Assembly of the *Drosophila* phototransduction cascade into a signalling complex shapes elementary responses

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The subcellular compartmentalization of signalling molecules helps to ensure the selective activation of different signal-transduction cascades within a single cell[1]. Although there are many examples of compartmentalized signalling molecules, there are few examples of entire signalling cascades being organized as distinct signalling complexes. In *Drosophila* photoreceptors, the InaD protein, which consists of five PDZ domains, functions as a multivalent adaptor that brings together several components of the phototransduction cascade into a macromolecular complex[2–5]. Here we study single-photon responses in several photoreceptor mutant backgrounds, and show that the InaD macromolecular complex is the unit of signalling that underlies elementary responses. We show that the localized activity of this signalling unit promotes reliable single-photon responses as well as rapid activation and feedback regulation. Finally, we use genetic and electrophysiological tools to illustrate how the assembly of signalling molecules into a transduction complex limits signal amplification *in vivo*.

Phototransduction in *Drosophila* occurs through a phosphoinositide-mediated and calcium-regulated G-protein-coupled transduction pathway, in which light-mediated activation of rhodopsin leads to the sequential activation of a heterotrimeric G protein, an eye-specific phospholipase C (PLC) and the gating of the transient receptor potential (TRP) and TRPL light-activated ion channels[6,7]. The InaD scaffold protein assembles several components of this cascade, including TRP, PLC and protein kinase C (PKC), into an organized protein–protein complex[2–5]. Null *inaD* mutants have a marked disruption of the subcellular distribution of signalling molecules, a loss of transduction complexes and severely impaired photoresponses[2].

Drosophila photoreceptors can 'report' activity with high sensitivity and specificity: photoreceptor cells are sensitive to single photons, and the signalling pathway can be turned on and off with millisecond kinetics (phototransduction in *Drosophila* is the fastest known G-protein cascade, taking just a few tens of milliseconds to go from light activation of rhodopsin to the generation of a receptor potential)[6,7]. In photoreceptor neurons, the elementary response to a single photon of light is known as a quantum bump[8–10]. A quantum bump results from the opening (or closing) of light-activated ion channels in response to one activated rhodopsin molecule, and reflects the amplification of the entire visual cascade. We postulated that the signalling kinetics of photoreceptor neurons may be a direct consequence of organizing signalling molecules into architecturally defined signalling units. We reasoned that InaD may function as a master scaffold to assemble the phototransduction machinery[42]; and therefore sought to determine whether individual InaD macromolecular complexes embody elementary responses.

We studied light-induced currents in wild-type and mutant photoreceptor neurons by performing whole-cell patch-clamp recordings under conditions that activate single rhodopsin molecules[11]. Mutants lacking InaD (*inaD*^{1} mutants) have single-photon responses that are grossly disorganized, with large defects in amplitude, latency and deactivation (Fig. 1 and Table 1). Quantum bumps in *inaD*^{1} mutants have amplitudes that are less than one-fifth those of controls, latencies that are roughly six times greater than wild-type latencies and responses that fail to terminate normally, with single photons producing several microbumps. These results demonstrate a critical role for InaD in quantum-bump generation and substantiate a requirement for the InaD signalling complex in both activation and feedback regulation.

If the InaD signalling complex provides the molecular framework of a quantum bump, it should be possible to manipulate quantum bumps by selectively manipulating the InaD complex. We therefore studied quantum bumps in *inaD*^{2} photoreceptors, a mutant defective in the fifth PDZ (postsynaptic density protein, discs-large, ZO-1) domain of InaD; this mutant fails to recruit PLC to the transduction complex[2]. As predicted, *inaD*^{2} mutants show severe bump defects, with latency times over eight times larger than those of wild-type controls (Fig. 1 and Table 1). This activation phenotype is consistent with a dramatic reduction of PLC levels in transduction complexes, and resembles the phenotype produced by *norpA* hypomorphic alleles (see below). However, *inaD*^{2} mutants, unlike *norpA* hypomorphs, have nearly normal levels of PLC[2], showing that it is not simply the presence of PLC that is important for function, but rather its location.

