Live cell micropatterning reveals the dynamics of signaling complexes at the plasma membrane

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Interactions of proteins in the plasma membrane are notoriously challenging to study under physiological conditions. We report in this paper a generic approach for spatial organization of plasma membrane proteins into micropatterns as a tool for visualizing and quantifying interactions with extracellular, intracellular, and transmembrane proteins in live cells. Based on a protein-repellent poly(ethylene glycol) polymer brush, micropatterned surface functionalization with the HaloTag ligand for capturing HaloTag fusion proteins and RGD peptides promoting cell adhesion was devised. Efficient micropatterning of the type I interferon (IFN) receptor subunit IFNAR2 fused to the HaloTag was achieved, and highly specific IFN binding to the receptor was detected. The dynamics of this interaction could be quantified on the single molecule level, and IFN-induced receptor dimerization in micropatterns could be monitored. Assembly of active signaling complexes was confirmed by immunostaining of phosphorylated Janus family kinases, and the interaction dynamics of cytosolic effector proteins recruited to the receptor complex were unambiguously quantified by fluorescence recovery after photobleaching.
We have here applied this approach to unravel the protein–protein interactions involved in the formation of the IFN signaling complex. The IFN receptor (IFNAR) is comprised of two subunits, IFNAR1 and IFNAR2 (Cohen et al., 1995; Uzé et al., 2007), which both independently interact with the ligand (Lamken et al., 2005; Li et al., 2008; Thomas et al., 2011), thus forming a ternary complex (Fig. 1 b). IFNs bind IFNAR2 with substantially higher binding affinity (lower nanomolar regimen) than IFNAR1 (micromolar regimen; Lamken et al., 2004; Jaks et al., 2007; Lavoie et al., 2011). IFN-mediated receptor dimerization leads to phosphorylation of the cytosolic Janus family kinases (JAKs) tyk2 and Jak1, which are noncovalently associated with IFNAR1 and IFNAR2, respectively (Prejean and Colamonici, 2000). Signal transducers and activators of transcription (STATs), STAT1 and STAT2, are recruited to the receptor and phosphorylated by tyk2 and/or Jak1 to form homodimers (pSTAT1/pSTAT1) and heterodimers (pSTAT1/pSTAT2), which translocate into the nucleus and regulate transcription. Recently, the USP18 (ubiquitin-specific protease 18) has been identified as a key regulator of IFN signaling, which was suggested to interact with IFNAR2 (Malakhova et al., 2006) and modulate receptor assembly (François-Newton et al., 2011). Although some of the protein–protein interactions involved in receptor assembly and effector recruitment have been characterized by pull-down experiments, these do not provide a mechanistic view of the spatiotemporal organization of the IFN transmembrane signaling complex. Open questions include a potential predimerization of IFNAR1 and IFNAR2, which has been suggested for other cytokine receptors (Remy et al., 1999; Kubatzyk et al., 2001; Grötzinger, 2002; Pang and Zhou, 2012), the dynamics of JAK association with the receptor subunits (Haan et al., 2006), and the role of the determinants of STAT1 and STAT2 recruitment (Prejean and Colamonici, 2000) as well as the mechanism of USP18 negative feedback for IFN signaling (François-Newton et al., 2011, 2012). We demonstrate here how micropatterning of the receptor subunits within the plasma membrane of live cells provides versatile means to address these fundamental questions in cytokine receptor research.

Figure 1. Strategies for assembly of functional signaling complexes into micropatterns. (a) Binary surface patterning by photochemical coupling of maleimido-RGD to a maleimide-functionalized PEG polymer brush (I) followed by reaction of the nonilluminated maleimide groups with a thiol-functionalized HTL (II). (b) Concept of spatial organization of IFN receptor signaling complexes in the plasma membrane of cells cultured on the surface of a micropatterned coverslide. (top) IFNAR2 fused to the HaloTag is captured into HTL-functionalized areas (HTL functionalities are depicted in red), whereas cell attachment via focal adhesions is mediated by RGD-functionalized areas (RGD functionalities are depicted in violet). (bottom) Upon addition of the IFN, functional complexes are formed by recruitment of IFNAR1 (green), leading to phosphorylation of the associated JAK kinases Jak1 and tyk2 as well as of tyrosine residues on the cytosolic domains of the receptor subunits. Thus, effector proteins such as STAT1 and STAT2 are locally recruited to the receptor.

