attributable to a change in $\delta^{18}O_{sw}$. This estimate yields a volume-weighted average for all oceans of 1.0 per mil, rather than 1.3 per mil, and requires the average $\delta^{18}O$ value of continental ice during the LGM to be −32 per mil rather than −42 per mil, consistent with estimates for the isotopic composition of continental ice during the LGM (*20*).

An alternative method for relating the 0.8 ± 0.1 per mil change in the tropical Atlantic to the global mean is to estimate how changes in the proportion of NADW and AABW would have affected the $\delta^{18}O_{sw}$ at Site 925 (*21*). On the basis of carbon isotopes in foraminifera from deep sea sediment (*13*) and the distribution of preformed phosphate in the modern ocean (*22*), the ratio of NADW to AABW in the region of Site 925 changed from 40:60 during the LGM to 90:10 today. If the difference between $\delta^{18}O$ values of AABW and NADW was the same during the LGM, the circulation changes at Site 925 during the LGM reduced the $\delta^{18}O_{sw}$ by 0.25 per mil. This reduction implies a mean ocean change of 1.05 per mil, in good agreement with the estimate based on deep water temperature changes.

A lower glacial-interglacial change in $\delta^{18}O_{sw}$ makes planktonic foraminiferal $\delta^{18}O$ records consistent with greater cooling of the tropics during the LGM. By assuming an ice-volume component of 1.3 per mil, $\delta^{18}O$ records of planktonic foraminifera yield tropical sea surface temperatures that are at most 2°C colder during the LGM (*1*), consistent with estimates from CLIMAP based on biodiversity (*16*). If the buildup of continental ice affects the carbonate oxygen isotope record for the LGM by only 1.0 per mil as a global average and by as little as 0.8 per mil for deep water in the Atlantic Ocean, then $\delta^{18}O$ records of tropical planktonic foraminifera can yield from 3° to 5°C of cooling since the LGM. Although it remains uncertain how the $\delta^{18}O$ of surface waters changed during the LGM as a result of changes in evaporation and precipitation, reducing the ice-volume component helps to reconcile tropical planktonic foraminiferal $\delta^{18}O$ records with the coral $\delta^{18}O$ and Sr/Ca measurements (*3*) and with terrestrial climate proxies, including snow-line elevations (*23*), noble gases in ground water (*24*), and pollen records (*25*).

