Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7551–7554, October 1990
Biochemistry

Antibody caging of a nuclear-targeting signal

(photolysis/fluorescein-anti-fluorescein/peptide signals/nuclear transport)

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Communicated by John Roth, July 16, 1990 (received for review May 9, 1990)

ABSTRACT **We have developed a technique for reversibly masking a peptide-targeting signal. A fluoresceinated derivative of the simian virus 40 large tumor antigen nuclear-targeting signal was synthesized and cross-linked to bovine serum albumin. The conjugated protein was efficiently transported into rat liver nuclei unless the peptide-targeting signal was sterically hindered by binding of an anti-fluorescein antibody. Addition of free 5-aminofluorescein competed for antibody binding and rapidly restored nuclear accumulation of the derivatized bovine serum albumin. General use of hapten derivatization and anti-hapten antibodies for caging portions of macromolecular surfaces can be extended to a variety of proteins, including antibodies themselves.**

The ability to initiate biochemical reactions by rapidly revealing hidden functional groups provides a powerful experimental tool with potential applications in many areas of biology. Just over a decade ago, Kaplan *et al.* (1) synthesized a nitrophenyl ester of the terminal phosphate on ATP. The derivatized ATP molecule is unable to participate in phosphorylation reactions unless the ester is cleaved by photolysis. Such reversible masking or caging has since been applied to a variety of small molecules including Ca^{2+} (2), H^+ (3), and other nucleotides (4–6). These compounds have, in turn, been used to study physiological processes ranging from bacterial chemotaxis to signal transduction and muscle contraction.

Studies of organelle biogenesis have established the general principle that short stretches of amino acids within a protein largely determine its intracellular location (7, 8). A technique for reversibly concealing such peptide signals would be a useful tool, permitting manipulation of the position as well as the function of proteins within cells. To develop such a procedure, we chose the nuclear-targeting signal (NTS) of the simian virus 40 (SV40) tumor antigen. When fused to a cytoplasmic enzyme such as pyruvate kinase, the SV40 NTS is sufficient to misdirect the chimeric protein to the nucleus (9). In addition, synthetic peptides containing the signal can be chemically cross-linked to large nonnuclear proteins, and the resulting conjugates then accumulate in nuclei (10, 11). Equally important, the ability to demonstrate nuclear transport in *Xenopus* egg extracts containing foreign nuclei (12) provides a convenient assay for caging. By reversibly masking the heptapeptide SV40 tumor antigen NTS, we show that the caging approach, once limited to small molecules, can be extended to functional regions on protein surfaces.

MATERIALS AND METHODS

Synthesis of the Fluorescein-Coupled NTS Peptide (F-NTS). The fluorescein isothiocyanate (FITC)-conjugated peptide, FITC-Lys-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Tyr-Gly-Cys, which includes the SV40 NTS, was prepared by solid-phase synthesis using 2-chlorobenzyloxycarbonyl protection of ε-amino groups of internal lysines and 9-fluorenylmethyloxycarbonyl (FMOC) protection of the ε-amino group of the N-terminal lysine. When synthesis was complete, FMOC was removed with 20% (vol/vol) piperidine in dichloromethane and the resin was treated with 250 mg of FITC (isomer I) for 1.25 hr, thereby fluoresceinating just the N-terminal lysine. After trifluoroacetic acid unblocking of the N terminus, the F-NTS peptide was deprotected and cleaved from the resin using HF. Crude peptide was purified by reverse-phase HPLC and the composition was verified by amino acid analysis.

