separately at 50°C for 10 min in reaction buffer [50 mM Hepes (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$], equilibrated to 37°C for 2 min, and then mixed at 37°C to start the cleavage reactions. Portions of the reaction mixtures were removed at various time points, added to a stop solution (10 mM EDTA), and the products separated on a 15% polyacrylamide gel in 8 M urea. With 500 nM ribozyme and 25 nM substrate, the half-time ($t_{1/2}$) for the reactions with either Hamβ1G or Hamβ2G was ~30 min. This $t_{1/2}$ value is approximately that expected for the conditions used (*5, 21*). No cleavage was observed with the Hamβ1D ribozyme.

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13. Plasmids encoding the N2A:Hamβ1G, N2A:Hamβ2G, N2A:Hamβ1D, and N2A retroviral vectors were introduced into the amphotropic packaging cell line AM12 (*22*) by electroporation with a Bio-Rad Gene Pulser. Stably transduced cells were selected with G418 (0.7 mg/ml), and the G418-resistant colonies were pooled. We determined the titer of *neo*^{r} virus in the supernatants from these cells by infecting NIH 3T3 cells with serial dilutions of these supernatants and then selecting with G418.
14. B. A. Sullenger, unpublished results.
15. The E86/B2A cells (10^4) infected with the various retroviral vectors were expanded in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum without G418 selection. In a separate experiment, selection with G418 further reduced the titer of β-gal virus released from active hammerhead-containing cells by a few percent, but had no effect on β-gal activity within the cells or on the titer of *neo*^{r} virus released.
16. The β-gal assay values obtained from infected E86/B2A cells were as follows: 160 U (1000 × A_{420}/hour) (N2A:Hamβ1D), 290 U (N2A), 205 U (N2A:Hamβ1G), and 220 U (N2A:Hamβ2G), where A_{420} is the absorbance at 420 nm. Untransduced E86 cells (*8*) were assayed simultaneously and were used to zero the A_{420} readings. The *neo*^{r} viral titers (per milliliter) were as follows: 6.5×10^4 (N2A:Hamβ1D), 7.0×10^4 (N2A), 6.0×10^4 (N2A:Hamβ1G), and 9.0×10^4 (N2A:Hamβ2G). The β-gal viral titers (per milliliter) were as follows: 8.1×10^3 (N2A:Hamβ1D), 6.0×10^3 (N2A), 6.5×10^2 (N2A:Hamβ1G), and 8.0×10^2 (N2A:Hamβ2G). The β-gal viral titer of the parental E86/B2A cell line was 9.0×10^3 per milliliter.
17. E86/B2A cells (10^4) were infected with N2A:Hamβ1G and N2A:Hamβ1D viruses at an MOI of 10 and expanded to 10^8. The cell medium was changed and was removed 24 hours later. Virion RNA was isolated as in (*23*). Total RNA was isolated from the cells by the guanidinium isothiocyanate method (*24*). Viral supernatants (1 ml) were used to infect NIH 3T3 cells (10^4) for 3 hours in the presence of polybrene (8 μg/ml). These cells were expanded to 10^8 and total RNA was isolated. RNase protection analysis was performed on 50 μg of packaging cell and infected cell RNAs and on 20% of the isolated virion RNAs with an RPA II ribonuclease protection assay kit (Ambion). Body-labeled RNA probes were transcribed by T7 RNA polymerase from templates generated by the polymerase chain reaction (PCR). The B2A and N2A:Hamβ1G plasmids were amplified with PCR primers specific for the *lacZ* and *neo*^{r} genes as described in the RPA II kit. The *lacZ* probe (190 nt) was complementary to 181 nt of RNA flanking the cleavage site for Hamβ1G on B2A transcripts. The *neo*^{r} probe (150 nt) was complementary to 140 nt of N2A:Hamβ1 RNA.
18. To determine the ratio of B2A and N2A:Hamβ1 RNAs, we added *lacZ* and *neo*^{r} RNA probes (6×10^4 cpm of each) to the samples and performed RNase protection analysis as above (*17*). Mixing the probes controlled for any differences in RNA loading. Products were quantified with an Image Acquisition and Analysis System (AMBIS, Inc., San Diego, CA) and with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). An internally controlled PCR approach confirmed the decrease in B2A retroviral RNA in infected NIH 3T3 cells; the substrate escape ratio was reproducibly between 0.09 and 0.14.
19. In this experimental system, we cannot directly test whether the presence of the packaging signal on the ribozyme-encoding transcripts is essential for inhibition of B2A titer, because it cannot be deleted from the N2A:Hamβ vectors if one is using retroviral vector–mediated gene transfer for introduction of N2A:Hamβ vectors into E86/B2A cells. Therefore, we cannot rule out the possibility that the packaging pathway is intrinsically more susceptible to inhibition than the mRNA pathway. For example, differences in ionic conditions or RNA-binding proteins associated with these two pathways may influence ribozyme cleavage activity or target accessibility.
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Activation of Exocytosis by the Heterotrimeric G Protein G_{i3}