REFERENCES AND NOTES

1. W. S. Broecker, *Quat. Res.* **26**, 121 (1986).
2. R. G. Fairbanks, *Nature* **342**, 637 (1989).
3. T. P. Guilderson, R. G. Fairbanks, J. L. Rubenstone, *Science* **263**, 663 (1994).
4. D. P. Schrag and D. J. DePaolo, *Paleoceanography* **8**, 1 (1993).
5. Samples were squeezed from whole rounds at 1.5-m intervals from the sea floor to a depth of 60 m and at approximately 30-m intervals down to 569 m.
6. Two splits of each sample were equilibrated with 100 μmol of CO_2 at 25°C for 24 hours before analysis on a VG Optima gas source mass spectrometer. After analysis, samples were equilibrated with a fresh injection of CO_2 and reanalyzed. This method provides four replicate analyses for each sample. Standard deviations based on the four replicates of each sample range from 0.01 to 0.05 per mil. The standard deviation (1σ) of 30 replicate analyses of standard water analyzed at the same time as the pore fluid samples is 0.03 per mil.
7. N. J. Shackleton and M. A. Hall, in *Proceedings of the Ocean Drilling Program: Scientific Results*, K. Becker and H. Sakai, Eds. (Ocean Drilling Program, College Station, TX, 1989), vol. 111, pp. 295–316.
8. N. J. Shackleton and N. G. Pisias, in *The Carbon Cycle and Atmospheric CO_2: Natural Variations Archean to Present*, E. T. Sundquist and W. S. Broecker, Eds. (AGU Monograph, American Geophysical Union, Washington, DC, 1985), pp. 303–317.
9. E. Bard, B. Hamelin, R. G. Fairbanks, A. Zindler, *Nature* **345**, 405 (1990).
10. J. H. Simpson and H. Y. Carr, *Phys. Rev.* **111**, 1201 (1958).
11. W. B. Curry *et al.*, *Proceedings of the Ocean Drilling Program: Initial Reports* (Ocean Drilling Program, College Station, TX, 1995), vol. 154.
12. N. J. Shackleton, *Quat. Sci. Rev.* **6**, 183 (1987).
13. W. B. Curry and G. P. Lohmann, *Paleoceanography* **5**, 487 (1990).
14. The oxygen isotope thermometer scale for calcite between 0° and 2°C is 0.26 per mil per degree [J. R. O'Neil, R. N. Clayton, T. K. Mayeda, *J. Chem. Phys.* **51**, 5547 (1969)].
15. R. G. Fairbanks and R. K. Matthews, *Quat. Res.* **10**, 181 (1978). This study determined the change in $\delta^{18}O_{sw}$ per meter of sea level change (0.011 per mil m^{-1}) from the change in $\delta^{18}O$ of coral aragonite in Pleistocene terraces, assuming that melting of glacial ice preceded changes in sea surface temperature.
16. CLIMAP Project Members, *Geol. Soc. Am. Map Chart Ser. MC-36* (1981).
17. A. Shemesh, C. D. Charles, R. G. Fairbanks, *Science* **256**, 1434 (1992).
18. S. Levitus, *Climatological Atlas of the World Ocean* (NOAA Prof. Pap. 13, National Oceanic and Atmospheric Administration, Rockville, MD, 1982).
19. J. C. Duplessy *et al.*, *Paleoceanography* **3**, 343 (1988); D. W. Oppo, R. G. Fairbanks, A. L. Gordon, *ibid.* **5**, 43 (1990); D. W. Oppo and R. G. Fairbanks, *ibid.*, p. 277.
20. E. Olausson, in *Progress in Oceanography*, M. Sears, Ed. (Pergamon, Oxford, 1965), vol. 3, pp. 221–252; N. J. Shackleton, *Nature* **215**, 15 (1967); W. Dansgaard and H. Tauber, *Science* **166**, 499 (1969); A. C. Mix and W. F. Ruddiman, *Quat. Res.* **21**, 1 (1984); C. Hillaire-Marcel and C. Causse, *ibid.* **32**, 132 (1989).
21. This approach resulted from conversations with W. Broecker. For a discussion of the use of phosphate and carbon isotopes as tracers of ocean circulation, see W. S. Broecker, *Paleoceanography* **8**, 137 (1993).
22. A. E. Bainbridge, *GEOSECS Pacific Final Hydrographic Data Report* (GEOSECS Operations Group, Scripps Institution of Oceanography, La Jolla, CA, 1976).
23. S. C. Porter, *Quat. Res.* **12**, 161 (1979); P. Webster and N. Streten, *ibid.* **10**, 279 (1978).
24. M. Stute, P. Schlosser, J. F. Clark, W. S. Broecker, *Science* **256**, 1000 (1992).
25. T. Van der Hammen, *J. Biogeogr.* **1**, 3 (1974).

26. We thank D. DePaolo for inspiration; N. Shackleton, W. Curry, and C. Richter for dedicating Hole 925E to this project; and the entire scientific party of ODP Leg 154 for their support. This manuscript benefited from comments by M. Delaney, N. Shackleton, J. Gieskes, and W. Broecker. This work was partly supported by a grant from the Joint Oceanographic Institutions.

23 January 1996; accepted 2 May 1996

Nuclear Encoding of a Chloroplast RNA Polymerase Sigma Subunit in a Red Alga

Kan Tanaka, Kosuke Oikawa, Niji Ohta, Haruko Kuroiwa, Tsuneyoshi Kuroiwa, Hideo Takahashi*

A chloroplast RNA polymerase sigma factor is encoded by a nuclear gene, *sigA*, in the red alga *Cyanidium caldarium* RK-1. The encoded protein functions as an RNA polymerase sigma factor in vitro and it is localized to the chloroplast in vivo. SigA shows high sequence similarity to the sigma factors of cyanobacteria, which is indicative of the ancestral endosymbiotic event and subsequent transfer of the *sigA* gene to the nuclear genome.

DNA sequence and phylogenetic analyses indicate that chloroplasts are close relatives of free-living cyanobacteria and are derived from an endosymbiotic event. Chloroplast RNA polymerase is closely related to that of cyanobacteria (*1*). Chloroplast genomes of several plant species encode RNA polymerase subunits corresponding to α, β, and β′ of the eubacterial core enzyme (*2*). However, although the σ subunit, a protein important for transcription initiation and promoter selectivity (*3*), has been found in chloroplasts (*4*), the corresponding gene has not been found in the chloroplast genome.