A quantum bump represents the opening of many ion channels[12]; therefore, an InaD signalling complex (transducisome) must contain several InaD molecules and ion channels assembled into a macromolecular complex. If a quantum bump represents the output of a localized signalling complex, then fundamental properties of a quantum bump, such as amplitude, latency, rise and decay[13], should reflect the activity of individual signalling complexes. A prominent feature of invertebrate quantum bumps is their significant variability in amplitude (Fig. 1). This broad distribution in amplitude has been thought to result from randomness in the amplification process, leading to stochastic fluctuations in the number of activated ion channels per rhodopsin molecule[13]. We presumed that the variability in bump amplitude may instead reflect variation in the composition of different signalling com-

Table 1 Kinetics of quantum bumps

Mutant	Number of bumps	Number of trials	Mean frequency of events	Relative light stimulus	Latency (ms)	Amplitude (pA)
WT	147	480	0.36	1	66 ± 24	36 ± 19
inaD^1	73	336	0.24	1	385 ± 374	8 ± 7
inaD^2	68	240	0.33	1	481 ± 389	19 ± 11
chp	65	192	0.41	1	160 ± 120	27 ± 16
norpA^{H52}	87	360	0.28	1	372 ± 270	29 ± 17
norpA^{P16}	274	ND	ND	1	~60,000	27 ± 13
WT pupae	139	239	0.87	1	62 ± 52	18 ± 23
Gaq' pupae	118	220	0.77	1,000	104 ± 96	18 ± 20

The table shows mean values (±s.d.) for latency, amplitude and relative light stimulus (defined as the amount of light required to generate a quantum bump normalized to wild-type stimulation) in several mutant backgrounds. Bumps were evoked by light flashes of 10 ms (log[*I*] = −6.5 for wild-type; this was set as 1 in the relative light calculations); only some of the flashes (or trails) generated a bump. The mean frequency of events was calculated by assuming a Poisson distribution in which the probability of zero bumps ($P_0 = e^{-m}$ = (1 − no. of bumps/no. of trials), and solving for m (ref. 8). The data for the $G\alpha q^1$ mutant were obtained from late-stage pupae; therefore, wild-type controls from the same stage are also included ($G\alpha q^1$ values were calculated from ref. 11).
chp, *chaoptin*; ND, not determined; WT, wild-type.

plexes (that is, variation in the number of channels contained in a complex). As a photoreceptor cell contains 10^8 molecules of rhodopsin[14], the probability of generating multiple single-photon responses from the same rhodopsin molecule is infinitesimal, and so the variance could result from activity of different signalling complexes. A prediction of this postulate is that if it were possible to stimulate the same rhodopsin molecule over and over (that is, the same signalling complex repeatedly), then quantum-bump amplitudes should be highly reproducible with only a small variance. This experiment could be done either by engineering flies that have one or a few rhodopsin molecules per cell, or by generating modified photoreceptors in which one rhodopsin molecule signals continuously after activation by a single photon of light. We showed previously that calmodulin is required for rhodopsin shut-off by

