Addition of Cu(II) to a solution containing the native sequence protein with bound fluorescein produced no metal-dependent perturbation of the bound fluorescein fluorescence (Fig. 4C). In contrast, a dramatic effect was again observed with QM 212 protein, as increasing Cu(II) produced a marked decrease in the excitation maxima at 508 nm, leaving a residual peak at 493 nm. Saturation behavior was observed (see Fig. 4B), and since no large increase in the fluorescence intensity at 493 nm was seen (compare A and B in Fig. 4), Cu(II) was apparently not simply displacing fluorescein (*23*). Nevertheless, the precise placement of the metal site must await determination of the x-ray crystal structure of the mutant protein. These results are most consistent with Cu(II) binding simultaneously with fluorescein in the QM 212 binding site. Such simultaneous binding can be viewed as a noncatalytic model for substrate binding to a metalloantibody.

Catalytic metalloantibodies offer an opportunity to combine the enormous potential for substrate recognition of antibodies with the distinctive chemical opportunities afforded by metals. In effect, a particular antibody derived from a combinatorial library might position a catalytically active metal next to a specific locus or face of a substrate. In so doing, the antibody would define the regio or enatio sense of a metal-induced transformation and by using different antibodies, examination or alternative reactive sites on the substrate can be explored.

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10. We examined Cys, Asp, Glu, and His residues within 20 Å of the designed metal binding site to address the possibility of other ligands coordinating the metal (Zn) coincidentally. The nearest possible ligands, Asp^{1}, His^{27D}, and His^{93} [Kabat numbering (*9*)], which have potential binding sites 13 to 16 Å from the Zn atom, could not form bonds to the metal jointly with one of the engineered His residues without major rearrangements in the antibody structure. These rearrangements would involve major changes in the antibody binding site, blocking fluorescein binding, and can therefore be ruled out. In the heavy chain, Glu^{6} and Glu^{46} have their potential Zn binding atoms 13 to 16 Å from the Zn atom. Glu^{6} and Glu^{46} are framework residues separated from the Zn site by β-sheet structure. Only Asp^{101} could be a potential ligand without destroying fluorescein binding. The oxygen atoms of the Asp^{101} side chain are about 4 Å from the Zn atom in our model, so rearrangements would be required.
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23. Alteration of the bound fluorescein excitation spectrum upon addition of Cu(II) is most consistent with a static mechanism implying a metal binding event (*21*, p. 265). Several features of this data in Fig. 4B deserve special comment. First, it is assumed that only properly folded protein can bind fluorescein, so the observed effect of Cu(II) on the bound fluorescein maxima at 508 nm must be occurring in a correctly folded binding pocket. Second, under the conditions chosen for the experiment with QM 212, 70% of the fluorescein was bound and 30% remained in solution, thus explaining the shoulder that remained relatively unchanged at 493 nm. At higher concentrations of Cu(II) and Zn(II) fluorescein was displaced. Third, the perturbed nature of the bound fluorescein excitation maxima indicates that the Cu(II) is bound in close proximity to the fluorescein, offering further evidence that the Cu(II) is binding in the intended three-histidine site.

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A G Protein Mutant That Inhibits Thrombin and Purinergic Receptor Activation of Phospholipase A_2

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The stimulation of phospholipase A_2 by thrombin and type 2 (P_2)–purinergic receptor agonists in Chinese hamster ovary cells is mediated by the G protein G_i. To delineate α chain regulatory regions responsible for control of phospholipase A_2, chimeric cDNAs were constructed in which different lengths of the α subunit of G_s (α_s) were replaced with the corresponding sequence of the G_i α subunit (α_{i2}). When a carboxyl-terminal chimera $\alpha_{s\text{-}i(38)}$, which has the last 38 amino acids of α_s substituted with the last 36 residues of α_{i2}, was expressed in Chinese hamster ovary cells, the receptor-stimulated phospholipase A_2 activity was inhibited, although the chimera could still activate adenylyl cyclase. Thus, $\alpha_{s\text{-}i(38)}$ is an active α_s, but also a dominant negative α_i molecule, indicating that the last 36 amino acids of α_{i2} are a critical domain for G protein regulation of phospholipase A_2 activity.

THE RELATED G PROTEINS G_s AND G_i stimulate and inhibit adenylyl cyclase, respectively. The α_s and α_i subunit polypeptides are ~65% homologous in primary sequence (*1–3*) and share common βγ subunits (*4*), but selectively couple to different receptors (*5*). Despite their initial identification as inhibitors of adenylyl cyclase, the primary function of G_i proteins may actually be the regulation of ion channels, phospholipase A_2, and possibly phospholipase C (*6–8*). It has been concluded that G_i-like proteins regulate receptor-coupled phospholipases because these pathways are inhibited by treatment of cells with pertussis toxin, which adenosine diphosphate (ADP) ribosylates and inhibits G_i and G_o proteins (*9–11*), and because guanosine triphosphate (GTP) analogs stimulate phospholipase activities in permeabilized cells and membranes (*7, 8*).