The unicellular red alga *Cyanidium caldarium* RK-1 is an acidophillic eukaryote with a small genome [13 mega–base pairs (Mbp)] (*5*). The cells contain a nucleus, a mitochondrion, and a chloroplast, each with its own genome. The chloroplast RNA polymerase and σ subunit of a related red alga,

K. Tanaka, K. Oikawa, H. Takahashi, Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan.
N. Ohta, Department of Basic Human Sciences, Waseda University, Mikajima 2-579-15, Tokorozawa, Saitama 359, Japan.
H. Kuroiwa and T. Kuroiwa, Department of Plant Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.
*To whom correspondence should be addressed.

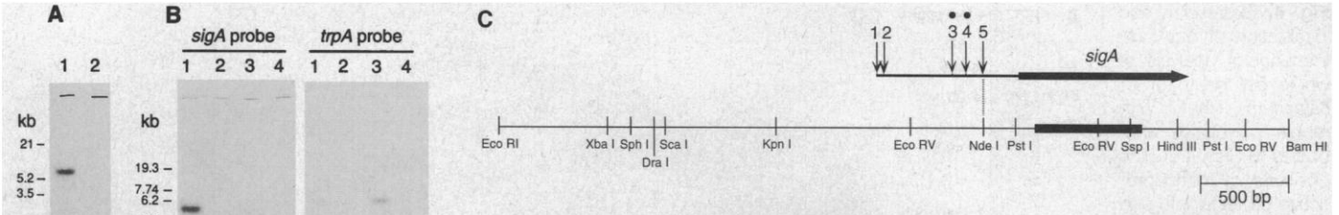
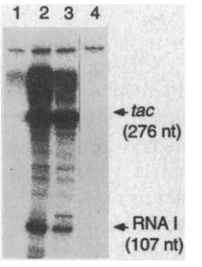


Fig. 1. (**A**) *Cyanidium caldarium* RK-1 total DNA was subjected to Southern analysis with an oligonucleotide probe corresponding to the *sigA* sequence amplified through use of PCR with degenerate primers (*21*). Lane 1, Eco RI digest; lane 2, Pst I digest. (**B**) *Cyanidium* nuclear and chloroplast DNAs were separately enriched (*22*) and subjected to Southern analysis with either a PCR-amplified *sigA* sequence (left) or the chloroplast DNA fragment containing *trpA* (*11*) (right) as a probe (*21*). Lane 1, Eco RI digest of nuclear DNA; lane 2, Pst I digest of nuclear DNA; lane 3, Eco RI digest of chloroplast DNA; lane 4, Pst I digest of chloroplast DNA. (**C**) Schematic representation of the 4.5-kb Eco RI–Bam HI fragment and the predicted *sigA* open reading frame. Representative restriction sites are shown, and the position of the expected open reading frame is indicated by a horizontal arrow above the restriction map; the thick portion of the arrow corresponds to the region of the encoded protein that shows homology to cyanobacterial σ factors. The numbered vertical arrows denote the five possible initiation codons, and the dotted numbers represent the probable initiation codons. The thick line on the physical map indicates the DNA region used as a probe in (B).