Results

Binary functional patterning of maleimide-functionalized surfaces

To obtain high-contrast micropatterns of target proteins while restricting focal adhesion outside these zones, an efficient binary patterning technique was implemented. To this end, we exploited the photoactivation of maleimides by UV illumination (Yamada et al., 1968) for spatially resolved surface functionalization. We have previously used this approach for micropatterning by maleimide photodestruction (Waichman et al., 2011) and by photochemical coupling of hydrophobic tethers via vinyl ether groups (Waichman et al., 2013). Here, we established cross-linking of photoactivated maleimide groups on the surface with maleimide-functionalized ligands in solution for binary patterning (Fig. 1 a). Surface modification by photochemical maleimide cross-linking was quantitatively assessed by solid-phase detection using reflectance interference spectroscopy.
with HaloTag-EGFP-IFNAR2 were cultured on a coverslide functionalized with RGD and HTL in subcellular dimension (compare Fig. 2 a) and imaged at the surface-proximal cellular membrane by total internal reflection (TIR) fluorescence (TIRF) microscopy. Strikingly, successful reorganization of IFNAR2 in the plasma membrane of a transfected cell following the line structure of the photomask used for patterning was observed with high contrast (Fig. 2 b). Upon incubation of the ligand, IFN\(\alpha\) site-specifically labeled with ATTO 655 (AT655IFN\(\alpha\)) binding into the same zones occupied by IFNAR2 was observed. Staining of the entire micropatterned substrate with a purified HaloTag-EGFP fusion protein confirmed IFN\(\alpha\) binding within HTL-functionalized areas (Fig. S2). In contrast, expression of IFNAR2 without a HaloTag resulted into homogeneous distribution of AT655IFN\(\alpha\) on the entire cell surface (Fig. S2). Thus, not only efficient micropatterning of IFNAR2 was achieved but also the functionality of its extracellular ligand binding domain and its accessibility by IFN was preserved. IFN\(\alpha\) binds with an affinity of \(\sim 5\) nM to IFNAR2, corresponding to a dissociation rate constant of \(\sim 0.02\) s\(^{-1}\) (Piehler and Schreiber, 1999; Strunk et al., 2008). For probing the dynamics of this interaction in a quantitative manner, we used the IFN\(\alpha\)2 mutant M148A in combination with the mutations N65A, L80A, Y85A, and Y89A (M148A-NLYY; Piehler et al., 2000b; Roisman et al., 2005), which binds IFN\(\alpha\)2 with \(\sim 50\)-fold
Formation of the ternary signaling complex

In the next step, we explored the assembly of the ternary signaling complex induced by IFN-α2, which can interact simultaneously with IFNAR1 and IFNAR2 via independent binding sites (Thomas et al., 2011). IFNAR1 binds IFN-α2 with a $K_d$ of 5 µM affinity, a binding affinity that is three orders of magnitude lower than the binding affinity toward IFNAR2 (Lamken et al., 2004). Owing to these very asymmetric binding affinities, IFN-α2 interaction with IFNAR1 is believed only to occur after binding to the high-affinity subunit IFNAR2. However, predimerization of the receptor subunits in its absence has been suggested for several cytokine receptors including the IFNAR (Remy et al., 1999; Kubatzky et al., 2001; Grötzinger, 2002; Krause et al., 2002, 2013; Brown et al., 2005; Pang and Zhou, 2012).