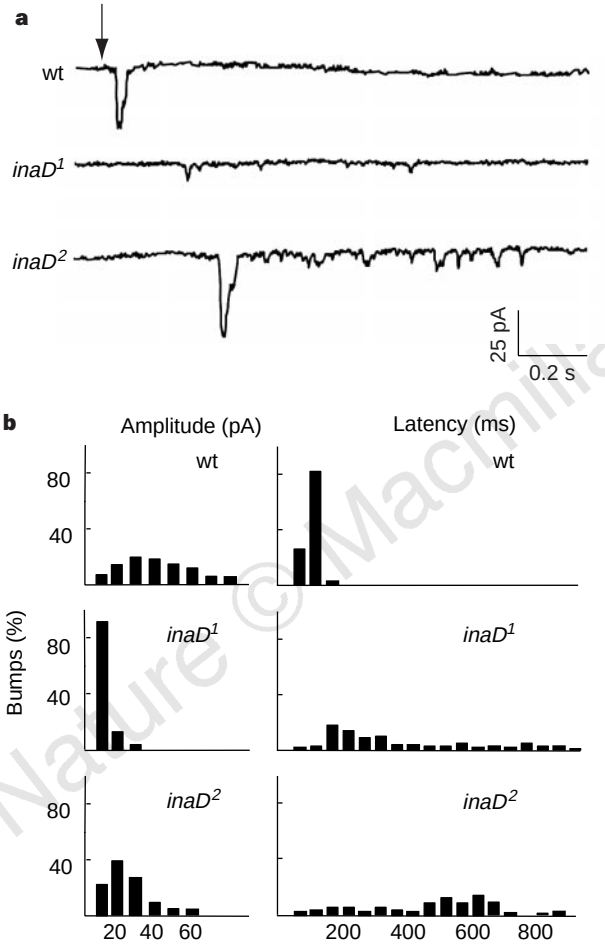

Figure 1 Disrupting the InaD signalling complex disrupts quantum bumps. **a**, Single-photon responses in *inaD* mutants. Cells were stimulated with a 10-ms flash of light of wavelength 580 nm ($\log[I] = -6.5$; see Methods) at the time indicated by the arrow. The *inaD*^{1} null mutant shows defects in amplitude, latency and deactivation of quantum bumps, consistent with a loss of signalling complexes. The *inaD*^{2} allele contains a mutation in its PLC-interaction site, and exhibits disruption of latency and deactivation. wt, wild-type. **b**, Amplitude and latency distributions of *inaD* quantum bumps. Responses were measured for 73 *inaD*^{1} bumps (from 6 cells) and 67 *inaD*^{2} bumps (from 5 cells). The probability of a bump occurring was 0.22 for *inaD*^{1} and 0.28 for *inaD*^{2} mutants (assuming a Poisson distribution of events and solving for P values $= 1 -$ (trials with zero quantum bumps/total trials))[8]. Because *inaD* mutants produce more than one response per flash, only the amplitude and latency of the first response were measured. Table 1 shows the quantification of response parameters.

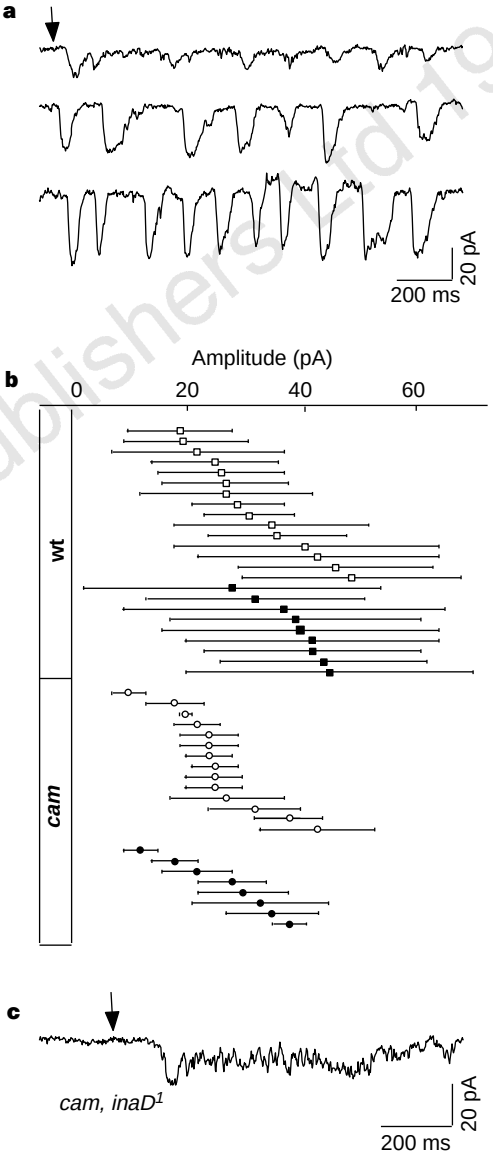

Figure 2 A quantum bump is an organized unit of signalling. **a**, Single-photon responses from a *cam* mutant photoreceptor. Each trace represents quantum bumps produced by a single activated photoreceptor; the three different traces represent the activity produced by three different rhodopsin molecules. Bath solution contained 0.4 mM $CaCl_2$. **b**, The spread of quantum bumps produced by different activated receptors compared with the spread of bumps produced by one activated receptor. Responses of 24 wild-type (wt) cells and 22 *cam* mutants are shown. Each trace represents the mean ± s.d. of seven bumps generated by consecutive flashes of light (wt), or the mean ± s.d. of seven bumps generated by a single photon of light (*cam*). Cells were stimulated with a 10-ms flash of 580-nm light, $\log[I] = -6.5$. The spread of all of the different *cam* responses mimics the variability seen in normal single-photon responses. The open symbols indicate that the bath solution contained 1.5 mM $CaCl_2$, and the filled symbols indicate that the bath solution contained 0.4 mM $CaCl_2$. **c**, Quantum bumps from *cam*, *inaD*^{1} double mutants show that loss of InaD disrupts localized signalling.