Fig. 2. Sequence comparison of the *Cyanidium* SigA protein with the principal σ factors of cyanobacteria (*23*). Amino acid sequences were aligned from region 1.2 to the COOH-terminus, with the conserved regions of σ factors (*12*) indicated above the sequences. C, A, and S correspond to *Cyanidium* SigA, the principal σ factor of *Anabaena* sp. strain PCC 7120 (the *sigA* gene product) (*7*), and the principal σ factor of *Synechococcus* sp. strain PCC 7942 (the *rpoD1* gene product) (*8*), respectively. For the *Cyanidium* sequence, amino acid residues were arbitrarily numbered from the beginning of region 1.2. The predicted NH_2-terminal sequence beginning from the first possible initiation codon of SigA was also described. Five possible initiation codons are underlined twice, and serine and threonine residues are underlined once. Dots in the alignment indicate identical amino acid residues among the three gene products. The boxed residues denote amino acid sequences used to design PCR primers for the cloning of *sigA*. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D83179.

```
C -250 MDVDYQPRSAYMIAAGSRRTQTHKCGTFEKLQEPGDPDSVLTGIILRSSE
C -200 GAHQERSDASGLDIIIGTWSPSHRTSALVDPRGLAAYADELLAAADIDIA
C -150 SLTEEGGLAPQNSANQPSRISFPGSLGAYVRGLEQQYRSERMIDRAPSAT
C -100 EIESRKSKKAALKSQNKMTSSRKETRSSSSQARARQQKGRKSEQAPGVPV
C  -50 HMRAGPRTKNTVTVRDPFADNQSANDGSTVPVEEVYSNSETEEAESQIES

          <---          1.2          --->
C    1 DTIRSYLREIGRYQLLHPGEEIELSKQVCILMDLEQFQRSFREEHGKSPT
A   80 ·S··L··Q····IR··RAD·····ARKIAD·LE··RVRERLS·KLERD·R
S   74 ·S··L··Q····IR··RAD·····ARQIAD·LA··RIRDELL·QLDRL·S

                                  <---      2.1      --->
C   51 ESEWAQGCGYGDDVEKLREHIRDGRKAKERMVTANLRLVVSIAKRYSNRG
A  130 DS···EA-V-QLPLPAF·YRLHI··R··DK··QS··········K·M···
S  124 DA···AA-V-DSPLDEF·RRLFR··R··DK··QS··········K·M···

          --    2.2     --><---    2.3    --><---    2.4
C  101 VALQDLIQEGSIGLIRGVEKFDAERGFRFSTYATWWIRQSITRAISDSSR
A  178 LSF·····L····AA····H·K·YK·····A·····A·Q··
S  172 LSF·····L····AA····H·K·YK·····A·····A·Q··

          --->                <---          3
C  151 SIRLPVHVHDTISLIRKQTKALQVELGRPPSEEEICESVGIDRAKYRLVM
A  228 T·····LYE···R·K·T··LLSQ·M··KPT····ATRMEMTIE·L·FIA
S  222 T·····LYE···R·K·T··LVSQ·M··KQT····ATRMEMTIE·L·FIA

                          --->
C  201 ECSRNIVSLETPLRSGDDVHFLGESLIAPEERAEENCSRDTLRESIEKVL
A  278 KSAQLPI·····IGKEE·SR-··DFIESDG·TP·DQVSKNL···DL·K··
S  272 KSAQLPI·····IGKEE·SR-··DFIEADG·TP·DEVAKNL···DL·G··

        <---   4.1   --->    <---        4.2          ---
C  251 HCLSTREREVIRMRFGLTDGRPRTLEEVGSRFNVTRERIRQIESKALKKL
A  327 DS··P···D·L·L·Y··D···MK····I·QI···········A···R··
S  321 ST··P···D·L·L·Y··D···MK····I·QL···········A···R··

       >
C  301 RTPAENNFLDEYLGEV
A  377 ·H·NR·SV·K··IR
S  371 ·H·NR·SI·K··IR
```

Fig. 3. The σ factor activity of SigA with eubacterial RNA polymerase: in vitro transcription with the *E. coli* core RNA polymerase reconstituted with SigA (*24*). Plasmid pKK223-3 (0.1 pmol) containing *tac* and RNA I promoters was tested in a single-round transcription assay in vitro. Lane 1, *E. coli* RNA polymerase core enzyme (3 pmol); lane 2, *E. coli* RNA polymerase core enzyme (3 pmol) reconstituted with *E. coli* σ^{70} (9 pmol); lane 3, *E. coli* RNA polymerase core enzyme (3 pmol) reconstituted with recombinant SigA (9 pmol); lane 4, SigA (9 pmol); nt, nucleotides.



Galdieria sulphuraria, have recently been analyzed (6).

To identify a gene encoding the RNA polymerase σ factor of the *Cyanidium* chloroplast, we adopted a polymerase chain reaction (PCR)–based strategy. We used synthetic DNA primers based on amino acid sequences conserved among the principal σ factors of cyanobacteria (*7*, *8*) to amplify total *Cyanidium* DNA (*9*). An oligonucleotide corresponding to a sequence amplified between the two PCR primers was synthesized and used as a probe for Southern (DNA) hybridization analysis with *Cyanidium* total DNA. A single DNA region corresponding to a 5.4-kb Eco RI fragment that hybridized with the probe (Fig. 1A) was cloned in *Escherichia coli* (*10*). Determination of the nucleotide sequence of the 4508-bp Eco RI–Bam HI region revealed an open reading frame that encodes a putative protein homologous to cyanobacterial principal σ factors. We thus named this gene *sigA*.

To determine in which organelle the *sigA* gene is located, we analyzed fractions enriched for nuclear and chloroplast DNAs separately. The *sigA* probe hybridized to a greater extent with the nuclear DNA than with the chloroplast DNA (Fig. 1B, left). Rehybridization of the same blot confirmed localization of the chloroplast *trpA* gene (*11*) to the chloroplast DNA (Fig. 1B, right). The high GC content of the *sigA* gene (50.47%) compared with the extremely low GC content of mitochondrial DNA makes it unlikely that *sigA* is encoded by mitochondria. Thus, *sigA* appears to be encoded by the nuclear genome.