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Recently, we (*12, 13*) and Masters *et al.* (*14*) have generated α_i-α_s chimeras in an attempt to define functional domains of α subunit polypeptides. One such chimera, $\alpha_{i\text{-}s(Bam)}$, encodes the NH_2-terminal 60% of α_{i2} and the COOH-terminal 40% of α_s, and behaves as a functional α_s that coupled to β-adrenergic receptors and activated adenylyl cyclase (*14*). Therefore, the COOH-terminal 40% of α_s must encode the structural domains required for receptor selectivity and effector enzyme regulation. The COOH-terminus involvement in receptor coupling has been further defined by pertussis toxin–catalyzed ADP ribosylation of a cysteine that is four residues from the end of the α_i chain, which results in the uncoupling of G_i from receptor activation (*2*). Furthermore, mutation of Arg^{389} to proline in α_s, which is six residues from the COOH-terminus, uncouples G_s from activation by β-adrenergic receptors (*15*). The chimera $\alpha_{s\text{-}i(38)}$, which has the last 38 amino acids of α_s substituted with the last 36 residues of α_{i2}, constitutively activates adenosine 3′,5′-monophosphate (cAMP) synthesis in Chinese hamster ovary (CHO) cells (*12*). The $\alpha_{s\text{-}i(38)}$ polypeptide produces both an accelerated rate and increase in maximal adenylyl cyclase stimulation by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), but has a normal guanosine triphosphatase (GTPase) activity (*12*). These observations indicated that mutations introduced by substitution of the α_{i2} sequence at the COOH-terminus of α_s dramatically altered the α_s regulation of the effector enzyme, adenylyl cyclase.

Pertussis toxin treatment of CHO cells inhibits thrombin- or purinergic receptor–stimulated arachidonic acid release by 70 to 80% (see below). The thrombin and purinergic receptor stimulation of phospholipase C in CHO cells was relatively small compared to that for phospholipase A_2, and pertussis toxin inhibited phospholipase C–mediated inositol lipid hydrolysis by only 40%. This indicated that pertussis toxin–sensitive G_i proteins are differentially involved in the regulation of phospholipases A_2 and C (*7, 8*).

We have examined phospholipase A_2 and C activities in CHO cells transfected with four α_s-α_i chimeras whose structures are shown in Fig. 1A. The $\alpha_{s\text{-}i(38)}$ chimera has approximately 10% of the polypeptide encoded by α_{i2} at the COOH-terminus. $\alpha_{i(54)\text{-}s}$ and $\alpha_{i\text{-}s(Bam)}$ are composed of approximately 10 and 60%, respectively, of the α_{i2} sequence at the NH_2-terminus with the remainder of the polypeptide being α_s. The $\alpha_{i(54)\text{-}s\text{-}i(38)}$ chimera is the combination of $\alpha_{i(54)\text{-}s}$ and $\alpha_{s\text{-}i(38)}$, where both ends of the polypeptide correspond to α_{i2} and the middle is α_s. Concentration-response and time-course analysis in representative CHO clones indicated that expression of $\alpha_{i\text{-}s(Bam)}$ or $\alpha_{i(54)\text{-}s}$ polypeptides had little effect on thrombin-stimulated arachidonic acid release (Fig. 1, B and C). In contrast, CHO cells expressing the $\alpha_{s\text{-}i(38)}$ chimera showed a diminution of thrombin-stimulated arachidonic acid release. The diminished arachidonic acid release in the $\alpha_{s\text{-}i(38)}$ clone relative to the wild-type, $\alpha_{i(54)\text{-}s}$ or $\alpha_{i\text{-}s(Bam)}$ clones was not due to an altered time course or thrombin concentration required for maximal phospholipase A_2 stimulation. This finding suggested that the chimeras having α_i COOH-terminal sequences inhibited thrombin regulation of phospholipase A_2 activity to levels similar to that achieved with pertussis toxin treatment (Fig. 1, B and C).