Here, we used spatial redistribution with micropatterned receptors to unambiguously probe receptor dimerization in living cells. Control experiments revealed that micropatterning caused only minor changes in the membrane-proximal cytoskeleton (Fig. S3) and no changes in the diffusion properties of membrane proteins (Fig. S3). To explore assembly of the ternary IFN–receptor complex, HeLa cells were transiently transfected with IFNAR1 fused to the HaloTag and monomeric EGFP (HaloTag-EGFP-IFNAR1) and with IFNAR2 fused to TagRFP-T (TagRFP-IFNAR2). Upon culturing these cells on a micropatterned support, IFNAR1 was captured into micropatterns, whereas homogeneous distribution of IFNAR2 was observed (Fig. 3 a). After incubation of AT655IFN-α2 for a few minutes, recruitment of IFNAR2 into the micropatterns was observed (Video 3 and Fig. 3 b). The excellent congruence of the increased dissociation rate constant, whereas binding to endogenous IFNAR1 is negligible (the binding properties of IFNα2 mutants are summarized in Table S1). Binding of IFNα2 M148A-NLYY labeled with DY-647 (DY647IFNα2 M148A-NLYY) to micropatterned IFNAR2 was monitored by single-molecule imaging. Transient binding specifically within the micropatterns was observed as expected for this ligand (Video 1 and Fig. 2 c) and confirmed by single-molecule bleaching at elevated laser power (Arant and Ulbrich, 2014). No lateral diffusion was observed for ligands bound within the micropattern as expected for immobilized IFNAR2. Thus, the lifetime of the complex could be determined reliably by the analysis of the residence time of individual molecules within the pattern (Fig. 2 d). An exponential decay was observed, yielding a dissociation rate constant of $1.25 \pm 0.23 \text{s}^{-1}$, which is in very good agreement with the rate constant found for this interaction in vitro by ensemble and single-molecule imaging techniques (Waichman et al., 2011, 2013). Specific and reversible ligand binding to micropatterned IFNAR2 was furthermore confirmed by chasing with unlabeled IFNα2-α8tail-YNS (H57Y, E58N, and Q61S), which binds IFNAR with substantially higher affinity (Levin et al., 2011). Rapid displacement of DY647IFNα2 M148A-NLYY was observed as was very low residual binding (Video 2 and Fig. 2 e). The fluorescence decay within the pattern was fitted by a single exponential, yielding a dissociation rate constant of $1.15 \pm 0.20 \text{s}^{-1}$ in good agreement with the single molecule data. These studies not only confirmed the high patterning efficiency and full functionality of micropatterned IFNAR2 but also the compatibility of this approach with quantitative binding assays by ensemble and single-molecule detection techniques.

**Figure 3.** Ternary complex assembly in micropatterns. (a and b) HaloTag-EGFP-IFNAR1 [green channel] expressed in HeLa cells together with TagRFP-IFNAR2 [yellow channel] and cultured on a micropatterned support before (a) and 20 min after (b) addition of AT655IFNα2 [red channel]. Bars, 10 µm. (c) Distribution IFNAR1 [green], IFNAR2 [orange], and IFNα2 [red] after formation of the ternary complex [representative of two cells analyzed]. For comparison, the distribution of IFNAR2 before addition of IFNα2 [orange, dotted line] is shown. Integrated line profiles were scaled to similar amplitudes and overlaid at an arbitrary ordinate scale. a.u., arbitrary unit. (d) Time-resolved increase of the IFNAR2 concentration within [black squares] and decrease outside IFNAR1 micropatterns [red dots]. Intensities normalized to the intensities outside the functionalized areas are plotted [representative of two cells analyzed].
stabilization is in line with binding experiments using radio-
labeled IFNs, which suggested an increase in binding affinity
upon coexpression of IFNAR1 and IFNAR2 versus IFNAR2
only (Cohen et al., 1995). These experiments confirmed intact
functionality of micropatterned IFNAR2 to recruit IFNAR1
into a dynamic ternary signaling complex in the plasma mem-
brane of live cells.