Several generally conserved domain structures, from NH_2-terminal region 1.1 to

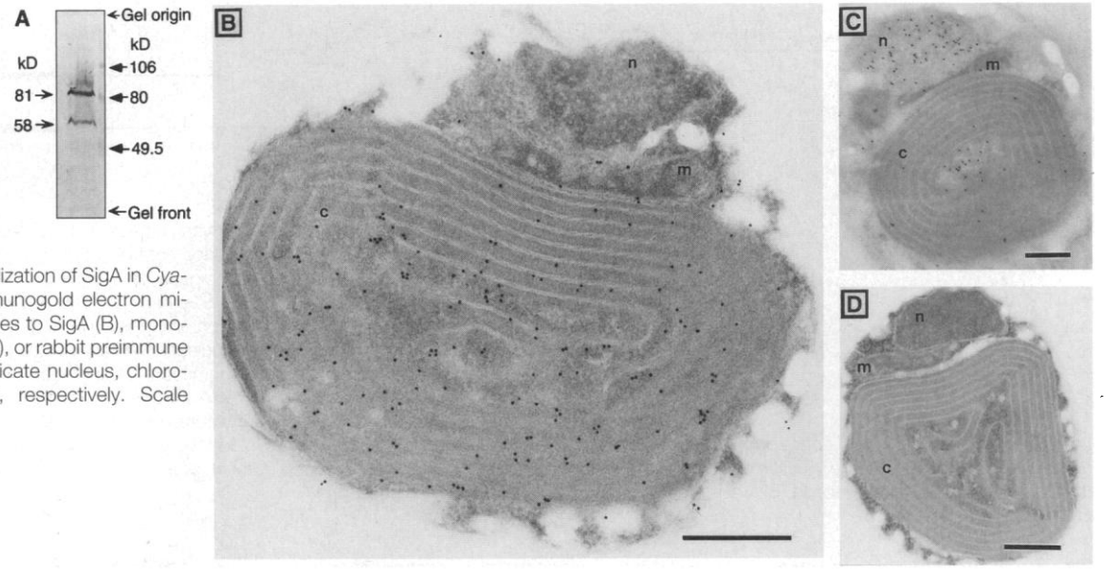


Fig. 4. Expression and localization of SigA. (**A**) Immunoblot analysis of whole cell lysate of *C. caldarium* RK-1 with rabbit polyclonal antibodies to SigA (*25*). The positions of immunoreactive proteins (left) and molecular size standards (right) are indicated. (**B** through **D**) Subcellular localization of SigA in *Cyanidium* determined by immunogold electron microscopy (*26*) with antibodies to SigA (B), monoclonal antibodies to DNA (C), or rabbit preimmune serum (D). n, c, and m indicate nucleus, chloroplast, and mitochondrion, respectively. Scale bars, 0.25 μm.

COOH-terminal region 4.2, have been identified in σ proteins (*3*, *12*). The putative amino acid sequence of SigA could be aligned from region 1.2 to the COOH-terminus with the sequences of principal σ factors of cyanobacteria (Fig. 2). No introns were found in *sigA*. Sequence similarity continued between regions 1.2 and 2, an area of greater divergence among eubacterial σ factors. Thus, the putative SigA protein appears to be a close relative of cyanobacterial principal σ factors. Because sequence similarity was low in region 1.1, we could not predict the NH_2-terminus of SigA. Most nuclear-encoded chloroplast proteins are synthesized as larger precursors containing an NH_2-terminal extension called a transit peptide (*13*). Among five possible translation initiation codons (Fig. 1C), the position 3 or 4 makes a serine- and threonine-rich leader sequence that may function as a transit peptide (*13*), suggesting that one of these sites is the in vivo initiation site.

To determine whether SigA can function as a eubacterial σ factor, we purified the recombinant protein after expression in *E. coli* (*14*). We arbitrarily chose one position 5 (Fig. 1C) for translation initiation. The expressed protein included a leader sequence and His tag derived from the expression plasmid. The purified protein was reconstituted with core RNA polymerase of *E. coli* and the transcriptional specificity of the resulting enzyme was examined in vitro. The heterologous holoenzyme recognized the consensus *E. coli* promoters for *tac* and RNA I (Fig. 3), confirming that SigA can function as a eubacterial principal or principal-type σ factor.