The inhibition of receptor-stimulated arachidonic acid release is a direct consequence of $\alpha_{s\text{-}i(38)}$ expression (Table 1). The lack of variability in thrombin-stimulated arachidonic acid release from multiple wild-type CHO clones indicated that clonal variation did not contribute to the inhibited response observed with $\alpha_{s\text{-}i(38)}$–expressing clones (Table 1). Furthermore, multiple independent clones expressing $\alpha_{i\text{-}s(Bam)}$ or $\alpha_{i(54)\text{-}s}$ revealed little or no effect of these chimeras on the thrombin-stimulated, phospholipase A_2–catalyzed release of arachidonic acid relative to the wild-type response. Similarly, transfection of plasmid without a cDNA insert (pCWneo) or expression of wild-type α_s was without effect, whereas expression of wild-type α_{i2} may have slightly enhanced the response. Expression of $\alpha_{s\text{-}i(38)}$ or $\alpha_{i(54)\text{-}s\text{-}i(38)}$, but not $\alpha_{i(54)\text{-}s}$ or $\alpha_{i\text{-}s(Bam)}$ also inhibited

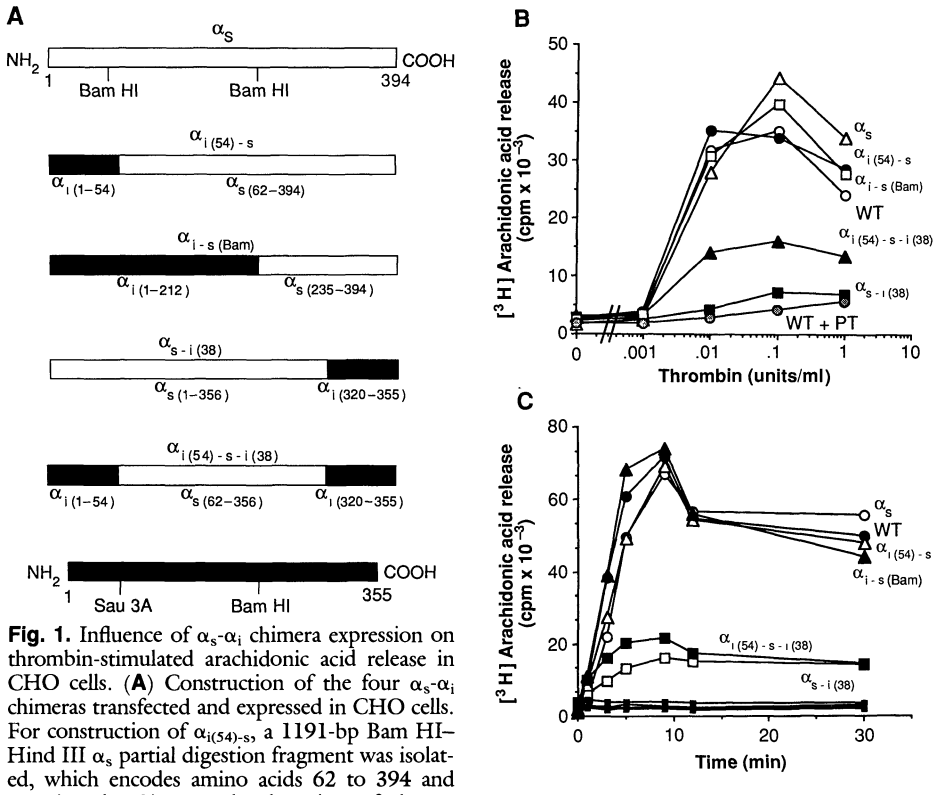

Fig. 1. Influence of α_s-α_i chimera expression on thrombin-stimulated arachidonic acid release in CHO cells. (**A**) Construction of the four α_s-α_i chimeras transfected and expressed in CHO cells. For construction of $\alpha_{i(54)\text{-}s}$, a 1191-bp Bam HI–Hind III α_s partial digestion fragment was isolated, which encodes amino acids 62 to 394 and contains the 3′-untranslated region of the α_s cDNA. A 318-bp α_{i2} fragment encoding the 5′-untranslated region and the first 54 codons of the α_{i2} cDNA was isolated from an Eco RI–Sau 3A digest. The chimeric $\alpha_{i(54)\text{-}s}$ cDNA was then assembled by ligation of the fragments and the identity verified by restriction analysis and DNA sequencing. The $\alpha_{i\text{-}s(Bam)}$ chimera was generated with the conserved Bam HI site in the α_s and α_{i2} cDNAs that encodes for a polypeptide having the first 212 residues of α_{i2} and the last 160 residues of α_s (*14*). The $\alpha_{s\text{-}i(38)}$ construct has been described (*12*), and $\alpha_{i(54)\text{-}s\text{-}i(38)}$ is the combination of the NH_2- and COOH-terminal chimeras. For expression, the α chain constructs were placed in the plasmid pCW1 (*12*) and transfected by electroporation of CHO cells (10^7) with 10 μg of plasmid DNA (Bio-Rad Gene Pulsar; 25 μF, 1 kV). G418-resistant colonies were isolated, subcloned, and screened for chimeric α_s-α_i expression by both Northern (RNA) blotting and immunoblotting (*12*). (**B** and **C**) Concentration-response and time-course analysis of thrombin-stimulated arachidonic acid release in transfected CHO cell clones. Representative clones expressing the indicated α chain construct or wild-type CHO cells were prelabeled with [^{3}H]arachidonic acid (*23*) and then incubated with (B) various concentrations of thrombin or (C) thrombin (0.1 unit/ml) for 9 min, and the [^{3}H]arachidonic acid release was determined (*23*). No significant difference in total [^{3}H]arachidonic acid incorporation or the phospholipid-labeling pattern was observed among clones in any experiment. The experiments are representative of two independent concentration-response and time-course analyses for each set of clones. Pertussis toxin (PT) treatment (20 ng/ml) of wild-type (WT) CHO cells was for 18 hours during the overnight [^{3}H]arachidonic acid labeling of cell lipids. Small solid symbols in (C) represent the basal arachidonic acid release for the clones in the absence of thrombin.