Functional association of the JAKs
To explore the capability of this micropatterned ternary com-
plex to activate intracellular signaling, we first probed the
interaction of the tyk2 and Jak1 with IFNAR1 and IFNAR2,
respectively. To this end, tyk2 fused to monomeric EGFP (tyk2-
EGFP) was coexpressed with IFNAR1 fused to an N-terminal
HaloTag (HaloTag-IFNAR1) in HeLa cells, which were cul-
tured on a micropatterned support (Fig. 5 a). Micropatterning
of tyk2-EGFP was observed, and colocalization with immobi-
lized IFNAR1 was confirmed by using the IFN
\( \alpha_2 \) variant YNS,
which binds IFNAR1 with
\( \sim 60 \) -fold increased binding affinity
compared with the wild type (Fig. 5 b; Kalie et al., 2007). To
exclude that the observed intensity distribution was caused by
an increased background excitation as a result of changes in
the membrane topography within HTL-functionalized zones,
control experiments with cells expressing EGFP and HaloTag-
IFNAR2 were performed (Fig. S3). No correlation between the
EGFP fluorescence intensity and the micropattern was observed
under these conditions, confirming specific association of tyk2
to micropatterned IFNAR1. The stability of this complex was
quantitatively probed by FRAP. For this purpose, tyk2-EGFP

Figure 4. Reversible IFN\( \alpha_2 \) binding to both IFNAR1 and IFNAR2. (a) HaloTag-IFNAR2 expressed in HeLa cells together with IFNAR1-EGFP (green chan-
nel) and cultured on a micropatterned support at different times after injection of AT655IFN\( \alpha_2 \) (red channel). Bars, 10 µm. (b) Micropatterns after binding
of 0.5 nM DY647IFN\( \alpha_2 \) M148A (left) and after addition of unlabeled IFN\( \alpha_2 \)α8tail-YNS (right). Dotted lines indicate the analyzed line pattern. Bars, 5 µm.
(c) Displacement kinetics in micropatterns after addition of unlabeled IFN\( \alpha_2 \)α8tail-YNS and a monoexponential fit of the curve (from the cell shown in b,
representative of three cells analyzed). The time regimen during injection and refocusing is marked by a gray bar.
For this purpose, HeLa cells were cotransfected with IFNAR1 fused to the HaloTag and TagRFP (HaloTag-TagRFP-IFNAR1) as well as IFNAR2 fused to the SNAP-tag (SNAP-IFNAR2) and cultured on a micropatterned support. After addition of AT655IFNα2, cells were fixed and immunostained via an anti-pJak1 antibody. Colocalization of AT655IFNα2 and HaloTag-TagRFP-IFNAR1 in micropatterns confirmed ternary complex formation. Strikingly, specific binding of the anti-pJak1 antibody into these micropatterns was confirmed (Fig. 5 e), which was not observed in the absence of IFNα2 (Fig. S4). Thus, spatially resolved activation of Jak1 was assayed by this micropatterning method.

Recruitment of STAT proteins

The mechanistic details of STAT recruitment to IFNAR have remained enigmatic. Based on pull-down experiments, STAT2 has been suggested to be constitutively associated with the cytosolic domain of IFNAR2 (Li et al., 1997). This interaction was confirmed by coexpression of HaloTag-IFNAR2 and STAT2 fused to monomeric EGFP (STAT2-EGFP; Fig. 6 a). Colocalization was bleached within a segment of the micropattern, and the recovery was followed (Video 5 and Fig. 5 c). A comparison of the changes in fluorescence intensity during the FRAP experiment in different regions of the micropattern is shown in Fig. 5 d: Although stable fluorescence signals were obtained both within and outside the micropattern of nonbleached control regions, fluorescence recovery was observed in the bleached area. The minor recovery outside the micropattern could be explained by interaction of tyk2 with other cytokine receptors. However, owing to the small amplitude of this control curve, the exchange kinetics within the micropattern was directly assessed from the recovery curve by fitting a monoeponential function, yielding a rate constant of 0.0025 ± 0.001 s⁻¹. As a large excess of tyk2-EGFP is available in the cytosol, this rate constant can be interpreted as the dissociation rate constant of the tyk2–IFNAR1 complex as a rate-limiting step of the exchange.