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Secretagogues of rat peritoneal mast cells, such as mastoparan and compound 48/80, induce mast cell exocytosis by activating directly the guanosine triphosphate–binding proteins that are required for exocytosis. The introduction of a synthetic peptide that corresponds to the carboxyl-terminal end sequence of $G\alpha_{i3}$ into the cells specifically blocked this secretion. Similar results were obtained when antibodies to this peptide were introduced. The $G\alpha_{i3}$ was located in both the Golgi and the plasma membrane, but only the latter source of $G\alpha_{i3}$ appeared to be essential for secretion. These results indicate that $G\alpha_{i3}$ functions to control regulated exocytosis in mast cells.

The nonhydrolyzable analog of guanosine triphosphate (GTP), guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), when introduced into patch-clamped (*1*) or streptolysin O (SLO)–permeabilized mast cells (*2, 3*), stimulates exocytosis independently of phospholipase C (PLC). This suggests that a GTP-binding protein, designated G_E, may act downstream of PLC in the control of regulated secretion (*4*). However, whereas both small GTP-binding proteins of the Ras (*5*) and Rab (*6*) families as well as heterotrimeric G proteins (*7*) have been implicated in exocytosis, the identity of G_E has remained obscure. Certain positively charged secretagogues of rat peritoneal mast cells, including mastoparan (*8*), substance P (*9*), compound 48/80 (*7*), neomycin (*10*), and a variety of kinins (*11*), induce exocytosis in a receptor-independent manner by interacting directly with heterotrimeric G proteins. Although they activate phosphoinositide metabolism, these secretagogues can also induce exocytosis independently of PLC, presumably by directly activating G_E (*10*). The finding that treatment with pertussis toxin (Ptx) inhibits exocytosis under these conditions indicated that G_E is a Ptx-sensitive heterotrimeric G protein (*10*). Therefore, we analyzed the Ptx-sensitive G proteins present in mast cells to identify the G protein that fulfills the functional role of G_E in these cells.

Mast cell proteins were adenosine diphosphate (ADP)–ribosylated by Ptx and separated by both two-dimensional gel electrophoresis and SDS–polyacrylamide gel electrophoresis (PAGE) in the presence of

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8 M urea, a condition that allows the separation of various α subunits of G proteins (*12*). Both methods revealed the presence of two Ptx substrates (Fig. 1A). These two substrates were identified by immunoblot analysis as $G\alpha_{i2}$ and $G\alpha_{i3}$ (Fig. 1B). Neither $G\alpha_{i1}$ nor $G\alpha_o$ could be detected in mast cells, whereas they were clearly detected in rat brain membranes (Fig. 1B).

Activation of G proteins by the positively charged secretagogues requires the insertion of the hydrophobic moiety of the activating molecule [α helices of the peptides (*13*) or the aromatic rings of compound 48/80 (*14*)] into the membrane and the interaction of the positively charged domain of the molecule with the COOH-terminus of the G protein α subunit (*13, 15*). This interaction facilitates nucleotide exchange, by a mechanism analogous to that of G protein–coupled receptors. Therefore, to determine whether $G\alpha_{i2}$ or $G\alpha_{i3}$ fulfills the function of G_E, we introduced synthetic peptides that correspond to either the COOH-terminal sequences of α subunits or antibodies to these peptides into permeabilized cells. Such peptides or antibodies prevent coupling of G proteins to their respective receptors (*16–19*).