type 2 (P_2)–purinergic receptor regulation of arachidonic acid release (Table 1B). The magnitude of P_2-purinergic receptor inhibition was similar to that observed with the thrombin receptor, indicating that the inhibition was not receptor-selective. The phospholipase A_2 response in independent $\alpha_{s\text{-}i(38)}$ and $\alpha_{i(54)\text{-}s\text{-}i(38)}$ clones was inhibited 35 to 80% relative to the wild-type CHO cells, which correlated with the levels of expression of the transfected cDNA in various clones (*12, 16*).

Because the $\alpha_{s\text{-}i(38)}$ polypeptide behaves as a constitutively active α_s and activates adenylyl cyclase in CHO cells (*12*), we determined whether cAMP was causing the inhibition of thrombin-stimulated arachidonic acid release. Expression of the α_s point mutants $\alpha_s Val^{49}$ or $\alpha_s Leu^{227}$, which constitutively activate adenylyl cyclase (*17–19*), had no effect on thrombin-stimulated arachidonic acid release (Table 1A). Similarly, treatment of wild-type CHO cells with cell-permeable cAMP analogs in order to activate cAMP-dependent protein kinase had no influence on the thrombin-stimulated phospholipase A_2 activation (*20*). Only pertussis toxin treatment of wild-type CHO cells mimicked inhibition of thrombin-stimulated arachidonic acid release caused by expression of the $\alpha_{s\text{-}i(38)}$ or $\alpha_{i(54)\text{-}s\text{-}i(38)}$ chimeras (Fig. 1B). Our findings indicate that the α_{i2} domain at the COOH-terminus of the α subunit is responsible for the phospholipase inhibition phenotype, which could be observed when the $\alpha_{s\text{-}i(38)}$ polypeptide expression was approximately 20 to 25% of the wild-type α_i chain.

The inhibition by $\alpha_{s\text{-}i(38)}$ of inositol lipid hydrolysis by phospholipase C was qualitatively different from that observed for phospholipase A_2. First, the magnitude of the thrombin response is small, and both pertussis toxin and $\alpha_{s\text{-}i(38)}$ inhibited the phospholipase C stimulation only 40% (Table 2) as compared to the 70 to 80% inhibition observed with the phospholipase A_2 enzyme (Fig. 1 and Table 1). Second, expression of the $\alpha_{i(54)\text{-}s\text{-}i(38)}$ polypeptide had little effect on the thrombin-stimulated phospholipase C response. This contrasts with the regulation of the phospholipase A_2 response, where $\alpha_{i(54)\text{-}s\text{-}i(38)}$ was similar to $\alpha_{s\text{-}i(38)}$ in its ability to blunt thrombin-stimulated arachidonic acid release. Analysis of α_s-like characteristics of the chimeras has demonstrated that NH_2-terminal mutations in the $\alpha_{i(54)\text{-}s}$ construct influence the activation of adenylyl cyclase by the COOH-terminal $\alpha_{s\text{-}i(38)}$ mutant when both mutations are within the same α chain (*21*). Thus, our results indicate that $\alpha_{s\text{-}i(38)}$ and $\alpha_{i(54)\text{-}s\text{-}i(38)}$ are similar, but phenotypically distinct in their regulation of adenylyl cyclase and phospholipases. G_i is probably directly involved in regulating arachidonic acid release, but additional "G_p" proteins may be involved in controlling inositol lipid hydrolysis (*7, 8*).

