TNF-α (30 ng/ml) or with PMA (1 μM).
13. Cell lysis, Pyk2 immunoprecipitations, and immunoblotting were carried out as previously described (*11*). Polyclonal antibodies to amino acids 684 to 1009 of human Pyk2 cDNA (anti-Pyk2) expressed as a GST fusion protein in pGEX-2T (Pharmacia Biotech) and phosphotyrosine antibodies were used. Equal amounts of total cell lysate (500 to 700 μg) as determined by Bradford assay (Bio-Rad) were subjected to immunoprecipitation.
14. GST–c-Jun(1–79) fusion proteins were produced and bound to agarose beads as described (*7*). Bound protein (2 μg) was used in solid-phase JNK assays. Lysates were incubated with the GST fusion protein, and after JNK proteins were bound, the beads were washed extensively, [γ-^{32}P]adenosine triphosphate was added, and the phosphorylation of the probe was analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. JNK activity was determined by quantitation of the amount of GST–c-Jun(1–79) phosphorylation with a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics).
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16. 293T cells grown on 10-cm plates were transiently transfected [lipofectamine reagent (Gibco BRL)] with various amounts (5, 10, or 15 μg) of expression vector alone (pRK5); 5 or 10 μg of Pyk2 expression vector together with various amounts of pRK5 (5 or 10 μg); or 5 μg of Pyk2 together with 10 or 20 μg of a dominant-negative mutant of Pyk2 (PKM). COS and PC-12 cells on 10-cm plates were transiently transfected with 10 or 20 μg of pRK5 and 10 or 20 μg of PKM. After 48 hours, the cells were treated as described (*11*, *13*).
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22. We thank M. Karin, A. Lin, A. Abo, J. Vilcek, and E. Skolnik for plasmids and reagents; S. Earp and members of the Schlessinger lab for their helpful discussions; M. Cleary and G. Rodrigues for comments on the manuscript; and J. Small for dedicated secretarial assistance. This work was supported by a grant from SUGEN (J.S.).

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Cooperative DNA Binding and Sequence-Selective Recognition Conferred by the STAT Amino-Terminal Domain

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STAT proteins (signal transducers and activators of transcription) activate distinct target genes despite having similar DNA binding preferences. The transcriptional specificity of STAT proteins was investigated on natural STAT binding sites near the interferon-gamma gene. These sites are arranged in multiple copies and required cooperative interactions for STAT binding. The conserved amino-terminal domain of STAT proteins was required for cooperative DNA binding, although this domain was not essential for dimerization or binding to a single site. Cooperative binding interactions enabled the STAT proteins to recognize variations of the consensus site. These sites can be specific for the different STAT proteins and may function to direct selective transcriptional activation.

Cytokines activate intracellular signaling pathways during immune and inflammatory responses. One of the signal-transduction pathways activated by these ligands involves the STAT family of proteins (*1*). STAT proteins are selectively recruited to various cytokine receptors by virtue of their peptide-binding specificities mediated by their SRC homology 2 (SH2) domains (*2*). Thus, part of the specificity of STAT activation relies on selective recognition of phosphotyrosine-containing peptides of cytokine receptors. After they are tyrosine phosphorylated, the STAT proteins dimerize and migrate to the nucleus where they bind to specific DNA sequences and elicit various programs of gene activation. The mechanisms that determine STAT specificity in transcriptional regulation are unclear because most of the STAT proteins have similar DNA binding preferences (*3*, *4*).

The STAT proteins contain a DNA binding domain that is located in the center of the protein, approximately from residues 350 to 500 (*3*). Other functional domains include the SH2 domain and a phosphotyrosine-containing region near the COOH-terminus that participate in dimerization. The transcription-activation domains are located at the COOH-terminal ends of the molecules (*5*). The NH_2-terminal region is also conserved among the STAT family. This region is required for phosphorylation of STAT2 in response to interferon-α (IFN-α) and may be required for receptor binding (*6*). It is not known whether this region plays a similar role in other STAT proteins or whether the NH_2-terminal domain takes part in the nuclear function of these proteins as transcription factors.