The effects of COOH-terminal peptides from $G\alpha_{i2}$ or $G\alpha_{i3}$ on mast cell exocytosis were tested. We used the free-acid form of adenosine triphosphate (ATP), ATP^{4-}, as the permeabilizing ligand because this technique allows the reversible permeabilization of the cells without loss of their responsiveness to external agonists (*20*). Permeabilized cells were exposed to various concentrations of the synthetic peptides KE and EC (*21*), resealed by the addition of Mg^{2+} (*20*), and stimulated with 48/80 in the absence of external Ca^{2+}. The KE peptide at concentrations of up to 100 μg/ml had no effect on histamine secretion induced by 48/80 (Fig. 2A). In contrast, the EC peptide inhibited secretion, with 50% inhibition at 80 μg/ml and maximal inhibition at 200 μg/ml. Both peptides inhibited release when introduced at concentrations greater than 200 μg/ml (*22*); at these high concentrations specificity may be lost because of the 70% identity between these peptides. An irrelevant peptide of a similar size (CPAGILNKLV) did not inhibit secretion at a similar range of concentrations (*22*).

The EC peptide was ineffective when added to intact cells (*22*), indicating that the target for the peptide was intracellular. Moreover, the EC peptide appeared to act by direct competition with the G_E protein in permeabilized mast cells exposed to both EC and neomycin (1 mM) to block phosphoinositide metabolism (*7*). Although neomycin by itself did not trigger exocytosis, the application of 48/80 to the cells containing neomycin induced histamine secretion that was completely inhibited by the EC peptide (Fig. 2B). Thus, $G\alpha_{i3}$ appears to mediate PLC-independent exocytosis in response to 48/80.

Secretion was also induced by AlF_4^-, which activates heterotrimeric but not small GTP-binding proteins (*23*), and by GTP-γ-S (Fig. 3A). When the cells were permeabilized with SLO, under conditions in which PLC is not involved (*24*) and Ca^{2+} is required (*2*), EGTA inhibited exocytosis induced by both types of stimuli (Fig. 3A). That is, secretion required the presence of Ca^{2+} that is presumed to mod-

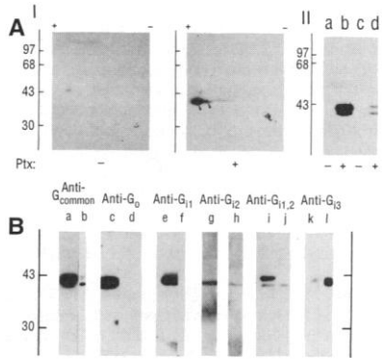

Fig. 1. Analysis of Ptx substrates in mast cells. (**A**) Purified mast cells (*7*) were solubilized in a lysis buffer containing 1% Triton X-100. The soluble mast cell proteins were subjected to ADP-ribosylation in the absence or presence of Ptx (*7*). On separation by two-dimensional gel electrophoresis (*29*), a 45-kD protein was labeled in the absence of Ptx (panel I). Ptx catalyzed the ADP-ribosylation of two additional proteins (panel I). Separation of the ADP-ribosylated proteins by SDS-PAGE (9% gels) in the presence of 8 M urea revealed no substrates in the absence of the toxin (panel II, lanes a and c). Ptx catalyzed ADP-ribosylation of two proteins (39 and 42 kD) in mast cell extract (panel II, lane d) and ADP-ribosylation of at least two proteins in rat brain membranes (panel II, lane b). (**B**) Solubilized mast cell proteins (75 μg per lane, lanes b, d, f, h, j, and l); rat brain membranes (100 μg per lane, lanes a, c, and i); a mixture of G_i and G_o, purified from rat brain membranes (4 μg per lane, lanes e and g); and an extract of HL-60 membranes (150 μg per lane, lane k) were separated by SDS-PAGE with 8 M urea, transferred to nitrocellulose membranes, and analyzed with the indicated antibodies to G proteins (*30*).