Radiolabeling and Conjugation. Radioiodinated F-NTS-bovine serum albumin (BSA) was freshly prepared for each experiment as follows: BSA [1×10^{-4} M in 5 μl of 150 mM potassium phosphate (pH 7.5)] was radioiodinated using Iodogen-coated tubes (Pierce) and 1 mCi of $Na^{125}I$ (1 Ci = 37 GBq). After 30 min, the reaction mixture was transferred to an uncoated tube and brought to pH 9 by the addition of 2 μl of 500 mM sodium borate (pH 9.2), and 1 μl of a solution of 5 mM succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) in dimethylformamide was added. After 30 min, the solution was titrated to pH 7 by the addition of 4 μl of 500 mM potassium phosphate (pH 6.6), and 1 μl of F-NTS peptide [4 mM in 20 mM sodium phosphate (pH 8)] was added. After 2–3 hr, 1 μl of a solution of 5 mM cysteine (pH 7.8) was added to scavenge unreacted cross-linker. Chromatography on Sephadex G-100 indicated that greater than 90% of the starting ^{125}I and greater than 65% of the starting FITC was associated with BSA. SDS/PAGE analysis indicated that BSA was conjugated to an average of four or five F-NTS peptides per molecule. For derivatization of ^{125}I-labeled BSA (IBSA) with FITC alone, 2 μl of a solution of 1.3 mM FITC (isomer I) in dimethyl sulfoxide were added to the reaction mixture described above after adjustment to pH 9. After 1.5 hr the reaction mixture was adjusted to pH 7 as described above.

Preparation of Anti-Fluorescein (anti-F) Antibodies. The anti-F-secreting hybridoma (clone 4-4-20) was a generous gift of Edward Voss (University of Illinois). Antibodies, obtained from the ascites fluid of BALB/c mice, were purified by 50% saturated ammonium sulfate precipitation followed by DEAE-cellulose chromatography.

Assay for Nuclear Transport. Extracts were prepared from *Xenopus* eggs (13) on the day of the experiment. Rat liver nuclei were prepared (14, 15) and stored at −70°C until use. Assay of nuclear targeting was essentially as described (12): 5 μl of nuclei (6×10^6 nuclei) was added to 20 μl of egg extract supplemented with 0.5 mM ATP, 7 mM creatine phosphate, and creatine kinase at 11 units/ml. The mixture was incubated at 25°C for 20 min before the addition of 1 μl of FITC-NTS-IBSA (final concentration, 45–60 ng/μl). At the designated times, nuclei and egg extract mixtures were

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Abbreviations: FITC, fluorescein isothiocyanate; F, fluorescein; BSA, bovine serum albumin; NTS, nuclear-targeting signal; SV40, simian virus 40; IBSA, ^{125}I-labeled BSA; 5AF, 5-aminofluorescein.

diluted with 1 ml of 80 mM KCl/20 mM NaCl/250 mM sucrose/5 mM EDTA/1 mM dithiothreitol/15 mM Pipes, pH 7.4, fixed with 10 mM ethylene glycol bis(succinimidyl succinate), and centrifuged through a 75% (vol/vol) glycerol cushion onto polylysine-coated coverslips (16). Coverslips were then washed in four changes of isotonic phosphate-buffered saline (PBS), dried, coated with Kodak NTB2 autoradiographic emulsion, and exposed for 12 hr. The developed slides were mounted in 50% glycerol/PBS, pH 8 containing Hoechst 33258 at 10 μg/ml and Dabco (1,4-diazabicyclo[2.2.2]octane, Aldrich) at 1 mg/ml. Photographs were taken on Kodak T-MAX P3200 Professional film using a Nikon Optiphot microscope equipped for epifluorescence (UV1A filter for Hoechst and B2A filter for fluorescein fluorescence).

Binding and Release of Anti-F Monoclonal Antibodies. For caging experiments, 1 μl of F-NTS-IBSA or photobleached F*-NTS-IBSA was mixed with 5 μl of anti-F monoclonal antibody [15 mg/ml in 20 mM sodium phosphate (pH 8)] before addition to extracts. For uncaging, 5 μl of 5-amino-fluorescein (5AF) [0.5 mM in 20 mM sodium phosphate (pH 8)] was added directly to extract–nuclei mixtures. To prepare photobleached F*-NTS-IBSA, 20 μl of F-NTS-IBSA was drawn into a capillary tube that was sealed and irradiated for 30 min in the beams of two opposing Kodak slide projectors.