Rabbit polyclonal antibodies to the recombinant SigA protein recognized two proteins from a *Cyanidium* whole cell extract (Fig. 4A). Similar results were obtained with antibody prepared independently from another rabbit. The two proteins may correspond to modified or processed forms of the same gene product. Immunogold electron microscopy with the rabbit polyclonal antibodies localized SigA to the chloroplast in *Cyanidium*, whereas antibodies to DNA reacted with the nucleus, mitochondria, and chloroplast (Fig. 4, B through D, and Table 1).

Thus, a red alga RNA polymerase σ factor that localized to the chloroplast is encoded by the nuclear genome. In *Cyanidium*, therefore, the nucleus may control chloroplast gene expression through modulation of expression or function of SigA. It is likely that higher plants resemble *Cyanidium* in this respect, although red algae may represent a separate evolutionary lineage. Understanding σ-factor regulation may contribute to our knowledge of not only the developmental processes of bacteriophages and sporulating bacteria but also the development and differentiation of plastids of higher plants.

Table 1. Density of labeling by antibodies to SigA (anti-SigA) and to DNA (anti-DNA) in cells of *C. caldarium* RK-1. Values are means ± SD. Densities were calculated as described (20); *n*, number of cell sections examined.

Cell type	Number of gold particles (× 16) (μm^{-2})		
	Anti-SigA (n = 57)	Anti-DNA (n = 46)	Control (n = 25)
Chloroplast	13.9 ± 2.8	4.4 ± 2.1	0.1 ± 0.1
Mitochondrion	1.1 ± 1.1	2.4 ± 2.1	0.1 ± 0.2
Nucleus	1.1 ± 0.8	25.9 ± 7.2	0.1 ± 0.1
Cytoplasm	2.0 ± 0.8	0.6 ± 0.5	0.2 ± 0.2
Background	0.4 ± 0.6	0.3 ± 0.5	0.1 ± 0.3

REFERENCES AND NOTES

1. J. Hu and L. Bogorad, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1531 (1990); J. Hu, R. F. Troxler, L. Bogorad, *Nucleic Acids Res.* **19**, 3431 (1991); G. J. Schneider and R. Haselkorn, *J. Bacteriol.* **170**, 4136 (1988); K. J. Bergsland and R. Haselkorn, *ibid.* **173**, 3446 (1991).
2. K. Shinozaki *et al.*, *EMBO J.* **5**, 2043 (1986); K. Ohyama *et al.*, *Nature* **322**, 572 (1986); J. Hiratsuka *et al.*, *Mol. Gen. Genet.* **217**, 185 (1989); R. B. Hallick *et al.*, *Nucleic Acids Res.* **21**, 3537 (1993); M. Reith and J. Munholland, *Plant Cell* **5**, 465 (1993).
3. J. D. Helmann and M. J. Chamberlin, *Annu. Rev. Biochem.* **57**, 839 (1988).
4. S. Lerbs, E. Bräutigam, R. Mache, *Mol. Gen. Genet.* **211**, 459 (1988); K. Tiller, A. Eisermann, G. Link, *Eur. J. Biochem.* **198**, 93 (1991).
5. N. Ohta, H. Nagashima, S. Kawano, T. Kuroiwa, *Plant Cell Physiol.* **33**, 657 (1992).
6. R. F. Troxler, F. Zhang, J. Hu, L. Bogorad, *Plant Physiol.* **104**, 753 (1994); B. Liu and R. F. Troxler, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3313 (1996).
7. B. Brahamsha and R. Haselkorn, *J. Bacteriol.* **173**, 2442 (1991).
8. K. Tanaka, S. Masuda, H. Takahashi, *Biochim. Biophys. Acta* **1132**, 94 (1992).
9. *Cyanidium* total DNA was prepared as described (6). Oligonucleotides were synthesized with an Applied Biosystems model 392 DNA synthesizer. DNA was sequenced through use of the dideoxy chain termination method with a Taq Dye Primer Cycle sequencing kit (Applied Biosystems, Japan) and an Applied Biosystems model 373A DNA sequencer. Degenerate primers containing deoxyinosine [5′-GGCCGGATCCGA(C/T)(C/T)TIATICA(A/G)GA(A/G)GG-3′ and 5′-AGATCGAT-

