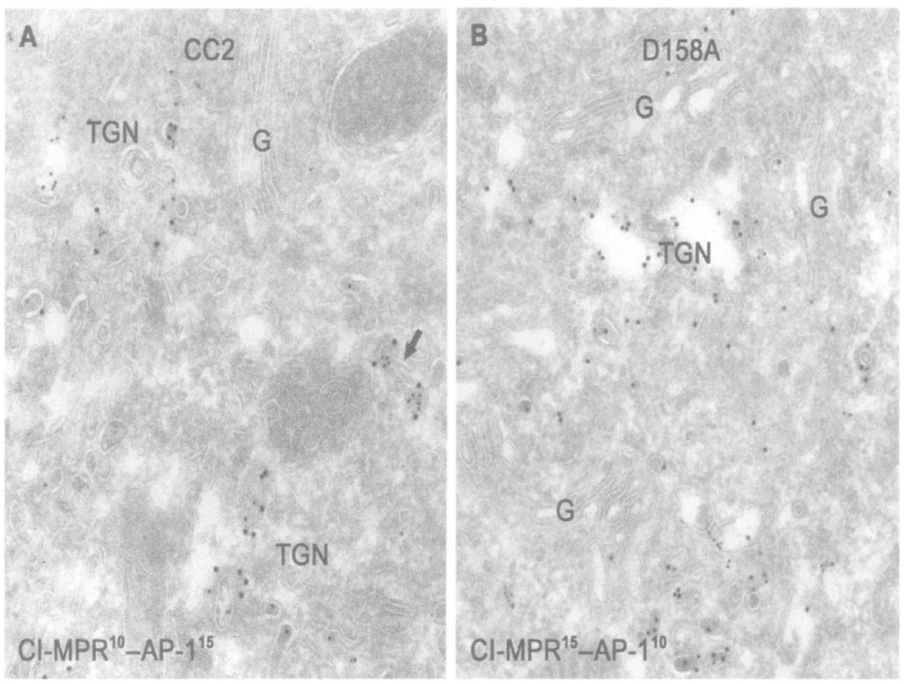

Fig. 4. CI-MPR and AP-1 localization at the TGN. Cryosections of L cells expressing wt CI-MPR (CC2) and mutant CI-MPR (D158A) were immunogold double-labeled for CI-MPR and AP-1 with gold sizes as indicated (*24*). (**A**) Fields of TGN adjacent to a Golgi stack (G) showing that CI-MPR and AP-1 colocalize in CCVs and a clathrin-coated bud (arrow) at the TGN. Magnification, 43,000×. (**B**) In contrast to (A), the mutant CI-MPR showed little colocalization with AP-1 but was found in noncoated TGN profiles near the Golgi. Magnification, 37,000×.

cargo molecules for incorporation into AP-1–CCVs at the TGN. This is similar to the role of phosphofurin acidic cluster–sorting protein 1 in presenting the MPRs and furin to AP-1 for packaging into CCVs in endosomes (*16*). In addition, it has recently been reported that a number of proteins, including AP180, epsin, β-arrestin, huntingtin-interacting protein 1 (HIP1), and disabled-2 (Dab2), function to recruit specific cargo molecules to forming AP-2–CCVs at the plasma membrane (*17*–*21*). Thus, cargo-specific adaptor proteins are implicated in protein sorting at multiple sites of the exocytic and endocytic pathways. In the case of GGA1 and GGA3, we suggest that cargo release might be initiated by an AP-1–associated CK-2 that phosphorylates them, resulting in autoinhibition. GGA2 is not subject to this form of regulation and therefore may release its cargo by another mechanism. Although these data indicate that the GGAs serve as connector proteins, they do not exclude the possibility that the GGAs also nucleate their own vesicles.