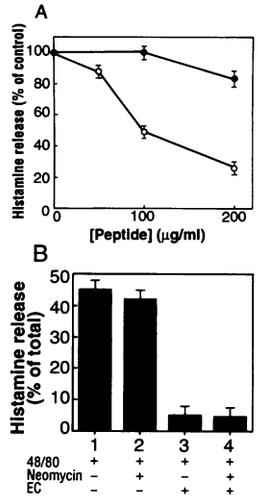

Fig. 2. Effect of EC and KE peptides on 48/80-induced histamine release. Purified mast cells were permeabilized by ATP (*31*) in the presence of the indicated concentrations of the KE (●) or EC (○) peptides (**A**) or in the presence or absence of EC peptide (250 μg/ml) and neomycin (1 mM) (**B**). The cells were subsequently resealed and treated with 48/80 (5 μg/ml). The reactions were quenched and the amount of secreted histamine was measured. Values in (A) are presented as percentage of the release measured in the absence of peptides which varied between 40 to 80% of total histamine. Each experimental point represents the mean of duplicate samples of a representative experiment that was repeated with similar results at least three times.

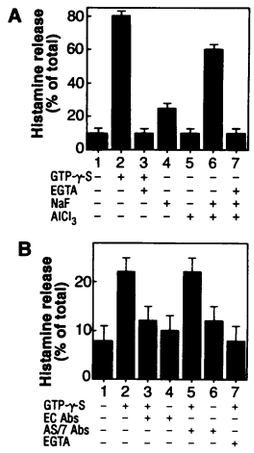

Fig. 3. Histamine release from SLO-permeabilized cells. (**A**) Purified mast cells were permeabilized by SLO in the presence of 100 μM GTP-γ-S (lanes 2 and 3); NaF (30 mM, lanes 4, 6, and 7); $AlCl_3$ (50 μM, lanes 5, 6, and 7); and EGTA (1 mM, lanes 3 and 7). (**B**) For the introduction of antibodies, cells were permeabilized for 10 min at 37°C in the presence of either AS/7 (33.5 μg/ml, lanes 5 and 6) or EC (33.5 μg/ml, lanes 3 and 4) antibodies before they were stimulated with GTP-γ-S (100 μM, lanes 2, 3, 5, and 7) or with GTP-γ-S (100 μM) and EGTA (1 mM, lane 7). After further incubation for 20 min, the reactions were quenched and the amount of secreted histamine was measured. For permeabilization, purified mast cells were incubated for 20 min at 37°C with reduced SLO (0.4 IU/ml, Wellcome Diagnostics) in a buffer containing 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, bovine serum albumin (BSA) (1 mg/ml), and 20 mM Hepes (pH 7.2).