Trypsin Digestion. To assay the accessibility of caged F-NTS to trypsin, 10 μl of F-NTS-BSA [0.32 mg/ml in 20 mM sodium phosphate (pH 8)] was added to 14 μl of either F-specific monoclonal antibodies or nonspecific mouse IgG [15 mg/ml in 20 mM sodium phosphate (pH 8)] before the addition of 5 μl of trypsin (Sigma, type III; 10 μg/ml in 20 mM sodium phosphate, pH 8/100 mM NaCl). After 30 sec at 4°C, the reaction was stopped by the addition of 5 μl of soybean trypsin inhibitor (Sigma; 10 μg/ml in 20 mM sodium phosphate, pH 8/100 mM NaCl). To restore the fluorescence quenched by the binding of anti-F, antigen–antibody complexes were dissociated by the addition of 80 μl of 70 mM NaOH followed by 5 μl of 20 mM sodium phosphate, pH 8/100 mM NaCl. The mixture was then applied to a 5-ml column of Sephadex G-100 and material was eluted with 10 mM NaOH.

RESULTS AND DISCUSSION

The scheme for caging the NTS is based on the principle of steric hinderance by a large removable antibody molecule (Fig. 1). The F antigen–antibody system (17) is well-suited for this purpose because of the photosensitivity of F and because of the existence of 5AF, a weakly fluorescent derivative of F that competes for antibody binding. We synthesized a peptide containing the SV40 NTS, Pro-Lys-Lys-Lys-Arg-Lys-Val, with an ε-amino fluoresceinated lysine residue placed in the N terminus of the signal. The F-labeled NTS was then cross-linked to BSA, a protein too large to enter the nucleus by itself but capable of transport when coupled to NTS (10, 11). Caging of the peptide signal would be accomplished by binding anti-F monoclonal antibodies to the conjugate, thus masking the targeting signal and inhibiting nuclear transport. However, excess 5AF added to the system should compete for binding of the anti-F, release the caged NTS, and allow transport of the conjugate to the nucleus.

The F-NTS was cross-linked to IBSA and the conjugate F-NTS-IBSA was added to a *Xenopus* egg extract containing rat liver nuclei. After 1 hr, nuclei were prepared for fluorescence microscopy and autoradiography. Hoechst staining was used to identify all nuclei in the microscope field (Fig. 2*A*). From the same field, those nuclei containing IBSA and those nuclei containing F-NTS are shown in Fig. 2 *B* and *C*, respectively. The perfect correspondence between the latter two signals indicates that nuclear fluorescence does not result

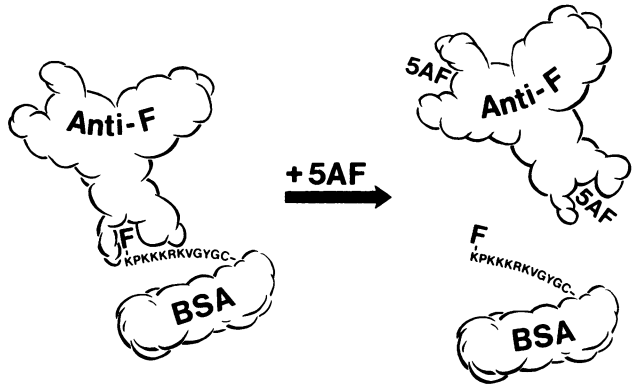

FIG. 1. Schematic representation of the technique used to cage the SV40 NTS. The SV40 NTS, Pro-Lys-Lys-Lys-Arg-Lys-Val, was synthesized with an N-terminal lysine that was derivatized by reaction with FITC (F-NTS) and coupled to IBSA (F-NTS-IBSA). Binding of anti-F monoclonal antibodies to F-NTS-IBSA masks the targeting signal and inhibits transport of the conjugate into the nucleus. Added 5AF competes for binding and releases the caged NTS thereby allowing transport of F-NTS-IBSA to the nucleus. The single-letter amino acid designation is used.

from free F-NTS that might have been cleaved from BSA. Fluoresceinated IBSA or IBSA alone did not accumulate within nuclei (data not shown). In agreement with the reports of others (12) using this transport system, between 30 and 60% of nuclei were capable of accumulating targeted proteins in any given experiment.