GC(C/T)TGIC(T/G)IATCCACCA-3′] were synthesized and used for PCR amplification of *Cyanidium* total DNA. The reaction was performed with TAKARA Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) for 30 cycles at 95°C for 1.5 min, 40°C for 2 min, and 72°C for 3 min. The amplified fragment was cloned with a pCR-Script SK cloning kit (Stratagene).
10. K. Tanaka, T. Shiina, H. Takahashi, *Science* **242**, 1040 (1988).
11. N. Ohta, N. Sato, S. Kawano, T. Kuroiwa, *Curr. Genet.* **25**, 357 (1994).
12. M. Lonetto, M. Gribskov, C. A. Gross, *J. Bacteriol.* **174**, 3843 (1992).
13. K. Keegstra and L. J. Olsen, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 471 (1989); M. Reith, *ibid.* **46**, 549 (1995).
14. To produce SigA in *E. coli* we inserted a 1733-bp Nde I–Bam HI fragment containing the *sigA* open reading frame into the linker site of the expression plasmid pET15b (Novagen) (*15*). The resultant plasmid, pETCA, was introduced into the host BL21(DE3) strain and the bacterial cells were incubated at 37°C in 300 ml of Luria broth (LB) medium until the optical density of the culture at 660 nm reached 0.5. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and after incubation for an additional 2 hours cells were collected by centrifugation. The recombinant protein was recovered as inclusion bodies, solubilized in TGED buffer [10 mM tris-HCl (pH 8.0), 5% (w/v) glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol] containing 6 M guanidine hydrochloride, and renatured by dialyzing against TGED buffer. The SigA protein was further purified in a fast protein liquid chromatography (FPLC) system (Pharmacia) on a POROS HQ column (4.6 mm × 100 mm; PerSeptive Biosystems, Cambridge, MA). Elution was performed with a linear gradient of 0 to 1.0 M NaCl in the same buffer, and the peak fraction was precipitated with ammonium sulfate, dialyzed against storage buffer [TGED containing 0.5 M NaCl and 50% (w/v) glycerol], and stored at −30°C. The calculated molecular weight of the protein is 46,794 (440 amino acids).
15. M. Studier, A. H. Rosenberg, J. J. Dunn, J. W. Dubendorff, *Methods Enzymol.* **185**, 60 (1990).
16. K. Igarashi and A. Ishihama, *Cell* **65**, 1015 (1991).
17. T. Nomura, N. Fujita, A. Ishihama, *Nucleic Acids Res.* **14**, 6857 (1986).
18. H. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
19. K. Tanaka and H. Takahashi, *Biochim. Biophys. Acta* **1089**, 113 (1991).
20. K. A. Johnson and J. L. Rosenbaum, *Cell* **62**, 615 (1990); H. Nozaki, H. Kuroiwa, T. Kuroiwa, *J. Phycol.* **30**, 279 (1994).
21. Hybridization experiments were performed as described (*10*), with the exception that incubation was at 55°C in Fig. 1A and at 65°C in Fig. 1B. Probes for Southern analysis were prepared as follows: in Fig. 1A, a synthetic DNA (5′-CGAAAGCCCCGTTCGGCATCAAAT-3′) was end-labeled with ^{32}P by T4 polynucleotide kinase; in Fig. 1B (left), a 616-bp DNA fragment corresponding to the internal region of the *sigA* open reading frame was amplified by PCR from the cloned *sigA* DNA with synthetic primers (5′-CAGGAAACAGCTATGACCTGTCCAAACAAGTGTGCA-3′ and 5′-TGTAAAACGACGGCCAGTGCAATTAACGATTCTCCC-3′), and the ^{32}P-labeled DNA probe was prepared by the random priming method; and in Fig. 1B (right), a 3270-bp Pst I fragment containing the *trpA* gene (*11*) was purified and labeled with ^{32}P by the random priming method.
22. Nuclear and chloroplast DNAs were enriched by density gradient centrifugation, first on ethidium bromide–CsCl and then on Hoechst 33258–CsCl (*5*). Mitochondrial, chloroplast, and nuclear DNAs banded separately in this order from the top.
23. Single-letter abbreviations for the amino acid residues are as follows: 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.
24. The purification of RNA polymerase core enzyme and the σ^{70} subunit has been described (*16*). To reconstitute the holoenzyme, we mixed the core enzyme with a threefold molar excess of either σ^{70} or SigA and then incubated the mixture for 30 min at 37°C. Single-round transcription reactions were performed under standard conditions (*17*). Transcripts separated by electrophoresis through a 7% polyacrylamide gel containing 8 M urea were analyzed with a Bioimage analyzer (BAS1000, Fuji Photo Film). The positions and the nucleotide length of transcripts initiated from the *tac* and RNA I promoters are indicated.
25. Rabbit antiserum to recombinant SigA was prepared as described (*18*). The antiserum was passed over a protein A column and the immunoglobulin G fraction was further passed over an affinity column prepared from SigA (10 mg) conjugated with HiTrap NHS-activated (1 ml; Pharmacia) as suggested by the supplier. *C. caldarium* RK-1 cells were ground in liquid nitrogen, and an amount corresponding to 10 μg of protein was fractionated by SDS–polyacrylamide gel electrophoresis in a 9.4% gel. Other procedures for immunoblot analysis were as described (*19*).
26. Immunogold electron microscopy was performed basically as described (*20*). *Cyanidium* cells were fixed in 2% glutaraldehyde, embedded in LR White resin, cut with an ultramicrotome, and treated with the primary antibody. The mouse monoclonal antibodies to human double-stranded DNA were from Chemicon. After the grids were washed, immune complexes were detected through incubation for 2 hours at room temperature with antibodies to rabbit or mouse immunoglobulin G labeled with 10-nm colloidal gold particles (BioCell Research Laboratories, Cardiff, UK). The grids were then stained with 3% aqueous uranyl acetate for 20 min and examined under an electron microscope.
27. We thank H. Hirokawa for encouragement and H. Takahashi for technical assistance. Supported in part by grants for scientific research from the Ministry of Education, Science, and Culture of Japan (05454069, 07760074, and 06101002), and by the Biodesign Research Program of RIKEN.