We investigated the mechanisms of STAT regulation in response to interleukin-12 (IL-12), a key cytokine that regulates cell-mediated immunity (*7*). Treatment of cells with IL-12 results in tyrosine phosphorylation and activation of STAT4 (*8*). A full-length STAT4 cDNA was cloned and expressed in baculovirus-infected insect cells (*9*). The DNA binding preference of STAT4 was determined by the random binding site selection method (*10*), and the optimal sequence for STAT4 was TTCCGGGAA (*11*). The sequence selected by STAT4 is identical to that of optimal sites for STAT1 and STAT3 (*3*). STAT5 also has a similar binding preference (*12*).

We also searched for natural STAT4 sites near the IFN-γ gene. IFN-γ is activated in response to IL-12 in T cells and natural killer cells, and many of the physiological effects of IL-12 appear to be mediated by IFN-γ (*7*). IL-12–mediated activation of IFN-γ expression is eliminated in STAT4-deficient mice (*13*). The human IFN-γ gene is organized on four exons (*14*). A 9-kb region spanning the gene contains the necessary cis-regulatory sequences for the correct T cell–specific and inducible transcription of IFN-γ (*15*). The first intron contains a deoxyribonuclease I (DNase I)–hypersensitive site, ~0.5 to 1 kb downstream of the transcription start site (*16*). Using DNase I footprinting (*17*), we detected binding sites for STATs 1, 4, 5, and 6 in the first intron (Fig. 1A). On the basis of our binding site selection results, we expected that STATs 1, 4, and 5 might have the same site preferences. Instead, we observed that each of the STAT proteins bound to a distinct pattern of adjacent sites in the first intron. The protected regions contain multiple sites that are variations of STAT consensus binding sequence (Fig. 1B).

The STAT4 footprint spans ~30 base pairs (bp) of DNA and contains two adjacent binding sites, designated 2 and 3 (Fig. 1B). Each binding site is a variant of the consensus sequence, and the two sites are spaced by 10 bp (*18*). The STAT4 binding sites vary from the optimum sequence in two ways. In both cases the sites are imperfect palindromes, with TTC in one half-site and TTA in the other half-site, and the central three nucleotides are different from those in the optimal site, which has CGG in the middle.

STAT1 also bound to site 3. STAT1 protected a large region that extends downstream and appears to contain at least four additional sites, 4, 5, 6, and 7. The other STAT proteins tested did not bind well to sites 4 through 7 (Fig. 1A). STAT5 bound to sites 2 and 3 and also to a third site that was immediately upstream, site 1. STATs 1 and 4 did not bind to site 1, and STAT6 bound only to site 1. The results indicate that STAT proteins can have selective

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DNA binding preferences when assayed on natural sites. These sites may be physiologically relevant because cytokines that induce each of these STAT proteins regulate IFN-γ expression (*19*).

The differences between the STAT binding sites in the IFN-γ first intron and the optimal site indicated that, individually, the natural sites that are selectively bound might be lower in affinity. To determine whether cooperative interactions between the adjacently bound STAT dimers might occur, we measured STAT4 binding to three different oligonucleotides: one that was mutated in site 2, another that was mutated in site 3, or an oligonucleotide that contained both sites (*18*). Mutation of either site reduced STAT4 binding, indicating that cooperative interactions contribute to STAT4 recognition of these adjacent sites (Fig. 2A).