To explore the formation of an active signaling complex within micropatterns, we probed tyrosine phosphorylation of Jak1, which is critically required for all further signaling cascades. For this purpose, HeLa cells were cotransfected with IFNAR1 fused to the HaloTag and TagRFP (HaloTag-TagRFP-IFNAR1) as well as IFNAR2 fused to the SNAP-tag (SNAP-IFNAR2) and cultured on a micropatterned support. After addition of AT655IFNα2, cells were fixed and immunostained via an anti-pJak1 antibody. Colocalization of AT655IFNα2 and HaloTag-TagRFP-IFNAR1 in micropatterns confirmed ternary complex formation. Strikingly, specific binding of the anti-pJak1 antibody into these micropatterns was confirmed (Fig. 5 e), which was not observed in the absence of IFNα2 (Fig. S4). Thus, spatially resolved activation of Jak1 was assayed by this micropatterning method.
active signaling complexes, IFNAR2-deficient cells (U5A) stably expressing SNAP-IFNAR1 and HaloTag-IFNAR2 were transfected with STAT1-EGFP and STAT2-tagRFP and cultured on micropatterned support. Upon addition of IFNα2, a steady increase of the fluorescence intensity at micropatterned IFNAR was observed for both STAT1 and STAT2 for \( \approx 10 \) min (Fig. 7 a), which was not observed for unstimulated cells. At the same time, the contrast of the micropattern substantially increased. Quantitative analysis of the fluorescence intensity within the micropattern revealed that a maximum intensity was obtained after 5–10 min of receptor stimulation followed by a relatively slow decay (Fig. 7 b). This temporal characteristic of STAT recruitment to IFNAR is in good agreement with the STAT phosphorylation kinetics. Efficient nuclear translocation of STAT1 and STAT2 corroborated the formation of active signaling complexes within micropatterns, assuming that the vast majority of IFNAR2 at the plasma membrane was captured to the surface. These results suggest that additional docking sites for STAT docking may be created by IFNAR phosphorylation, thus enhancing binding of STAT1 and STAT2.

Role of the negative feedback regulator USP18

USP18 has been shown to be an important negative feedback regulator of IFN signaling (Malakhova et al., 2006), which moreover plays a key role in differential IFN signaling (François-Newton et al., 2011, 2012). Because USP18 was shown to bind to IFNAR2 (Malakhova et al., 2006) and has been proposed to affect receptor assembly (François-Newton et al., 2011), we here applied the novel features of cell micropatterning for exploring its role in modulating protein interactions involved in the...
assembly of the IFNAR signaling complex. Binding of USP18 fused to mEGFP (EGFP-USP18) to micropatterned IFNAR2 independent on receptor dimerization was confirmed (Fig. 8, a and b). To unravel potential inhibition of IFNAR1 recruitment into the ternary complex by USP18 binding, we devised a competition assay for receptor dimerization (Fig. 8 c). For this purpose, HaloTag-IFNAR1 was cotransfected with IFNAR2 and USP18-EGFP as potentially competing interaction partners. In the absence of IFN, homogeneous distribution of USP18 was observed as expected for its interaction with IFNAR2, but not IFNAR1 (Fig. 8 c). Upon ternary complex formation by addition of IFNα2, translocation of USP18 together with IFNAR2 into micropatterns was clearly discerned (Fig. 8, c and d). These experiments revealed that USP18 does not compete with ternary complex formation but rather may act as an allosteric modulator.

We further tested the role of USP18 in assembly of the signaling complex by probing effector interactions in the presence of USP18. Unexpectedly, we unveiled a modulatory role of USP18 in STAT2 recruitment. Upon coexpression of USP18 and STAT2 with the micropatterned IFNAR2, we observed an increased contrast of STAT2. More detailed analysis of the interaction dynamics of the STAT2–IFNAR2 interaction in the presence of USP18 by FRAP (Fig. 8, e and f) revealed a dissociation rate constant of $0.015 \pm 0.005$ s$^{-1}$. Thus, USP18 binding to IFNAR2 stabilizes the constitutive STAT2–IFNAR2 interaction by a factor of $\approx 8$. These observations suggest that USP18 may also play a strong regulatory role in STAT signaling.