References and Notes

1. S. Kornfeld, *Annu. Rev. Biochem.* **61**, 307 (1992).
2. J. Klumperman *et al.*, *J. Cell Biol.* **121**, 997 (1993).
3. R. Puertollano, R. C. Aguilar, I. Gorshkova, R. J. Crouch, J. S. Bonifacino, *Science* **292**, 1712 (2001).
4. Y. Zhu, B. Doray, A. Poussu, V.-P. Lehto, S. Kornfeld, *Science* **292**, 1716 (2001).
5. H. Takatsu, Y. Katoh, Y. Shiba, K. Nakayama, *J. Biol. Chem.* **276**, 28541 (2001).
6. E. C. Dell'Angelica *et al.*, *J. Cell Biol.* **149**, 81 (2000).
7. R. Puertollano, P. A. Randazzo, J. F. Presley, L. M. Hartnell, J. S. Bonifacino, *Cell* **105**, 93 (2001).
8. S. Misra, R. Puertollano, Y. Kato, J. S. Bonifacino, J. H. Hurley, *Nature* **415**, 933 (2002).
9. T. Shiba *et al.*, *Nature*, **415**, 937 (2002).
10. H. J. Chen, J. Yuan, P. Lobel, *J. Biol. Chem.* **272**, 7003 (1997).
11. S. Höning, M. Sosa, A. Hille-Rehfeld, K. von Figura, *J. Biol. Chem.* **272**, 19884 (1997).
12. M. W. Black, H. R. Pelham, *J. Cell Biol.* **151**, 587 (2000).
13. J. Hirst *et al.*, *J. Cell Biol.* **149**, 67 (2000).

14. B. Doray, K. Bruns, P. Ghosh, S. Kornfeld, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 8072 (2002).
15. S. Meresse, T. Ludwig, R. Frank, B. Hoflack, *J. Biol. Chem.* **265**, 18833 (1990).
16. L. Wan *et al.*, *Cell* **94**, 205 (1998).
17. W. Hao, Z. Luo, L. Zheng, K. Prasad, E. M. Lafer, *J. Biol. Chem.* **274**, 22785 (1999).
18. A. Benmerah *et al.*, *J. Cell Biol.* **140**, 1055 (1998).
19. S. A. Laporte, R. H. Oakley, J. A. Holt, L. S. Barak, M. G. Caron, *J. Biol. Chem.* **275**, 23120 (2000).
20. S. K. Mishra *et al.*, *J. Biol. Chem.* **276**, 46230 (2001).
21. S. K. Mishra *et al.*, *EMBO J.*, in press.
22. B. Doray, S. Kornfeld, *Mol. Biol. Cell* **12**, 1925 (2001).
23. L. H. Wang, T. C. Südhof, R. G. Anderson, *J. Biol. Chem.* **270**, 10079 (1995).
24. Mouse L cells (LSS) stably expressing GGA2-hemagglutinin (HA) were grown in α–minimum essential medium (MEM) with 10% fetal bovine serum (FBS), and HeLa cells stably expressing myc-GGA1 were maintained in Dulbecco's minimum essential medium (DMEM) with 10% FBS. They were treated with 5 mM sodium butyrate for 8 hours before fixation for immuno-electron microscopy. The wild-type and D158A mutant bovine CI-MPR were expressed in receptor-negative mouse D9 cells (*10*).
25. H. J. Geuze, J. W. Slot, P. A. van der Ley, R. C. T. Scheffer, *J. Cell Biol.* **89**, 653 (1981).
26. G. Raposo, M. J. Kleijmeer, G. Posthuma, J. W. Slot, H. J. Geuze, in *Weir's Handbook of Experimental Immunology*, L. A. Herzenberg, D. M. Weir, L. A. Herzenberg, C. Blackwell, Eds. (Blackwell Science, Malden, MA, ed. 5, 1997), pp. 208.1–208.11.
27. GST fusions of γ-adaptin ear (residues 703 to 822) and γ-adaptin hinge (residues 595 to 683) were expressed and purified as described (*22*). GST-GGA2 ear (residues 473 to 613) was prepared as reported (*4*). The construct encoding murine GST–AP-2–α ear (residues 701 to 938) was provided by R. G. W. Anderson (*23*). For the in vitro phosphorylation assays, GST fusion peptides of GGA1 hinge (residues 342 to 367, inclusive of the sequence Ser^{355}-Leu-Leu-Asp^{358}) and the cytoplasmic tail of the bovine CI-MPR were expressed and purified as described (*14*). The mutagenesis of the GST-GGA1 peptide fusion (S355A, D358A) was performed using primers incorporating the desired mutations with the QuickChange system.
28. Y. Zhu, L. M. Traub, S. Kornfeld, *Mol. Biol. Cell* **9**, 1323 (1998).
29. Affinity-purified rabbit polyclonal antibody to CI-MPR was obtained from W. Gregory.
30. We thank H. Bai for the construct His-myc-GGA1 and L. Traub, D. Ory, K. Kornfeld, and R. Kornfeld for critical reading of the manuscript. Supported in part by NIH grant RO1 CA-08759 to S.K.