Neither $\alpha_{s\text{-}i(38)}$ nor pertussis toxin influenced the intracellular free calcium ($[Ca^{2+}]_i$) rise in response to thrombin (Fig. 2), a finding consistent with the small effect of these agents on inositol lipid hydrolysis. This result also indicates that the $\alpha_{s\text{-}i(38)}$ or pertussis toxin inhibition of phospholipase A_2 activation was not the result of a blunted intracellular Ca^{2+} response to thrombin. Expression of the constitutively active $\alpha_s Leu^{227}$ polypeptide had no influence on thrombin-stimulated inositol phosphate release, indicating that the α_s-like property of the $\alpha_{s\text{-}i(38)}$ chimera was not responsible for the inhibition of phospholipase C (Table 2).

The $\alpha_{s\text{-}i(38)}$ chimera polypeptide is a dom-

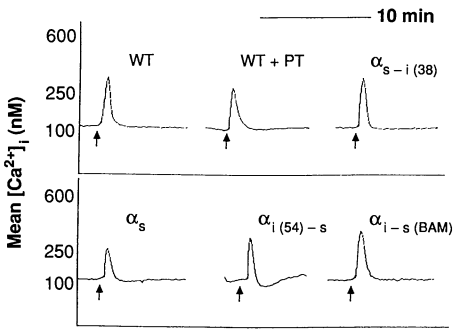

Fig. 2. Thrombin-stimulated elevation of intracellular Ca^{2+} in representative CHO cell clones. Wild-type (WT) and the designated CHO cell transfectants were grown to 50% confluency on plastic tissue culture dishes, washed in phosphate-buffered saline (PBS), and then suspended by incubation in PBS containing 450 μM EDTA. The suspended cells were washed in Ham's F-12 containing 5% fetal bovine serum (FBS), incubated for 1 hour at 37°C, and then pelleted and resuspended in Hank's balanced salt solution (HBSS, pH 7.0) containing 5 μM indo-1-acetoxymethyl ester (indo-1-AM) (Molecular Probes, Eugene, Oregon). After incubation for 30 min at 37°C to effect loading of indo-1-AM, cells were diluted 1:1 with HBSS (pH 7.4) containing 5% FBS, and incubated an additional 30 min at 37°C. The cells were then washed twice and resuspended in HBSS (pH 7.4) containing 5% FBS, and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by flow microfluorimetry with an Ortho System 50H with a 2150 computer analyzer with a 5-W argon laser (Ortho Diagnostic Systems, Westwood, Massachusetts) with 364-nm excitation (*27*). Fluorescence emissions were collected with a magnification objective lens and separated by a 465-nm long-pass dichroism steering filter (Ortho) into violet and blue emissions by passage through 390- or 490-nm band-pass filters, respectively (Oriel Corporation, Stratford, Connecticut). Ratio of linear emissions (390:490) for each cell was determined, and a histogram of fluorescence ratio as a function of cell number was created during 9-s increments. Measurements were at a flow rate of 600 to 700 cells per second at 37°C. Data represent means of $[Ca^{2+}]_i$ plotted as a function of time.

Table 1. Thrombin and purinergic receptor stimulation of phospholipase A_2 activity in CHO cell transfectants. (A) Wild-type CHO cells (CHO-WT) were subcloned and seven individual clones were tested for their [^{3}H]arachidonic release (means ± SEM) in response to thrombin (0.1 unit/ml) per 9 min (A). Independent CHO cell transfectants expressing the indicated α chain constructs were tested similarly for [^{3}H]arachidonic acid release in response to thrombin (A) or 0.5 mM of ATP (B). The pCWneo clone was transfected with the expression plasmid lacking a cDNA insert. α_s is a representative clone transfected with the wild-type α_s cDNA (*12*); $\alpha_s Val^{49}$ and $\alpha_s Leu^{227}$ are α_s chain mutants that constitutively stimulate cAMP synthesis resulting from inhibition of the α subunit GTPase (*17–19*). When more than one independent clone was tested, the number is designated in brackets next to each $\alpha_{s\text{-}i}$ construct; the response range for the clones is shown in parentheses next to the percent CHO-WT response.