An NH_2-terminal deletion mutant that lacks the first 88 amino acids of the protein was active for DNA binding when com-

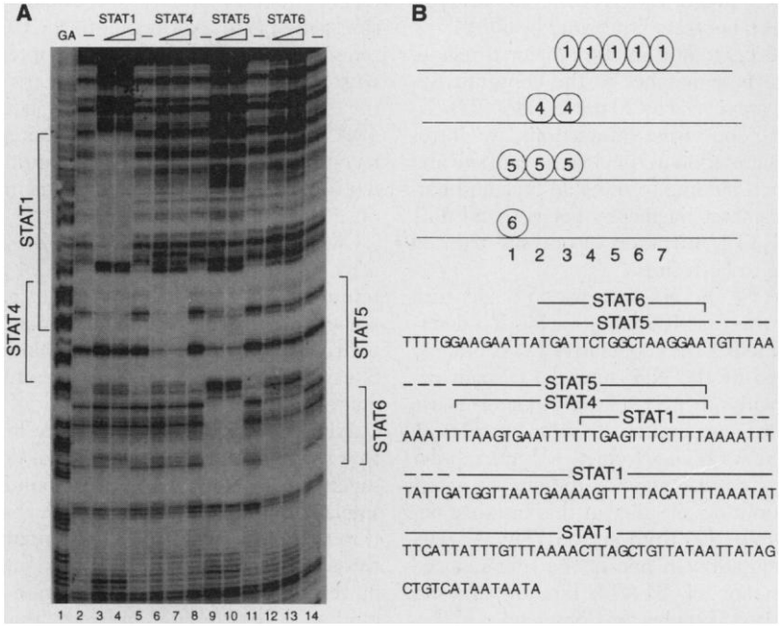

Fig. 1. Selective DNA binding by STAT proteins to clustered sites. (**A**) The DNA binding of STATs 1, 4, 5, and 6 was compared on a fragment located in the first intron of the IFN-γ gene. Each one of the STATs produced a distinct pattern of binding to adjacent sites. The positions of the protected regions are indicated at the sides of the gel. GA indicates the purine sequence of the fragment. (**B**) Sequence of the region containing the STAT binding sites. The footprinted regions indicated above the sequence are composed of multiple adjacent sites. The exact number of STAT1 sites within the protected region is unclear. Higher resolution methods are required to precisely determine the contact sites within the DNase I footprinted regions.

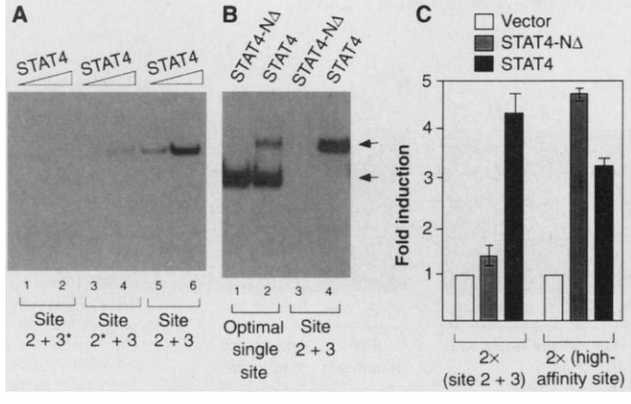

Fig. 2. Requirement of interactions mediated by the NH_2-terminal domain for cooperative DNA binding by STAT proteins. (**A**) Oligonucleotides corresponding to the STAT4 binding sites from the first intron were tested for binding. Reactions in lanes 1 and 2 contained a labeled oligonucleotide mutated in site 3, for lanes 3 and 4 binding site 2 was mutated, and for lanes 5 and 6 the wild-type sequence was used. Reactions in lanes 1, 3, and 5 contained 3 ng of STAT4, and those in lanes 2, 4, and 6 contained 10 ng of STAT4. (**B**) Binding to a single high-affinity site (lanes 1 and 2) but not adjacent sites in IFN-γ first intron (lanes 3 and 4) in the absence of the STAT4 NH_2-terminal domain. The protein-DNA complex that appears to be a tetramer is indicated by the upper arrow. The position of a dimer bound to the DNA is indicated by the lower arrow. (**C**) Requirement of the NH_2-terminal domain for transcriptional activation by STAT4 through the IFN-γ double sites but not through high-affinity STAT sites. Values shown are the averages of at least three independent transfections. Error bars indicate the standard deviation.