regulating arrestin function, and that calmodulin (*cam*) mutants produce many quantum bumps in response to a single photon of light[15]. Therefore, we used a hypomorphic allele of *cam* to analyse quantum-bump variance following activation of discrete transducisomes. Figure 2a shows sample traces of trains of quantum bumps from one *cam* mutant photoreceptor, illustrating both the high reproducibility of bump amplitudes following activation of the same rhodopsin molecule and the wide variability produced by the activation of different rhodopsin molecules. These results indicate that differences in bump amplitude do not simply stem from stochastic fluctuations in the number of activated channels. Instead, because a given receptor can consistently activate a similar number of ion channels (Fig. 2b), these results indicate that a quantum bump reflects the activation of a reliable unit of signalling. If the InaD transducisome represents this signalling unit, then *cam* mutants lacking InaD should no longer exhibit discrete trains of quantum bumps. As predicted, *cam*, $inaD^1$ double mutants generate a noisy, continuous response to a single photon of light, and do not produce individual bumps (Fig. 2c).

As *Drosophila* phototransduction takes place in a microvillar organelle (each photoreceptor cell contains ~60,000 microvilli, and each microvillus has ~1,000 rhodopsins)[6], we wondered whether the anatomical structure of a microvillus places physical boundaries on the organization of signalling complexes. Thus, we studied quantal responses in mutant photoreceptors that lack organized microvilli (*chaoptin* mutants)[16,17]. Quantum-bump amplitudes in *chaoptin* mutants are not significantly different from wild-type amplitudes (Table 1), showing that the microvillar architecture does not contribute to the structural organization underlying a quantum bump.

It is thought that the primary function of signalling cascades is to promote amplification. However, the assembly of signalling molecules into a protein–protein macromolecular complex would be expected to severely limit amplification, as the number of downstream elements in the cascade would be physically limited. Because phototransduction takes place in InaD complexes, we wondered whether there is any amplification upstream of the light-activated ion channels. To investigate this issue *in vivo*, we used the following logic: if a rhodopsin molecule activates many G proteins, then a reduction in the cellular levels of the G protein should result in fewer channels opening and a corresponding reduction in bump amplitude (as is seen in mutants that contain fewer light-activated channels[12]). However, if there is no amplification between rhodopsin and the G protein, then reducing the levels of the G protein should not affect bump size but should instead affect the frequency or latency of the response. Similarly, if there is no amplification at the G-protein–PLC interface, reducing the levels of PLC should not affect bump amplitude.

We used genetically engineered flies that express low levels of G protein (~1% of normal levels; see ref. 11) and severe hypomorphic alleles of *norpA* (which produce levels of PLC that are ~10% of wild-type levels)[18,19] to reduce the levels of these signalling molecules *in vivo*. Figure 3 and Table 1 show the distributions of quantum-bump amplitudes, latencies and relative bump frequencies for wild-type, $G\alpha q^1$ and *norpA* photoreceptors, demonstrating that a marked reduction in the levels of the G protein[11] or PLC does not significantly affect the size or shape of a quantum bump, although latency or frequency are significantly altered. These results demonstrate that there is little amplification upstream of second-messenger production, and suggest that one rhodopsin activates one G protein which in turn activates one PLC, leading to the opening of many ion channels. Interestingly, the G-protein hypomorphs exhibit nearly normal latencies but a 1,000-fold reduction in sensitivity (that is, ~1,000 rhodopsin molecules must be activated to generate a bump), whereas the most severe PLC hypomorphs exhibit a ~1,000-fold increase in latency but normal sensitivity. Why does a reduction in G protein levels mainly affect frequency, whereas a reduction in PLC levels affects latency? These findings can be explained easily by assuming that the lifetime of an activated rhodopsin is very short (being limited by arrestin-dependent shut-off) and so it is necessary to excite many rhodopsins before activating one in close proximity to a G protein. In contrast, the lifetime of an activated G protein is very long in the absence of PLC, possibly because PLC acts as a GTPase-activating protein to deactivate G_q, and so a G protein stays active until it encounters a PLC. However, once a rhodopsin encounters a G protein, or once a G protein finds a PLC, the generation of a bump occurs normally.