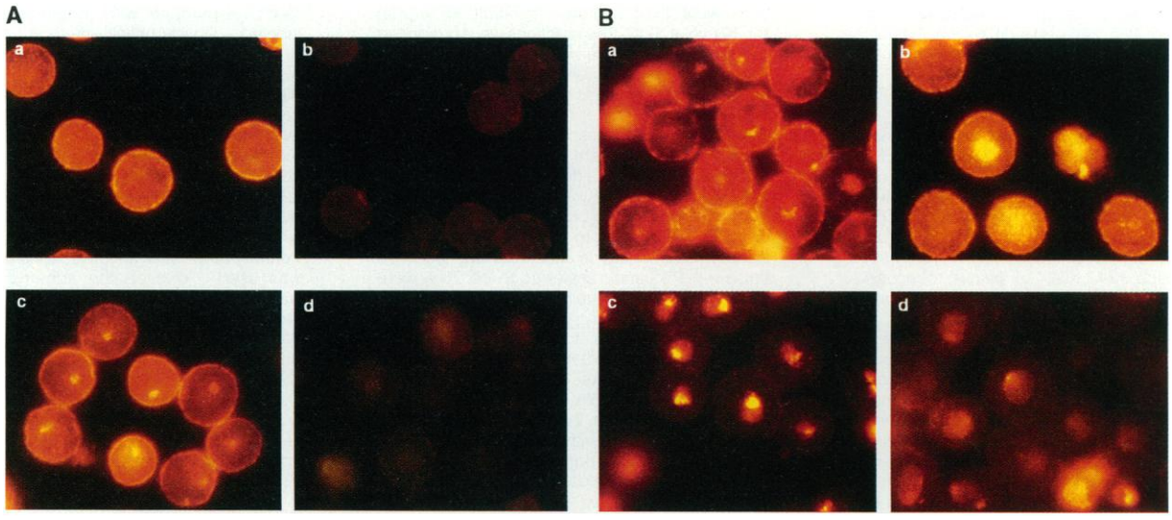

Fig. 4. Subcellular localization of $G\alpha_{i2}$ and $G\alpha_{i3}$ in rat peritoneal mast cells. (**A**) Purified mast cells were incubated with either affinity-purified AS/7 (33.5 μg/ml, a) or affinity-purified EC (50 μg/ml, c). To show specificity of staining, we also incubated the cells with antibodies that had been incubated with the respective immunizing peptide (100 μg/ml, b and d, respectively). (**B**) Staining of control (a and c) or BFA-treated (b and d) cells with EC (a and b) and with antibodies to the Golgi marker mannosidase II (c and d). Staining was done with lissamine rhodamine–conjugated antibodies (*32*).

ulate the activity of the G_E protein by binding to a Ca^{2+}-binding protein (C_E) with characteristics of an intracellular pseudoreceptor (*3*, *4*). Permeabilization with SLO, unlike permeabilization with ATP^{4-}, allowed the introduction of the antibodies AS/7 and EC that specifically bind to the COOH-terminal ends of $G\alpha_{i2}$ and $G\alpha_{i3}$, respectively. Of these two antibodies, only EC inhibited GTP-γ-S–induced release to the same extent as did EGTA (Fig. 3B). These results indicate that activation of G_E is eliminated either by preventing the activation of C_E with EGTA or by blocking the interaction between activated C_E and G_E with EC antibodies. This finding further supports the notion that the putative C_E protein activates $G\alpha_{i3}$ in a receptor-like manner (*3*).

The protein G_{i3} exists exclusively in the Golgi compartment in LLC-PK1 cells, where it regulates intra- and trans-Golgi trafficking (*25*). Therefore, we studied the subcellular localization of G_{i2} and G_{i3} in mast cells. Whereas the $G\alpha_{i2}$-specific AS/7 antibodies were localized to plasma membrane exclusively (Fig. 4A), the $G\alpha_{i3}$-specific EC antibodies stained both the plasma and the Golgi membranes (Fig. 4A). The latter membrane was also stained by antibodies to the Golgi marker mannosidase II (Fig. 4B). Hence, in mast cells G_{i3} would be accessible to interaction with secretagogues that insert into the plasma membrane.

The probable participation of both G_{i3} and members of the Rab family (*6*) in the late stages of exocytosis in mast cells indicates that there are similarities between regulated and constitutive secretion. However, the fungal drug brefeldin A (BFA), which disrupts the Golgi by influencing the small molecular size GTP-binding protein ARF (*26*), had no effect on secretion induced by 48/80 (*22*). BFA abolished the staining of the Golgi membrane with antibody to mannosidase II or EC, whereas staining of the plasma membrane with EC was not affected (Fig. 4B). Therefore, Golgi-bound G_{i3} may not participate in the control of regulated secretion. Furthermore, unlike Golgi-bound G_{i3} which inhibits constitutive secretion (*25*), the plasma membrane–bound form of G_{i3} appears to facilitate regulated exocytosis. Similarly, peptides that correspond to the effector domain of the Rab3a protein stimulate the exocytotic process (*6*), whereas they inhibit transport from the endoplasmic reticulum to Golgi (*27*). These differences might be related to the multicycle nature of the constitutive Golgi trafficking as compared with the single fusion event that occurs during the process of regulated exocytosis.

In conclusion, our data suggest that like constitutive secretion regulated exocytosis is regulated by at least one heterotrimeric G protein (G_{i3}) and at least one small GTP-binding protein of the Rab family. These GTP-binding proteins seem to communicate through a yet undefined mechanism to evoke membrane fusion and exocytosis in a Ca^{2+}-independent manner.