Clone	Arachidonic acid release (cpm)		Percent CHO-WT response (range)
	Basal	Thrombin	
	(A) Thrombin-stimulated release		
CHO-WT [7]	1,940 ± 176	45,650 ± 1,650	100 (84–110)
$\alpha_{s\text{-}i(38)}$ [3]	2,557 ± 354	18,778 ± 5,859	41 (21–65)
$\alpha_{i(54)\text{-}s\text{-}i(38)}$ [3]	1,984 ± 86	20,347 ± 5,894	44 (19–53)
$\alpha_{i(54)\text{-}s}$ [3]	2,156 ± 93	46,578 ± 6,094	102 (88–102)
$\alpha_{i\text{-}s(Bam)}$ [3]	2,564 ± 313	48,398 ± 940	106 (102–109)
α_{i2} [4]	2,078 ± 242	56,379 ± 8,993	124 (80–162)
pCWneo	1,033	41,121	90
α_s	1,813	42,141	92
$\alpha_s Val^{49}$	2,997	47,369	104
$\alpha_s Leu^{227}$	2,138	47,617	104
	(B) ATP-stimulated release		
Clone	Basal	ATP	
CHO-WT	4,092	42,254	100
α_s	4,076	41,316	98
$\alpha_{i\text{-}s(Bam)}$	4,070	58,399	138
$\alpha_{i(54)\text{-}s}$	3,464	48,730	115
$\alpha_{s\text{-}i(38)}$	4,604	13,409	32
$\alpha_{i(54)\text{-}s\text{-}i(38)}$	2,966	26,736	63
α_{i2} [3]	3,689 ± 518	67,105 ± 3,518	158 (148–172)

inant negative G_i α subunit that inhibits thrombin and purinergic receptor activation of phospholipase A_2 and C pathways in CHO cells. Generally, dominant negative mutations have been defined in polypeptides that have multiple functional domains required for activity, where the function of one domain can be inhibited by mutation without influencing the activity of the other domains (*22*). In fact, G proteins have multiple domains including GTP binding, GTPase activity, βγ subunit association, receptor recognition, and effector interaction and regulation. The phenotype of CHO cells transfected with the $\alpha_{s\text{-}i(38)}$ chimera indicates that the α chain COOH-terminal phospholipase A_2 interaction domain is functional but the mutant polypeptide is unable to activate the enzyme. Experimental evidence for this conclusion is that expression of $\alpha_{s\text{-}i(38)}$ or pertussis toxin treatment inhibited ionomycin (a Ca^{2+} ionophore)-stimulated arachidonic acid release from CHO cells (Table 3), indicating that an α_i-like protein is required for maximal Ca^{2+}-stimulated phospholipase A_2 activity in CHO cells similar to that observed in guinea pig neutrophils (*23*). The inhibition of phospholipase A_2 activity by the $\alpha_{s\text{-}i(38)}$ polypeptide is thus distal to receptor–G protein coupling and the rise in $[Ca^{2+}]_i$. The $\alpha_{s\text{-}i(38)}$ inhibition of thrombin and purinergic receptor stimulation of phospholipase A_2 activity must be due to either its direct interaction with the phospholipase A_2 or a regulatory polypeptide required for both G_i and Ca^{2+} activation of the enzyme.

Additional experimental evidence supports the dominant negative character of the $\alpha_{s\text{-}i(38)}$ inhibition of phospholipase A_2 activation. First, immunoblotting analysis demonstrated that the expression of the endogenous α_i (α_{i2} and α_{i3}) polypeptides was not altered by expression of $\alpha_{s\text{-}i(38)}$ (*12*). The pertussis toxin–sensitive α_o polypeptide and transcript are not expressed in CHO cells (*20*), indicating that a change in expression of the wild-type α chains that are pertussis toxin substrates does not account for the $\alpha_{s\text{-}i(38)}$ phenotype. Second, cholate extracts from $\alpha_{s\text{-}i(38)}$–expressing CHO cells reconstituted the somatostatin-stimulated inhibition of adenylyl cyclase when mixed with membranes from S49 mouse lymphoma cells (*16*), indicating that the $\alpha_{s\text{-}i(38)}$ polypeptide does not inhibit wild-type G_i proteins or receptors coupled to G_i. Third, expression of $\alpha_{s\text{-}i(38)}$ did not influence the $[Ca^{2+}]_i$ rise in response to thrombin, indicating that this second messenger response was normal but that phospholipase A_2 activation was inhibited. Finally, the $\alpha_{s\text{-}i(38)}$ chimera activates adenylyl cyclase in CHO cells because of its character as a constitutively active α_s (*16*), which cannot be mimicked by pertussis toxin inhibition of G_i (*16*). The constitutive active nature of $\alpha_{s\text{-}i(38)}$ also indicates that it is not tightly associated with the βγ subunit complex, which would attenuate its activated state (*24*).