Collectively, our results illustrate how the organization of signalling molecules into discrete signalling units provides fundamental advantages, such as rapid responses and feedback regulation, to a transduction cascade. Our study of single-photon responses and their relationship to the InaD macromolecular complex also uncovered several unexpected findings. First, we showed that a physiologically described signalling event, such as a quantum bump, has a well-defined molecular framework, and that the organization of signalling molecules into a macromolecular complex ensures stable and reliable unitary responses. Second, we demonstrated that this organization limits the amplification of the cascade *in vivo*. Why

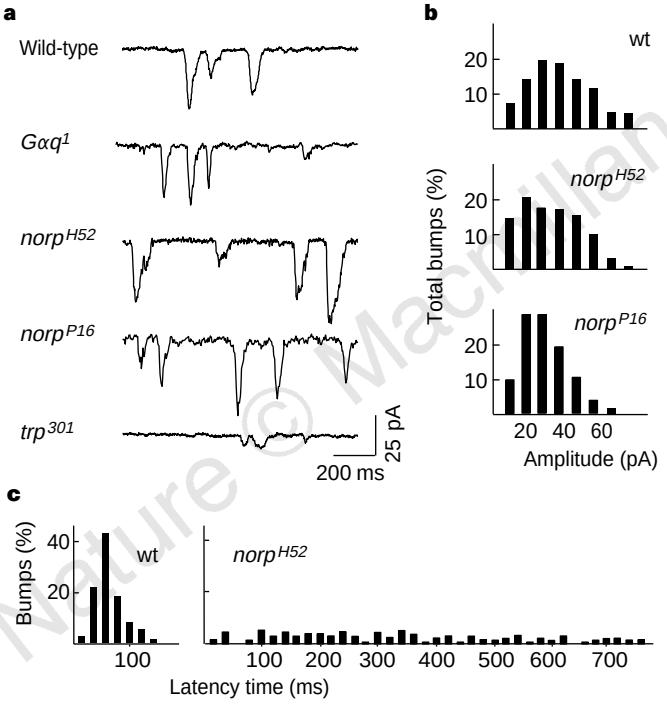

Figure 3 Amplification occurs downstream of PLC activation. **a**, Quantum bumps from a wild-type photoreceptor, from a photoreceptor containing ~1% of the normal levels of the eye-specific Gα subunit ($G\alpha q^1$), and from photoreceptors that express reduced levels of PLC ($norpA^{H52}$ and $norpA^{P16}$) are shown. Quantum bumps from a photoreceptor with low levels of the light-activated ion channel TRP (trp^{301}) are also shown; this photoreceptor exhibits significant reduction in bump size[12]. These results indicate that signal amplification occurs at the terminal signalling events, downstream of PLC activation. Stimulation was with continuous light of $\log[I] = -7$ for wild-type, $norpA^{H52}$ and trp^{301}, and $\log[I] = -4$ for $G\alpha q^1$. **b**, Amplitude distributions of quantum bumps in the *norpA* hypomorphs. For studies with wild-type and $norpA^{H52}$ alleles, cells were stimulated with a 10-ms flash of 580-nm light of $\log[I] = -6.5$. The probability of a bump occurring (P) was 0.30 for wild-type and 0.28 for $norpA^{H52}$ photoreceptors. **c**, Distributions of latency times for the $norpA^{H52}$ hypomorph. Latency was measured as the time from the onset of the light stimulus (10 ms, $\log[I] = -6.5$) to the peak amplitude of the response. For the $norpA^{P16}$ allele, the mean latency time was estimated as ~60 s, derived from the exponential decay of the bump rate following stimulation with a 10-ms flash of light of $\log[I] = -4.5$. See Table 1 for quantification of response parameters.

have multiple components in this transduction pathway if there is little amplification? Having many steps in the same signalling cascade allows fine control by providing several points of regulation. In the case of phototransduction, this may allow for regulatory mechanisms that endow photoreceptor cells with high temporal resolution, refined adaptation and a broad dynamic range of response[20,21]. □

Methods

We isolated photoreceptors from adult flies (>6 h after eclosion) and performed whole-cell, voltage-clamp recordings. The bath solution contained 124 mM NaCl, 4 mM KCl, 10 mM HEPES, 5 mM proline and 25 mM sucrose, pH 7.1. Unless otherwise stated, the bath solution also contained 1.5 mM $CaCl_2$. The pipette solution contained 95 mM potassium gluconate, 40 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$ and 0.2 mM EGTA, pH 7.15.