21 June 2002; accepted 29 July 2002

Inhibition of Retroviral RNA Production by ZAP, a CCCH-Type Zinc Finger Protein

Guangxia Gao,[1,2] Xuemin Guo,[2] Stephen P. Goff[1]*

Cells have evolved multiple mechanisms to inhibit viral replication. To identify previously unknown antiviral activities, we screened mammalian complementary DNA (cDNA) libraries for genes that prevent infection by a genetically marked retrovirus. Virus-resistant cells were selected from pools of transduced clones, and an active antiviral cDNA was recovered. The gene encodes a CCCH-type zinc finger protein designated ZAP. Expression of the gene caused a profound and specific loss of viral messenger RNAs (mRNAs) from the cytoplasm without affecting the levels of nuclear mRNAs. The finding suggests the existence of a previously unknown machinery for the inhibition of virus replication, targeting a step in viral gene expression.

Vertebrate cells have evolved many defense mechanisms to prevent or inhibit viral replication after an infection (*1*). A remarkable array of such antiviral proteins is induced by interferon (*2*), including a double-stranded RNA-dependent protein kinase (PKR) that phosphorylates eIF-2α and inhibits translation (*3*); the Mx proteins, which are guanosine triphosphatases that block viral gene expression (*4*); and oligoA synthetases (*5*, *6*) that activate ribonuclease (RNase) L to degrade mRNAs and ribosomal RNAs (rRNAs). In some cases, the antiviral state involves a drastic shutoff of host functions; in other cases, there is a more selective block of viral replication or gene expression. Although many parallel pathways have been uncovered, it is likely that more antiviral proteins remain to be found (*7*).

To identify previously unknown antiviral proteins, we conducted a directed search for genes that would block infection by a genetically marked retroviral genome. A library of expressed cDNAs was constructed in a retroviral vector, termed pBabe-HAZ, with several key features (Fig. 1A). Randomly primed cDNAs from wild-type Rat2 fibroblasts were inserted into the vector under the control of the constitutive SV40 early gene promoter, with a hemagglutinin (HA) epitope tag fused at their 5′ ends and a Zeocin resistance gene at the 3′ ends (*8*). A LoxP sequence, recognized by the Cre recombinase, was inserted into the U3 region of the 3′ long terminal repeat (LTR). During reverse transcription of the vector, this region becomes duplicated, generating LoxP sites in both LTRs of the integrated provirus and so allowing efficient excision of the provirus from the genome by the Cre recombinase. The complexity of the library was 2×10^7, with inserts ranging from 0.2 to 3.5 kb.

A strategy was developed to select and recover antiviral genes in the library (*8*) (fig. S1). Aliquots of the library DNAs were used to transform 293T cells, along with DNAs encoding the Moloney MuLV Gag-Pol and VSV G envelope proteins, producing 20 pools of transducing viruses. These pools collectively carry most of the expressed genes of Rat2 cells. The transducing viruses were used to infect thymidine kinase–negative (TK–) Rat2 cells, and recipient clones were selected by culture in zeocin. Thus, each clone in the pooled cells overexpressed a single member of the cDNA library.

To identify genes in the pooled cells that conferred retrovirus resistance, we used a powerful selection for virus-resistant cells (*9*) (fig. S2). The pools of transduced TK– Rat2 cells were challenged by repeated infection with both ecotropic and amphotropic retroviruses expressing the TK gene driven by the viral promoter, so as to transduce as many of the susceptible cells as possible. Most of the virus-sensitive cells, having now become TK-positive, were then killed by growth in the toxic thymidine analogue trifluorothymidine (TFT), and the rare TK-negative clones were recovered as candidate virus-resistant cells. Out of 5×10^5 transduced lines put through the selection, 200 TFT-resistant clones were isolated. Retesting showed that one line, L1D3, was dramatically resistant to

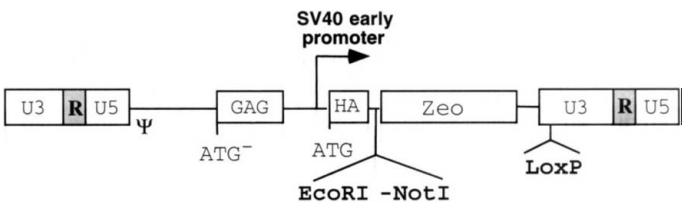

Fig. 1. Schematic representation of the pBabe-HAZ construct. Ψ, MuLV viral RNA packaging signal; Eco RI–Not I, linker sequence containing Eco RI and Not I sites; LoxP, LoxP sequence for recognition by Cre recombinase.

[1]Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University, College of Physicians and Surgeons, 701 West 168th Street, New York, NY 10032, USA. [2]Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China.

*To whom correspondence should be addressed. E-mail: goff@cancercenter.columbia.edu