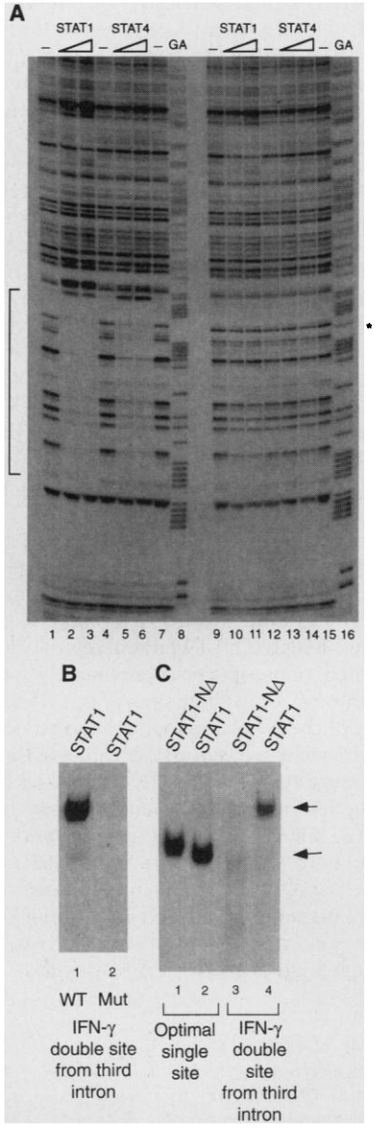

Fig. 3. Cooperative binding of STAT1 to adjacent sites. (**A**) Fragments spanning the IFN-γ promoter (−350 to +50) were labeled on the upstream end and incubated with either STAT1 or STAT4. Lanes 1 to 8, wild-type fragment; lanes 9 to 16, mutant fragment. A double point mutation was created in the downstream site (asterisk). (**B**) Requirement of the NH_2-terminal domain of STAT1 for cooperative binding. Binding of STAT1 to the wild-type (WT) probe (lane 1), which contains a double site from the downstream intron, or a probe with point mutation (Mut) in one of the sites (lane 2). (**C**) Requirement of the NH_2-terminal domain of STAT1 for cooperative binding (lanes 3 and 4) but not for binding to a single site (lanes 1 and 2). Arrows indicate the positions of the dimeric and tetrameric complexes.

pared with the full-length protein for binding to an optimal single site (Fig. 2B). This result indicated that the NH_2-terminal region of STAT4 is not required for tyrosine phosphorylation by JAK protein kinases, dimerization, or DNA binding to a single site. In contrast, the STAT4 deletion mutant was completely inactive for binding to the double site from the IFN-γ first intron (Fig. 2B). On the double-site fragment, the full-length STAT4 produced a protein-DNA complex with lower mobility, consistent with the size of a tetramer. On the single-site probe this larger complex was also detectable, suggesting that the protein-protein interaction can occur even when only one of the dimers is bound specifically to DNA. The inability of the deletion mutant to recognize the STAT4 sites from the IFN-γ gene indicates that the NH_2-terminal region is required for cooperative binding interactions between adjacently bound STAT4 dimers.

To determine whether the STAT4 protein could function through the sites from the IFN-γ gene to activate transcription, we did cotransfection experiments with STAT4 in COS cells. Two reporter genes were tested containing either the STAT4 binding sites from the IFN-γ first intron or high-affinity STAT sites from the IRF-1 promoter (*20*). The full-length STAT4 protein reproducibly activated transcription approximately fourfold through the IFN-γ sites (Fig. 2C). Deletion of the NH_2-terminal domain reduced STAT4-mediated activation through these sites, consistent with the DNA binding experiments. The reduced activity was not due to lower expression or phosphorylation; both proteins were expressed and active for DNA binding to a single site (*21*). Furthermore, the NH_2-terminal domain was not essential for activation through high-affinity STAT sites that do not require cooperative binding (Fig. 2C).