Photoreceptors were clamped at a holding potential of −70 mV. Signals were sampled at 1 kHz, then filtered at 500 Hz. In all experiments, light was filtered through a bandpass filter ($\lambda = 580 \pm 10$ nm) and neutral-density filters, and focused onto the photoreceptor cells through a 0.5 numerical aperture, ×40 objective. Photoreceptors were stimulated with a 75 W xenon source; I_O is the maximum intensity at 580 nm produced by the light source (0.04 mW cm^{-2}).

Single-photon responses were generated and analysed as described[8,11]. For analysis of quantum bumps in the temperature-sensitive *norpA*^{H52} allele, flies were heat-shocked for 1 h at 37 °C and set at 22 °C for 30 min; patch-clamp analysis was done as usual. For *norpA*^{P16}, bumps were continuously produced immediately after break-in (probably induced by the microscope light required for patching), with the bump rate declining over a period of several hundred seconds. Amplitudes were measured when the bump rate declined to <1 bump per second.

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The G protein Gα12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain

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Heterotrimeric guanine-nucleotide-binding proteins (G proteins) are signal transducers that relay messages from many receptors on the cell surface to modulate various cellular processes[1–4]. The direct downstream effectors of G proteins consist of the signalling molecules that are activated by their physical interactions with a Gα or Gβγ subunit. Effectors that interact directly with Gα12 G proteins have yet to be identified[5,6]. Here we show that Gα12 binds directly to, and stimulates the activity of, Bruton's tyrosine kinase (Btk) and a Ras GTPase-activating protein, Gap1^m, *in vitro* and *in vivo*. Gα12 interacts with a conserved domain, composed of the pleckstrin-homology domain and the adjacent Btk motif, that is present in both Btk and Gap1^m. Our results are, to our knowledge, the first to identify direct effectors for Gα12 and to show that

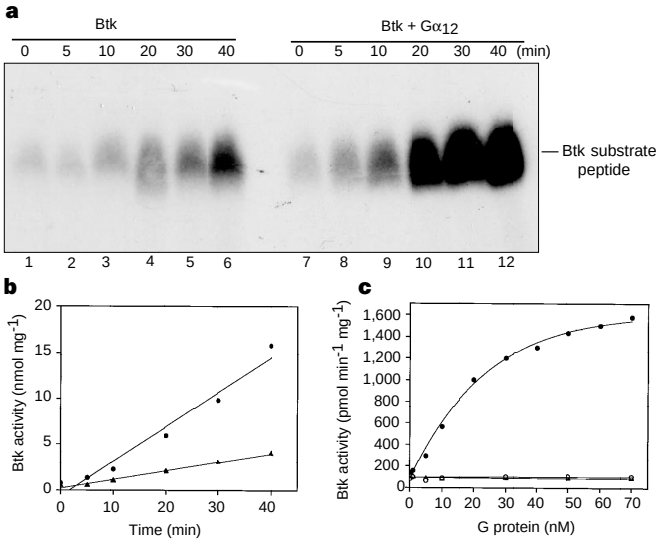

Figure 1 Stimulation of Btk kinase activity by Gα12. **a**, **b**, Time course of Btk kinase activity. **a**, Btk was incubated with substrate peptide for various times in the absence (lanes 1–6) or presence (lanes 7–12) of 5 nM Gα12-GTPγS. **b**, After longer exposure, the corresponding bands on the gel were cut out and phosphorylation was quantified by scintillation counting. Btk activity was expressed as nmoles PO_4 incorporated into peptide substrate per mg Btk. Data shown are representative of three similar experiments. Circles, activity in the presence of Gα12-GTPγS; triangles, activity in the absence of Gα12. **c**, Stimulation of Btk kinase activity by different concentrations of Gα12-GTPγS. The indicated concentrations of α-subunits were reconstituted with 1 ng Btk for 20 min at 30 °C. Increasing amounts of Gα12-GTPγS (filled circles) increased Btk activity. GαsGTPγS (open circles) or Gα12-GDP (filled triangles, barely visible) had no effect on Btk activity. Data are representative of 4–6 experiments.