Caging was tested by complexing F-NTS-IBSA with excess mouse monoclonal antibody 4-4-20 (anti-F), which binds F with an affinity constant of 3.4×10^{10} M^{-1} (18). The antibody–conjugate mixture was added to egg extract containing rat liver nuclei and nuclear uptake of F-NTS-IBSA was assayed (Fig. 3). Although binding of the anti-F antibody quenches F fluorescence, autoradiography allowed us to determine that nuclear accumulation of F-NTS-IBSA did not occur (Fig. 3*C*), indicating that the targeting signal was functionally inactive.

It might be argued that F-NTS-IBSA was excluded from nuclei because anti-F reacts with a component of the transport machinery rather than the derivatized BSA. To test this possibility, we first photobleached a portion of F-NTS-IBSA thus destroying its recognition by anti-F. The photodestroyed F*-NTS-IBSA was then mixed with excess anti-F and added to egg extract containing nuclei (Fig. 3). An identical reaction mixture was prepared using unirradiated F-NTS-IBSA. By comparing Fig. 3 *C* and *D*, it is apparent that photobleaching of F allowed nuclear transport of F-NTS-IBSA even in the presence of the anti-F monoclonal antibody. Thus, anti-F

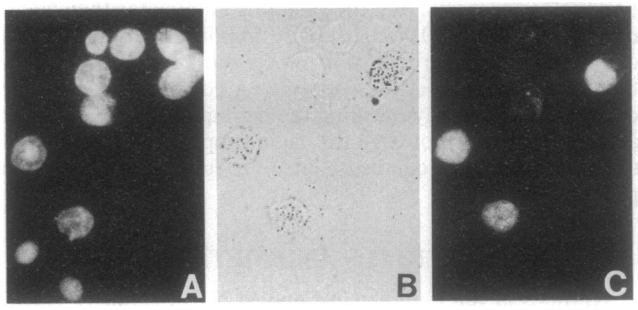

FIG. 2. Nuclear transport of F-NTS-IBSA. Rat liver nuclei were incubated in a *Xenopus* egg extract containing F-NTS-IBSA for 1 hr before being fixed and centrifuged onto a coverslip and processed for autoradiography. Hoechst staining of nuclear DNA (*A*), autoradiogram (*B*) and F fluorescence (*C*) of the same field of nuclei. Note that the three nuclei that accumulate IBSA are also fluorescent.