We have identified iterated STAT sites in other locations near and within the IFN-γ gene, including the 5′ flanking region and in the downstream intron. To determine whether cooperative DNA binding was also required for other sites, we compared binding of STAT1 and STAT4 to an adjacent pair of sites located in the promoter (−280 to −240) region of the IFN-γ gene. These sites were recognized by STAT4 and STAT1 (Fig. 3A), but were not bound by STAT5 or STAT6 (*21*). Within the protected region, the two best matches to the consensus sequence are spaced by 20 nucleotides (*22*). To test for cooperative interactions, we introduced a mutation in the downstream site and compared binding to the wild-type and mutant promoter fragments. For both STAT1 and STAT4, alteration of one site reduced binding to both sites.

STAT4 and STAT1 are 55% identical within the first 100 amino acids. To determine whether the cooperative DNA binding mediated by the NH_2-terminal domain occurred with other STAT family members, we examined cooperative binding by STAT1 (*23*). STAT1 also formed a higher order complex consistent with the size of a tetramer on a double site, in this instance derived from the third intron (Fig. 3B). A point mutation in one of the sites reduced the amount of STAT1 binding and no dimer-sized complex was observed, indicating that mutation of one of the sites affected binding to the neighboring site (Fig. 3B). Deletion of 44 NH_2-terminal residues of STAT1 did not affect binding to a single high-affinity site, but the mutant did not bind to a pair of low-affinity STAT1 sites (Fig. 3C). Thus, the requirement of the NH_2-terminal domain for cooperative DNA binding appears to be conserved among various members of the STAT family.

We examined the effects of antibodies directed against either the NH_2-terminal or COOH-terminal regions of STAT4 on DNA binding. Antibodies to the NH_2-terminal region (amino acids 10 to 27) did not supershift the STAT4 protein–DNA complex on the double-site probe (Fig. 4). Indeed, they appeared to disrupt the interaction between STAT4 dimers. These NH_2-terminal antibodies did supershift the STAT4 protein when it was bound to the single-site probe. Antibodies to the COOH-terminal region of the protein interacted with the STAT4-DNA complex on either the single-site or double-site probes (Fig. 4A). These results suggest that the epitope recognized by the NH_2-terminal antibody is not accessible when STAT4 dimers interact on the DNA.

We expressed and purified a 124–amino acid polypeptide from the STAT4 NH_2-terminus (*24*). Addition of increasing amounts of the NH_2-terminal domain competitively inhibited cooperative binding of STAT4 (Fig. 4B). The NH_2-terminal domain is not a nonspecific inhibitor of DNA binding, because it did not reduce binding to a single site. The NH_2-terminal domain supershifted a STAT4 dimer bound to a single site and enhanced DNA binding (Fig. 4B), suggesting that interaction through the NH_2-terminal domain may stabilize dimer formation and facilitate DNA binding. These data indicate that the STAT4 NH_2-terminal domain participates in protein-protein interactions resulting in tetramerization required for cooperative binding.