Our results define a role for α_{i2} in the G protein and Ca^{2+} control of receptor-regulated phospholipase A_2 activity. Dominant negative α chains offer distinct advantages over experimental treatment of cells with pertussis toxin because specific α chain sequences can be used to selectively inhibit pathways regulated by different G proteins. For example, cDNAs encoding the COOH-terminus of α_o or α_z could be used to test the postulated role of these α chains in phospholipase regulation (*25*). The demonstration of a dominant negative G protein α subunit construct, therefore, provides a genetic strategy for domain shuttling to dissect the role of specific G protein α chains, including those that are not toxin substrates, in the regulation of effector enzyme systems in hormone-responsive intact cell systems.

Table 3. Influence of pertussis toxin and $\alpha_{s\text{-}i(38)}$ expression on thrombin- and ionomycin-stimulated phospholipase A_2 activity. Cells were seeded, labeled with [^{3}H]arachidonic acid, and incubated with pertussis toxin (PT) as for Fig. 1. Cells were exposed to thrombin (0.1 unit/ml) or 2 μM ionomycin, and [^{3}H]arachidonic release was determined after a 9-min incubation. Each value is the mean of duplicate determinations and is representative of two experiments with each clone. Values in parentheses represent the percent CHO-WT response for each condition.

Clone	Arachidonic acid release (cpm)		
	Basal	Thrombin	Ionomycin
CHO-WT	2420	69,082 (100)	75,239 (100)
CHO-WT + PT	2054	13,778 (20)	41,341 (55)
$\alpha_{s\text{-}i(38)}$	4441	17,119 (25)	25,788 (34)
$\alpha_{i(54)\text{-}s\text{-}i(38)}$	2090	35,113 (50)	40,062 (53)

Table 2. Thrombin-stimulated inositol phosphate release in CHO cell transfectants. Wild-type and transfected CHO cells were seeded in 2.2-cm wells and allowed to reach 60% confluency. The cells were then labeled with *myo*-[2-^{3}H]inositol (2 μCi/ml, 23 Ci/mmol) for 24 hours in serum-free Dulbecco's modified Eagle's medium combined with Ham's F-12 (1:1) containing 0.1% bovine serum albumin (BSA). After labeling, the cells were washed three times and then incubated for 5 min in Dulbecco's modified Eagle's medium with Ham's F-12 and BSA containing 20 mM LiCl. The cells were stimulated with thrombin (0.1 unit/ml) for 30 min, fixed in ice-cold methanol:HCl (100:1), scraped, and placed into a tube containing chloroform (2 ml), H_2O (0.8 ml), and methanol:HCl (1 ml, 100:1). After mixing and centrifugation, the aqueous phase was passed over an AGI-X8 (200 to 400 mesh) formate anion-exchange column to isolate inositol phosphates (*26*). Total [^{3}H]inositol incorporation (means ± SEM) was determined by measuring the radioactivity in the organic phase. When more than one independent clone was tested, the number is shown in brackets next to the $\alpha_{s\text{-}i}$ construct. PT, pertussis toxin.

Clone	Total inositol phosphates per well (cpm)		Thrombin/ basal ratio
	Basal	Thrombin	
CHO-WT [4]	1247 ±77	3893 ± 123	3.2
CHO-WT + PT [3]	1094 ± 10	2106 ± 52	1.9
$\alpha_{s\text{-}i(38)}$ [3]	1309 ± 175	2495 ± 292	1.9
$\alpha_{i(54)\text{-}s}$ [3]	999 ± 164	3163 ± 522	3.2
$\alpha_{i(54)\text{-}s\text{-}i(38)}$ [3]	1073 ± 210	3173 ± 508	3.0
$\alpha_{i\text{-}s(Bam)}$ [3]	894 ± 92	3018 ± 556	3.4
α_s	1028 ± 16	3437 ± 25	3.3
α_i [2]	1162 ± 22	3418 ± 233	2.9
pCWneo	804 ± 52	3109 ± 127	3.2
$\alpha_s Leu^{227}$ [2]	1586 ± 75	4659 ± 155	2.9

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Failure to Phosphorylate the Retinoblastoma Gene Product in Senescent Human Fibroblasts

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Heterokaryon studies suggest that senescent and quiescent human diploid fibroblasts (HDF) contain a common inhibitor of entry into S phase. DNA synthesis can be induced in senescent and quiescent HDF by fusing them with cells containing DNA viral oncogenes such as SV40 T antigen, adenovirus E1A, or human papillomavirus E7. Both senescent and quiescent HDF contained the unphosphorylated form ($p110^{Rb}$) of the retinoblastoma protein, a putative inhibitor of proliferation. After serum stimulation, senescent HDF did not phosphorylate $p110^{Rb}$ and did not enter S phase, whereas quiescent HDF phosphorylated $p110^{Rb}$ and entered S phase. These findings, combined with the observations that T antigen, E1A, and E7 form complexes with, and presumably inactivate, unphosphorylated $p110^{Rb}$, suggest that failure to phosphorylate $p110^{Rb}$ may be an immediate cause of failure to enter S phase in senescent HDF.