12 February 1996; accepted 23 April 1996

Requirement for the Adapter Protein GRB2 in EGF Receptor Endocytosis

Zhixiang Wang and Michael F. Moran*

Activated epidermal growth factor (EGF) receptors induce the formation of various complexes of intracellular signaling proteins that are mediated by SRC homology 2 (SH2) and SH3 domains. The activated receptors are also rapidly internalized into the endocytotic compartment and degraded in lysosomes. EGF stimulation of canine epithelial cells induced a rapid and transient association of the SH3-SH2-SH3 protein GRB2 with dynamin, a guanosine triphosphatase that regulates endocytosis. Disruption of GRB2 interactions by microinjection of a peptide corresponding to the GRB2 SH2 domain or its phosphopeptide ligand blocked EGF receptor endocytosis; other SH2 domains that bind EGF receptors or antibodies that neutralize RAS did not. Both activation and termination of EGF signaling appear to be regulated by the diverse interactions of GRB2.

Sites of tyrosine autophosphorylation in activated EGF receptors (EGFRs) bind and thereby activate signaling proteins that contain phosphotyrosine (pTyr)-binding domains such as SH2 and PTB domains (*1*). For example, the adapter proteins GRB2 and SHC bind activated EGFRs and participate in EGF-induced activation of RAS (*2*). Concomitant with their binding to PTB and SH2 proteins, activated EGFRs interact with adaptins (*3*) and are internalized by way of clathrin-coated pits into the endocytotic compartment (*4*). EGFR association with the adaptin AP-2 is not required for EGFR internalization (*5*), but receptors that lack kinase activity or pTyr sites are poorly internalized or show abnormal intracellular trafficking (*6–8*). Therefore, EGFR substrates or EGFR-binding signaling proteins, or both, may be required for receptor internalization and trafficking. GRB2 binds to the RAS guanine nucleotide exchange factor SOS (*9*) and interacts through its SH3 domains with polyproline motifs located in the COOH-terminal regulatory region of the dynamin guanosine triphosphatase (GTPase) and with other proteins (*10*). Dynamin plays an essential role in coated vesicle formation (*11*) and localizes at the plasma membrane around the neck of emerging coated pits (*12*). We now show that GRB2 is required for endocytosis of the EGFR, and demonstrate a transient EGF-induced association of GRB2 with dynamin.

EGFRs are located at the surface of Maden Darby canine kidney (MDCK) cells deprived of serum. However, 30 min after stimulation with EGF, the receptors were internalized and accumulated in perinuclear vesicles characteristic of endosomes and lysosomes (Fig. 1A). When cells were microinjected (*13*) with a mixture of eight glutathione-S-transferase (GST) fusion peptides containing the SH2 domains of signaling proteins that bind activated EGFRs [GRB2, SHC, and two each from p120 RAS–GTPase-activating protein (RAS-GAP), the p85α subunit of phosphatidylinositol 3-kinase (PI3K), and phospholipase C–γ1 (PLC-γ1)] and then

Banting and Best Department of Medical Research and Department of Molecular and Medical Genetics, University of Toronto, 112 College Street, Toronto M5G 1L6, Canada.

*To whom correspondence should be addressed.