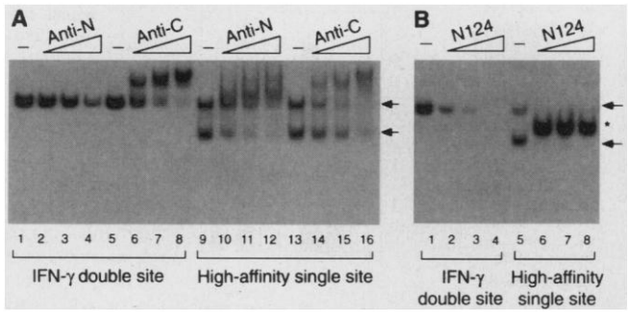

Fig. 4. Mediation of tetramerization required for cooperative DNA binding by the NH_2-terminal domain of STAT4. (**A**) Inhibition of cooperative binding but not binding to a single site by antibodies directed against the NH_2-terminal domain. STAT4 protein was tested for binding to the IFN-γ double site (lanes 1 to 8) or a high-affinity single site (lanes 9 to 16). Antibodies (100, 200, or 400 ng) specific for a peptide from the NH_2-terminal domain (residues 10 to 27) or the COOH-terminal domain (residues 729 to 748) were added to the binding reactions as indicated above the gel. Lower arrow, dimeric complex; upper arrow, tetrameric complex. (**B**) Interaction of the NH_2-terminal domain with full-length STAT4. The NH_2-domain of STAT4 (N124) was expressed in *Escherichia coli* and purified. Inhibition of STAT4 binding to the IFN-γ double-site probe by the polypeptide (lanes 1 to 4). Either 50, 100, or 200 ng of the N124 were added. Complex formation between the NH_2-terminal domain and the full-length DNA-bound STAT4 (lanes 5 to 8). The dimer complex was shifted to a slower mobility indicated by the asterisk.

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the baculovirus expression vector pVL1393 (Invitrogen) with a His tag at the COOH-terminal end. Proteins were purified by Ni-affinity chromatography (Qiagen). STATs 1, 5, and 6 were expressed and purified by identical methods. For DNA binding studies, insect cells were infected with recombinant viruses encoding JAK1 and one of the STATs.
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17. The binding reactions were carried out in 50 mM KCl, 12.5 mM Hepes (pH 7.9), 0.1 mM EDTA, 5% glycerol, 0.01% NP-40, 1 mM $NaVO_4$, 1 mM NaF, 1 mM β-glycerophosphate, and 1 μg of poly(dI-dC). The amount of STAT proteins in the binding reactions was between 50 and 500 ng. Only a small fraction of the purified STAT proteins was tyrosine phosphorylated and active for DNA binding. A Hind III to Sac I fragment that was labeled at the Hind III site was used for the footprints.
18. Gel mobility–shift reactions were done with the same buffer as used for the footprint reactions with the addition of 2 mM $MgCl_2$. The sequences of the oligonucleotides were as follows: the high-affinity single site, TTATGTTTCCGGGAAATGAG; site 2 + 3, CGCGAAAATTTTAAGTGAATTTTTTGAGTTTCTTTTAAAATTTT; site 2* + 3, CGCGAAAATTgAGTGgTTTTTTTGAGTTTCTTTTAAAATTTT; and site 2 + 3*, CGCGAAAATTTTAAGTGAATTTTTTGAGTccCTTTTttAATTTT. The positions of the consensus binding sites are underlined, and the positions of the altered bases are in lowercase letters. The antibodies used in Fig. 4A were obtained from Santa Cruz Biotechnology.
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20. COS-7 cells were transfected by the calcium phosphate precipitation method. Full-length STAT4 and the NH_2-terminal deletion mutant were cloned into the cytomegalovirus vector pRK-5. JAK1 was expressed from the plasmid pSRα. Luciferase reporter plasmids contained either two copies of the site 2 + 3 oligonucleotide or two copies of a high-affinity STAT site upstream of the herpes simplex virus thymidine kinase basal promoter (−50 to +10) in pGL2-basic. The high-affinity STAT site was derived from the IRF-1 promoter, GCCGTCATTTCGGGGAAATCA [S. H. Sims, Y. Cha, M. F. Romine, P. E. Gao, K. Gottleib, A. B. Deisseroth, *Mol. Cell. Biol.* **13**, 690 (1993)]. Each transfection contained 1.5 μg of the STAT4 and luciferase plasmids and 1 μg of Rous sarcoma virus β-galactosidase. The transfections with the IFN-γ sites also included 0.5 μg of the JAK1 expression vector. Transfection efficiencies were standardized by measurement of β-galactosidase activity.
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22. The sequence within the region protected by STAT1 and STAT4 is AGTCCTTGAATGGTGTGAAGTAAAAGTGCCTT*CAAAG*AATCCCC. The positions with the best match to the consensus binding site are underlined. Two nucleotides in the downstream site indicated by the asterisks were changed in the mutant fragment. The T was deleted and the G was changed to C.
23. Multiple STAT binding sites were detected by DNase I footprint analysis within a Kpn I to Acc I fragment from the downstream intron. Among these sites was a pair of sites that was specifically recognized by STAT1. The sequence with the protected region was ACCTTCTTTGCTCCAAAACTCTACAATGCAAAG*AATAGA, which corresponds to the sequence for the gel shift in Fig. 3C. A single point mutation was made by changing the G (indicated by the asterisk) to C.
24. A portion of the STAT4 cDNA encoding amino acids 1 to 124 with a His tag at the COOH-terminal end was subcloned into the T7 promoter expression vector pET-3A. Protein was expressed in the strain BL21(DE3) and purified by Ni-affinity chromatography.
25. We acknowledge K. Williamson for DNA sequencing and M. Rothe for help with cloning human STAT4. We thank U. Schindler, M. Rothe, T. Mikita, and S. McKnight for comments on the manuscript and helpful discussions, and Genetics Institute for IL-12. B. Schreiber and D. Goeddel provided valuable advice and reagents.