Discussion

We have here developed a micropatterned surface architecture as a robust platform for spatial organization of proteins within the plasma membrane. Based on a highly biocompatible PEG polymer brush support, nonspecific interaction with the surface is efficiently minimized. We designed a novel photochemical functionalization approach, which yielded highly orthogonal binary patterning, thus ensuring cell attachment via focal adhesions outside the areas functionalized for capturing membrane proteins. Thus, functional receptor micropatterning with good accessibility to the ligand was achieved, providing the capability to probe ligand interaction down to the single-molecule level with extremely low background of nonspecific binding to the coverslip surface. Notably, capturing via the HaloTag as compared with previous approaches based on antibodies ensures high contrast and long-term stability of substrates as well as more robust and generic application, as no suitable antibody toward the bait protein is required.

Based on these systematically engineered surface properties, the assembly of functional transmembrane signaling complexes within micropatterns was possible, including ligand-induced receptor dimerization and the association as well as the phosphorylation of cytosolic effector proteins. Local enrichment of functional bait proteins in the plasma membrane by micropatterned immobilization allows to unambiguously probe cytosolic interaction partners even in the case of promiscuous interactions, e.g., JAKs and STATs, which bind multiple cytokine receptors, and to discriminate membrane binding from background fluorescence. Moreover, immobilization of the micropatterned bait protein excludes lateral diffusion and therefore enables versatile quantitative assays for protein interaction analysis in the context of an intact plasma membrane within live cells. Thus, FRAP experiments in micropatterns allow for quantitatively probing the stability of transient interactions with high specificity. Based on this approach, we succeeded in addressing several fundamental concepts in cytokine signaling for the IFNAR as a model system: (a) ligand-induced heterodimerization of IFNAR1 and IFNAR2 in contrast to currently debated modes of preassembled subunits (Krause et al., 2013) was clearly demonstrated; (b) relatively stable association of the corresponding Jaks with the cytosolic receptor domains was directly shown; (c) stimulation-independent, transient binding of STAT2 to IFNAR2 and the recruitment of STAT1 via STAT2 could be shown as well as increased STAT recruitment to the activated signaling complex; and (d) the interaction of the negative feedback regulator USP18...
interaction partners and posttranslational modifications will be possible as demonstrated here for Jak1 phosphorylation. Thus, a broad application for unraveling protein–protein interactions at the plasma membrane can be envisaged for this generic, tag-based approach.

Materials and methods

Proteins and plasmids

IFNα2 and mutants fused to an N-terminal ybbR-tag (Yin et al., 2005; IFNα2, IFNα2-YNS, IFNα2 M148A, IFNα2 M148A-NYY, and IFNα2-α8tail-YNS) for site-specific posttranslational labeling were cloned by insertion of an oligonucleotide linker coding for the ybbR peptide (DSLEFIASKLA) into the NdeI restriction site upstream of the corresponding genes in the plasmid pT7T3-U18cis (Piehler and Schreiber, 1999). Proteins were expressed in Escherichia coli (TG1 strain) at 37°C. However, overexpression of interaction partners is required for optimum contrast, which requires careful interpretation of the biological relevance of experimentally observed interactions. Although we used here a photochemical micropatterning approach, the concept is well compatible with microcontact printing, which allows higher sample throughput. By combination with immunofluorescence labeling, identification of interaction partners and posttranslational modifications will be possible as demonstrated here for Jak1 phosphorylation. Thus, a broad application for unraveling protein–protein interactions at the plasma membrane can be envisaged for this generic, tag-based approach.