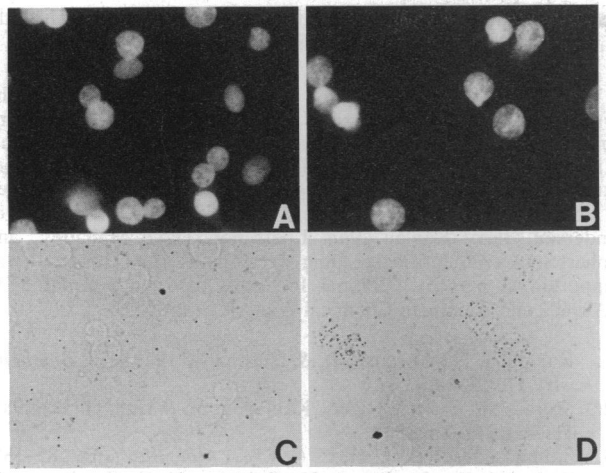

FIG. 3. Photodestruction of F prevents caging. Nuclei were incubated in frog egg extracts as described in Fig. 2. Anti-F monoclonal antibodies were either mixed with F-NTS-IBSA (*A* and *C*) or with photobleached F*-NTS-IBSA (*B* and *D*) before addition to extract–nuclei mixtures. After 1 hr of incubation, nuclei were fixed, centrifuged onto coverslips, and processed for autoradiography. (*A* and *B*) Hoechst fluorescence. (*C* and *D*) Autoradiographs. Note that caged F-NTS-IBSA was not transported into the nuclei shown in *A* and *C* (grains per nucleus = 0; n = 40). Photodestruction of F prevents antibody masking of the NTS; hence F*-NTS-IBSA was transported into the nuclei in *B* and *D* (grains per nucleus = 11.7; n = 40).

prevents nuclear entry of the conjugate by specifically binding to F on the derivatized targeting peptide and not by binding to a *Xenopus* protein.

We had hoped to use photodestruction of F to uncage the peptide signal by destroying the epitope to which anti-F binds. However, binding of anti-F significantly reduces both fluorescence and photodestruction of F. Consequently, we were unable to bleach antibody-bound F by using available light sources. Fluorometric measurements after addition of excess 5AF, however, showed that the antigen–antibody complex dissociates with $t_{1/2}$ of 1–2 min at 25°C (data not shown), thus providing an alternative to uncaging by light. When 5AF was added to nuclear transport mixtures containing F-NTS-IBSA caged by anti-F, nuclear accumulation of the conjugate was quickly restored (Fig. 4). Transport of F-NTS-IBSA in assay mixtures lacking anti-F was unaffected by the addition of 5AF (Fig. 4). Since nuclear accumulation of targeted protein can be detected within 5–10 min, uncaging by competition for antibody binding sites is thus entirely compatible with the kinetics of nuclear transport.

The cartoon in Fig. 1 is drawn such that anti-F covers the adjacent NTS. It is conceivable, however, that the antibody-bound NTS is still exposed but the antibody–BSA complex is prevented from accumulating in nuclei due to its excessive size. To test this possibility, Fab fragments of anti-F were prepared and substituted for whole antibody in a caging experiment similar to that shown in Fig. 3 *A* and *C*. As before, Fab-bound F-NTS-IBSA was excluded from nuclei whereas F-NTS-IBSA was transported (data not shown). Use of Fab fragments reduces the size of the antibody–BSA complex from a maximum of 750 kDa to 300 kDa since there were an average of four or five F-NTS peptides per BSA molecule. On the basis of size alone, such complexes should not be excluded from nuclei because molecules as large as ferritin (465 kDa) enter nuclei when coupled to transport signals (11). These results indicate that antibody–F-NTS-BSA complexes remain in the cytosol due to masking of the transport signal rather than their excessive size.

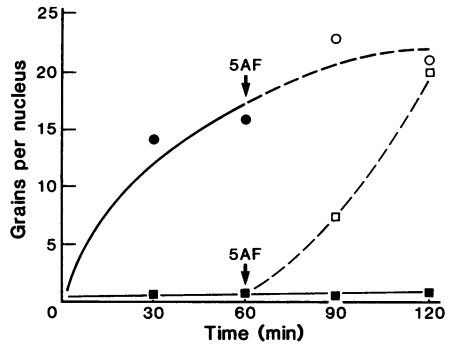

FIG. 4. Reversal of F-NTS-IBSA caging by 5AF. Nuclei were incubated in egg extracts containing F-NTS-IBSA (circles) or F-NTS-IBSA plus a 2.5-fold molar excess of anti-F Mab (squares). After 1 hr at 25°C, a 5-fold molar excess of 5AF was added to both reaction mixtures and incubation was continued for another hour. Samples were removed at the times indicated and nuclei were fixed and centrifuged onto coverslips. After processing and 7 or 12 hr of autoradiographic exposure, grains were counted over 40 nuclei per sample. These data represent the average and are plotted against the time of incubation. Solid symbols indicate that no 5AF was added; open symbols denote samples taken after the addition of 5AF. Note that in the presence of monoclonal antibody 4-4-20, F-NTS-IBSA was excluded from nuclei even after a 2-hr incubation (■). However, addition of 5AF was followed by nuclear accumulation of F-NTS-IBSA (□). Similar results were obtained in four experiments but, due to differences in the specific activity of labeled BSA and in exposure times, these data were not pooled.