HUMAN DIPLOID FIBROBLASTS (HDF) have a finite proliferative life-span at the end of which the cells remain alive in a G_1-arrested senescent state (*1*). Previous studies of the interaction of senescent HDF and replicating HDF in heterodikaryons have suggested that senescent HDF contain an inhibitor of entry into S phase because (i) they cannot be induced to synthesize DNA by fusion to replicating HDF and (ii) entry into S phase was inhibited in the replicative nuclei in these heterokaryons (*2*). Carcinogen-transformed HDF and several human tumor-derived cell lines were similarly inhibited from initiating DNA synthesis in heterodikaryons formed with senescent HDF (*3*). However, SV40-transformed HDF, adenovirus 5 early region–transfected human kidney cells (line 293), and HeLa cells, which express human papillomavirus (HPV) 18 DNA sequences (*4*), were not sensitive to inhibition by senescent HDF; rather, they were able to induce DNA synthesis in the senescent nuclei in heterodikaryons (*3, 5*). When young HDF were made quiescent by either serum deprivation or high cell density, they exhibited a similar inhibitory activity in the heterokaryon assay and were also induced to synthesize DNA by fusion with SV40-transformed HDF, 293 cells, and HeLa cells (*6, 7*). These data suggest the possibility (*3, 6*) that a common inhibitor was operating in senescent and quiescent cells and that cells transformed by certain DNA tumor viruses have a transforming factor that can override the putative inhibitor of DNA synthesis. Is the hypothesis of a common inhibitor consistent with the fact that the inhibition is reversible by serum stimulation in quiescent HDF but not in senescent HDF? This could occur because of changes in the ability of the senescent cells to respond to mitogens such that they are functionally mitogen-deprived even when they are fed with fresh serum.

SV40 T antigen, adenovirus E1A, and HPV E7 each can form a complex with the retinoblastoma susceptibility gene product (RB) (*8–10*). RB is considered to be an inhibitor of cell proliferation on the basis of its absence or inactivation in retinoblastoma and a subset of other human tumors and the ability of the wild-type gene (*RB1*) to suppress tumorigenicity when reintroduced into retinoblastoma and osteosarcoma cells (*11*). RB, which is unphosphorylated in G_0 or G_1 phase cells, becomes phosphorylated at the G_1/S boundary, suggesting that phosphorylation may be necessary for entry into S phase (*12–15*). As SV40 T antigen binds only the unphosphorylated form of RB (*16*), a simple hypothesis is that unphosphorylated RB inhibits entry into S phase and that inhibition can be relieved by either phosphorylation or binding to one of the three aforementioned viral oncogenes.

As an initial step in our study of the role of RB in cellular senescence, we investigated whether or not *RB1* is overexpressed in senescent HDF. Northern blots containing equal amounts of polyadenylated RNA from replicating, quiescent, and senescent HDF were analyzed for expression of *RB1* (*17*) and the cytoplasmic β-actin gene (*18*). The intensity of the RB band relative to the band representing β-actin was ~35% less for senescent HDF than for replicating HDF or quiescent HDF. This comparison may underestimate the difference in *RB1* expression because a recent report (*19*) suggests that β-actin expression in senescent HDF is about half of that in replicating HDF or quiescent HDF. In any case, our data clearly show that *RB1* is not overexpressed in senescent HDF.

The synthesis of RB in senescent HDF was examined by immunoprecipitation of ^{35}S-labeled proteins with the C36 monoclonal antibody (*8*) to human RB (Fig. 1A). The senescent cells, which had failed to achieve even one population doubling (PD) in 3 weeks with weekly refeeding, had predominantly the unphosphorylated main form of RB ($p110^{Rb}$). There was little or no phosphorylated main band, which can be seen as $pp112–116^{Rb}$ in the replicating cell profile. In addition, there was no incorporation of ^{32}P in this region in immunoprecipitates prepared from senescent HDF metabolically labeled with [^{32}P]orthophosphate (*20*). In other studies, phosphorylation of the 112- to 116-kD bands in replicating cells has been shown by metabolic labeling with [^{32}P]orthophosphate and by sensitivity to alkaline or acid phosphatase (*12, 14–16*). The phosphorylated character of these bands in replicating IMR-90 was confirmed by showing that they were sensitive to digestion with alkaline phosphatase (Fig. 1B).

In a few studies, additional lower molecular weight forms of RB ($p98^{Rb}$ and $pp100–104^{Rb}$) have been described that were attributed to translation of RB beginning at a

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