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21. The synthetic decapeptide KE (KENLKDCGLF) (*28*) replicates the COOH-terminal end of the α subunit of transducin and has 90% identity to $G\alpha_{i2}$. The EC peptide (KNNLKECGLY) corresponds to the COOH-terminal end of $G\alpha_{i3}$.
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28. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
29. The 2D gel electrophoresis was done as de-

scribed [L. M. Traub and R. Sagi-Eisenberg, *J. Biol. Chem.* **266**, 24642 (1991)] except that for the first dimension, 3.5% polyacrylamide rods containing 2% ampholytes (Pharmalyte 3–10 and 5–8, 1:4) were used and focusing was for 12,000 *V*·hours.

30. Antibodies used included affinity-purified AS/8 antibody to the α common peptide: CGAGESGKSTIVKQM (anti-G_{common}); AS/6 antibody to the $G\alpha_o$ peptide: CNLKEDGISAAKDVK (anti-G_o); AS119 antibody to the $G\alpha_{i1}$ peptide: LDRIAQPNYI (anti-G_{i1}); AS/64 antibody to the $G\alpha_{i2}$ peptide: CTGANKYDEAAS (anti-G_{i2}); AS/7 antibody to the $G\alpha_1$ peptide: KENLKDCGLF (known to recognize both $G\alpha_{i1}$ and $G\alpha_{i2}$) (anti-$G_{i1,2}$); EC antibody to the $G\alpha_{i3}$ peptide: KNNLKECGLY (anti-G_{i3}).
31. Permeabilization was done in the absence of divalent cations in a buffer (buffer A) containing 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, bovine serum albumin (BSA) (1 mg/ml), and 20 mM Hepes (pH 7.5). The cells were permeabilized for 6 min at 30°C with 50 μM ATP in the presence of EGTA (15 μM). The cells were resealed by the addition of 3 mM $MgCl_2$ for 10 min, transferred and diluted in new tubes containing buffer A, and incubated for 10 min. The cells were then treated with 48/80 (5 μg/ml) for 20 min at 37°C. The reactions were quenched by the addition of ice-cold buffer without BSA. The cells were sedimented and the amount of secreted histamine was measured as described (*7*).
32. Purified mast cells suspended in buffer A (*31*) were allowed to adhere to glass cover slips for 90 min at 37°C under a humidified atmosphere of 93% air and 7% CO_2. The cells were washed twice with phosphate-buffered saline (PBS) and fixed for 30 min at room temperature in 3% paraformaldehyde. The cells were washed three times with PBS and transferred to ice. They were subsequently permeabilized on ice for 3 min with a buffer containing 100 mM NaCl, 300 mM sucrose, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Pipes (pH 7.0), and 0.5% Triton X-100. After one wash with PBS and two washes with 0.05% Tween in PBS (Tween-PBS), the cells were incubated for 30 min at room temperature with affinity-purified AS/7 (33.5 μg/ml), affinity-purified EC (50 μg/ml), or antiserum to mannosidase II (1:200) diluted in Tween-PBS. After three washes with Tween-PBS buffer, the cells were incubated for 30 min at room temperature with lissamine rhodamine–labeled antibody [affinity-purified goat antibody to rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA), 1:40 dilution in Tween-PBS buffer]. Subsequently, the cells were washed once with Tween-PBS and twice with PBS. The cells were viewed on a Zeiss Axioskop microscope with a 100× Plan-Neofluar objective and photographed on Fujichrome 3200 film. For peptide inhibition, each antibody was incubated for 2 hours at room temperature with the respective peptide (100 μg/ml). For treatment with BFA, a similar labeling procedure was used. However, the adhered cells were washed and treated with BFA (10 μg/ml) for 10 min in PBS at 37°C before fixation.
33. We thank Y. Zick and J. R. Gillette for helpful discussions and a critical reading of this manuscript and A. M. Spiegel, G. Schultz, and J. G. Donaldson for their generous gift of antibodies. Supported by grants from the Israel Cancer Research Fund (R.S.E.), the Israel Academy of Sciences (R.S.E.), and the Israel Cancer Association (R.S.E.).