To assay caging independent of nuclear transport, we examined the accessibility of masked NTS to a small proteolytic enzyme. F-NTS-BSA bound to anti-F was exposed to trypsin and examined on a Sephadex G-100 column. After gel filtration of the reaction mixture, virtually all the F fluorescence was in the excluded volume (Fig. 5). By contrast, when nonspecific mouse IgG was substituted for anti-F in an otherwise identical protease reaction mixture, 35% of the total fluorescence appeared in the included volume, indicating that within 30 sec one-third of the unmasked signals were cleaved to produce free F-linked peptides. Clearly, the F-specific monoclonal antibody severely restricts access to the NTS since it was able to prevent peptide cleavage by the

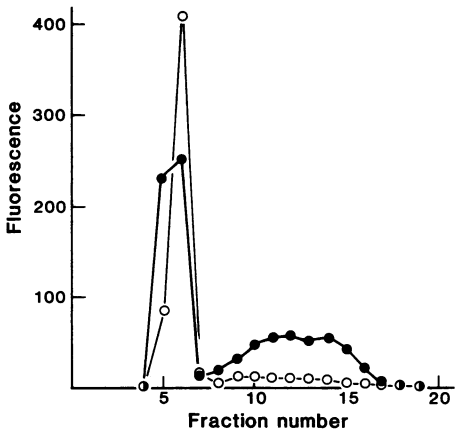

FIG. 5. Anti-F protects F-NTS from proteolysis. F-NTS-BSA, mixed with either anti-F (○) or an equivalent amount of nonspecific mouse IgG (●), was treated on ice for 30 sec with trypsin (1.6 μg/ml) before chromatography on Sephadex G-100. The column was developed in 10 mM NaOH at pH 12 to dissociate antigen–antibody complexes and restore fluorescence to samples quenched by the binding of anti-F. Under these conditions, the gel-filtration profile of F-NTS-BSA protected by the binding of anti-F during trypsinization (○) was identical to that of F-NTS-BSA not treated with trypsin (data not shown).

relatively small protease trypsin (24 kDa). The known cellular NTS-binding proteins are composed of polypeptide chains of 55–140 kDa (19, 20). It is likely, therefore, that caging also prevents interaction of NTS with these cellular transport factors, thereby inhibiting nuclear accumulation by interfering with signal recognition rather than transport. Whether masking is entirely due to steric hinderance will be resolved by application of the technique to cage other peptide sequences, such as Pro-Glu-Ser-Thr (PEST) proteolytic signals (21), where destruction of the targeted protein presumably does not require transport through membranes.

We have shown that a F antigen–antibody system can be used to mask a NTS. Such a technique should prove particularly advantageous in conjunction with microinjection studies. Caged domains, introduced into the cytoplasm of cells, would remain inactive until unveiled, either by flooding the cell with F or by irradiation with light (see below). For example, by exposing HeLa cells to 25 μM fluorescein diacetate for 1 min at 37°C, one can obtain an intracellular F concentration (45 μM) that is 10 times the anti-F concentration used in the experiments described here (data not shown). Introduction of antibody-caged proteins would thus allow time for recovery or synchronization of cells prior to activation of the injected molecule. Since fluorescein diacetate uptake and antigen–antibody complex dissociation take place on the order of 1–2 min, unveiling of masked reactants by this procedure is compatible with the kinetics of many biological processes such as receptor internalization, protein synthesis, and degradation. In addition, unmasking of caged domains on proteins involved in more rapid reactions, say neurotransmission or muscle contraction, should soon be attainable. The development of "break-away" F groups, compounds that contain photosensitive nitrobenzyl linkages to F (22), will allow uncaging by light.