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Diffusional Mobility of Golgi Proteins in Membranes of Living Cells

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The mechanism by which Golgi membrane proteins are retained within the Golgi complex in the midst of a continuous flow of protein and lipid is not yet understood. The diffusional mobilities of mammalian Golgi membrane proteins fused with green fluorescent protein from *Aequorea victoria* were measured in living HeLa cells with the fluorescence photobleaching recovery technique. The diffusion coefficients ranged from 3×10^{-9} square centimeters per second to 5×10^{-9} square centimeters per second, with greater than 90 percent of the chimeric proteins mobile. Extensive lateral diffusion of the chimeric proteins occurred between Golgi stacks. Thus, the chimeras diffuse rapidly and freely in Golgi membranes, which suggests that Golgi targeting and retention of these molecules does not depend on protein immobilization.

The Golgi complex contains a large number of resident components that play important roles in the processing and sorting of secretory and membrane proteins, but how these components are maintained in the Golgi despite a continuous flow of protein and lipid through the secretory pathway is currently a topic of debate (*1*). Several mechanisms of Golgi protein retention have been suggested: oligomerization into structures too large to enter transport vesicles (*2*), lateral segregation into lipid microdomains (*3*), and recognition of retention or retrieval signals (*4*). In these models specific protein-protein or protein-lipid interactions underlie Golgi protein retention. Whether such interactions affect the dynamic properties of Golgi membrane proteins in vivo, including the diffusional mobility of Golgi proteins and trafficking of these proteins between Golgi stacks, has not been addressed.

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To probe for interactions that might underlie the retention of Golgi membrane proteins, we examined the diffusional mobility of Golgi membrane components using fluorescence photobleaching recovery (FPR). This technique is a powerful tool for investigating the environment of membrane proteins and has revealed several types of interactions that constrain the lateral diffusion of proteins in the plasma membrane (*5*). The dynamic properties of intracellular membrane proteins have not been thoroughly explored by FPR because of a lack of appropriate fluorescent labels. Here, we used the *Aequorea victoria* green fluorescent protein (GFP) (*6*) as a tag to investigate the diffusional mobility of four Golgi membrane proteins (*7*), mannosidase II (Man II), β-1,4-galactosyltransferase (GalTase), and wild-type and mutant forms of KDEL receptor (KDELR), within the Golgi and the endoplasmic reticulum (ER). We also used fluorescence loss in photobleaching to examine the extent of lateral membrane continuity between Golgi stacks, and within the ER.

Man II and GalTase are "resident" enzymes of the Golgi complex, which function in carbohydrate processing. In contrast, KDELR is an itinerant Golgi component, which recycles to the ER when it binds KDEL ligand (*8*). Substitution of asparagine for aspartic acid at position 193 in KDELR, denoted $KDELR_m$, prevents it from redistributing into the ER in the presence of high concentrations of ligand (*9*). GFP was fused