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A Link Between Cyclin A Expression and Adhesion-Dependent Cell Cycle Progression

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Cell adhesion has an essential role in regulating proliferation during the G_1 phase of the cell cycle, and loss of this adhesion requirement is a classic feature of oncogenic transformation. The appearance of cyclin A messenger RNA and protein in late G_1 was dependent on cell adhesion in both NRK and NIH 3T3 fibroblasts. In contrast, the expression of Cdc2, Cdk2, cyclin D1, and cyclin E was independent of adhesion in both cell lines. Transfection of NRK cells with a cyclin A complementary DNA resulted in adhesion-independent accumulation of cyclin A protein and cyclin A–associated kinase activity. These transfected cells also entered S phase and complete multiple rounds of cell division in the absence of cell adhesion. Thus, cyclin A is a target of the adhesion-dependent signals that control cell proliferation.

Adhesion to substratum is required for the proliferation of most mammalian cell types; nonadherent cells fail to proliferate despite the presence of growth factors and nutrients (*1*, *2*). In NRK and NIH 3T3 fibroblasts, this adhesion requirement can be explained in terms of a discrete cell cycle transition that is manifest in late G_1 and prior to the rise in histone H1 kinase activity characteristic of cells entering S phase (*2*–*6*). G_1/S histone H1 kinase activity likely results from the activation of the Cdc2 or Cdk2 cyclin-dependent kinase (Cdk) by cyclin A or cyclin E (*6*–*8*). Thus, we asked whether the synthesis of these Cdk's or cyclins was anchorage-dependent.

Adherent and nonadherent fibroblasts synchronized in G_0 were exposed to growth factors (fetal calf serum and epidermal growth factor) (*9*) and collected at times corresponding to transit through G_1 (*10*). RNA blot hybridizations (*11*) showed that the accumulation of Cdc2 and cyclin D1 mRNAs was independent of cell anchorage, whereas the accumulation of cyclin A mRNA was strictly anchorage-dependent in both NRK and NIH 3T3 cells (Fig. 1A). In adherent cells, cyclin A mRNA was first detected in late G_1 (Fig. 1A), suggesting a possible role for cyclin A in mediating anchorage-dependent cell cycle progression from late G_1 to S.

Adherent and nonadherent NRK and NIH 3T3 cells synchronized at early S phase by incubation with hydroxyurea (*2*) were analyzed for Cdk2, cyclin E, and cyclin A protein (Fig. 1B). Immunoblotting (*12*) showed that the overall amounts of Cdk2 and cyclin E proteins were anchorage-independent, although the distribution

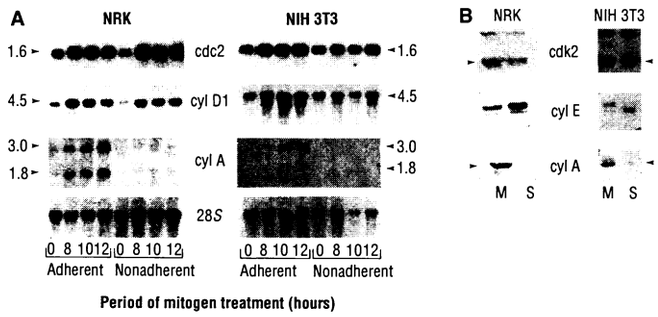

Fig. 1. Effect of cell adhesion on cell cycle–dependent expression of cyclin A. (**A**) Attachment-dependent expression of cyclin A mRNA. Adherent and nonadherent NRK and NIH 3T3 fibroblasts synchronized at G_0 were exposed to growth factors. Similar amounts of isolated total RNA (see 28*S*) were fractionated and hybridized to cDNA probes for $p34^{cdc2}$, cyclin D1, and cyclin A. Flow cytometry confirmed that both the adherent and nonadherent cells remained in G_1 throughout the time points tested. Molecular size markers are indicated at left (in kilobases). (**B**) Attachment-dependent expression of the cyclin A protein. Adherent (M, monolayer) and nonadherent (S, suspension) hydroxyurea-synchronized NRK and NIH 3T3 cells were prepared, collected, and extracted. Equal amounts of protein from each extract were subjected to immunoblot analysis. Cell viability was 90%, as determined by trypan blue exclusion, throughout the experiments.

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