Theoretically, caging could be accomplished using any set of haptens and antibodies, providing the molecule to be caged is easily derivatized and the antibody can subsequently be removed. The technique can also be used for reversibly masking binding sites on a variety of proteins. For example, to prepare caged antibodies, one could fluoresceinate antibodies bound to a column of antigen, elute the derivatized antibodies, coat them with anti-F, and rechromatograph them on the antigen column. Those antibodies failing to bind on the second pass would presumably have their antigen-binding sites masked by bound anti-F antibodies. Similar strategies could be applied to enzymes by using substrate affinity columns. Thus, the procedure described above should find general application since caged enzymes and antibodies will permit the synchronous initiation and termination of a wide variety of biochemical reactions within living cells.

We thank William R. Gray for advice and assistance during the synthesis of the peptide, James Herron for helpful discussions concerning the structure of monoclonal antibody 4-4-20, Dennis Winge for suggesting that masking might result from nuclear pore exclusion, and Tom Alber for suggesting that trypsin could be used to assay the accessibility of bound NTS. We also thank Trevor Anderson, Jerry Kaplan, Pius Loetscher, Dave Mahaffey, and Robert Schlegel for critical reading of the manuscript. This study was supported in part by National Institutes of Health Grant 5T32 CA09602 and American Chemical Society Grant CD 395.

1. Kaplan, J. H., Forbush, B. & Hoffman, J. F. (1978) *Biochemistry* **17**, 1929–1935.
2. Morad, M., Davies, N. W., Kaplan, J. H. & Lux, H. D. (1988) *Science* **241**, 842–844.
3. Shimada, K. & Berg, H. C. (1987) *J. Mol. Biol.* **193**, 585–589.
4. Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P. & Trentham, D. R. (1987) *Nature (London)* **327**, 249–252.
5. Karpen, J. W., Zimmerman, A. L., Stryer, L. & Baylor, D. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1287–1291.
6. Schlichting, I., Rapp, G., John, J., Wittinghofer, A., Pai, E. F. & Goody, R. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7687–7690.
7. Blobel, G. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1496–1500.
8. Garoff, H. (1985) *Annu. Rev. Cell Biol.* **1**, 403–445.
9. Kalderon, D., Roberts, B. L., Richardson, W. D. & Smith, A. E. (1984) *Cell* **39**, 499–509.
10. Goldfarb, D. S., Gariepy, J., Schoolnik, G. & Kornberg, R. D. (1986) *Nature (London)* **322**, 641–644.
11. Lanford, R. E., Kanda, P. & Kennedy, R. C. (1986) *Cell* **46**, 575–582.
12. Newmeyer, D. D., Finlay, D. R. & Forbes, D. J. (1986) *J. Cell Biol.* **103**, 2091–2102.
13. Newport, J. (1987) *Cell* **48**, 205–217.
14. Blobel, G. & Potter, V. (1966) *Science* **154**, 1662–1665.
15. Newport, J. & Spann, T. (1987) *Cell* **48**, 219–230.
16. Miake-Lye, R. & Kirschner, M. W. (1985) *Cell* **41**, 165–175.
17. Kranz, D. M. & Voss, E. W. (1981) *Mol. Immunol.* **18**, 889–898.
18. Gibson, A. L., Herron, J. N., He, X.-M., Patrick, V. A., Mason, M. L., Lin, J.-N., Kranz, D. M. & Voss, E. W. (1988) *Proteins* **3**, 155–160.
19. Adam, S. A., Lobl, T. J., Mitchell, M. A. & Gerace, L. (1989) *Nature (London)* **337**, 276–279.
20. Yamasaki, L., Kanda, P. & Lanford, R. E. (1989) *Mol. Cell. Biol.* **9**, 3028–3036.
21. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science* **234**, 364–368.
22. Yen, H.-R., Kopecek, J. & Andrade, J. D. (1989) *Makromol. Chem.* **190**, 69–82.