Figure 8. Role of the negative feedback regulator USP18 for IFNAR dimerization and STAT recruitment. (a and b) USP18 constitutively binds to IFNAR2: (a) HeLa cells transfected with HaloTag-tagBFP-IFNAR2 (cyan channel) and EGFP-USP18 (green channel) after incubation of AT655IFNα2 (red channel); cell boundaries are indicated by dotted lines. The squares indicate the analyzed sectional distribution of fluorescence intensity shown in b. Bars, 10 µm. (b) Contrast of USP18 before and after incubation of AT655IFNα2 compared with HaloTag-tagBFP-IFNAR2 (representative of three cells analyzed). [c and d] Ternary complex formation and USP18 binding to IFNAR2 are noncompetitive: USA cells transfected with HaloTag-IFNAR1, SNAP-IFNAR2, and EGFP-USP18 (green channel) before and after addition of IFNα2 (red channel); representative of three cells analyzed. Cell boundaries are indicated by dotted lines. The rectangles indicate the analyzed sectional distribution of fluorescence intensity shown in d. Bars, 10 µm. (d) Intensity pattern in the area indicated in c. [e and f] The stability of constitutive STAT2 binding to IFNAR2 is affected by USP18: (e) STAT2-tagRFP (yellow channel) bound to micropatterned HaloTag-IFNAR2 in cells coexpressing USP18 was bleached with a 405-nm laser in the indicated area (dotted circles), and recovery was monitored. Bars, 5 µm. (f) Comparison of the fluorescence recovery of STAT2 bound to IFNAR2 in the absence and in the presence of ectopic USP18 (representative of 10 cells analyzed in the absence of USP18 and five cells analyzed in the presence of USP18). rel., relative.
via enzymatic phosphopasteinyltransfer and purified by size exclusion chromatography as described previously (Waichman et al., 2010). A degree of labeling >90% was obtained for all IFNα2 proteins as determined by UV/visible spectroscopy. The binding affinities of different mutants are summarized in Table S1. HaloTag-EGFP with a His tag cloned into pET28b was produced in E. coli (BL21 strain) at 37°C and purified by immobilized metal ion affinity chromatography followed by size exclusion chromatography as described previously (Waichman et al., 2011). The HaloTag ligand was produced by D. Sieve (University of Osnabrück, Osnabrück, Germany). Protein expression in Hela cells was performed under control of the cytomegalovirus promoter using the pDisplay (Invitrogen) and the pSEM•Snap-1-26m (Covales Bioscience) vector backbones. The mouse Ig κ-chain leader sequence was used for protein targeting into the plasma membrane. IFNα1-EGFP was expressed in an S. cerevisiae strain by using an S. cerevisiae HaloTag promoter using the pMET7 (Takebe et al., 1998) vector backbone. Cloning details of all constructs used in this study are summarized in Table S2.

Synthesis of maleimido-RGD
Maleimido-functionalized RGD synthesis was carried out using a 1–4 bis(maleimido)butyric linker and a short peptide sequence, Ac-CGGRGDSCOOH. 6.5 mg Ac-CGGRGDSDCOOH (Coring System Diagnomix) in 0.5 ml 1-M Hepes buffer, pH 8.0, was mixed with 10 mg 1,4-bis(maleimido)butane in 1 ml DMSO [Sigma-Aldrich] for 1 h at room temperature. The mixture was diluted in water and loaded on a C18 reverse-phase HPLC column for purification using a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid [Sigma-Aldrich] water. Purified maleimido-RGD was lyophilized as a powder and stored at −20°C. Electrospray ionization mass spectrometry was a mass per charge of 883.1; calculated [M]+ was 883.

Surface modification and photopatterning
Surface silanization, covalent attachment of a thin PEG polymer brush (2,000 g/mol; Rapp Polymere), and further functionalization with maleimide groups were performed as detailed previously (Piehler et al., 2000a; Waichman et al., 2010). Surface chemistry was performed on standard glass coverslides (1.5 mm) for fluorescence microscopy or on reflectance interference spectroscopy transducers for functional surface characterization (see following section). After surface cleaning in freshly prepared Piranha solution (one part 30% H2O2 and two parts concentrated H2SO4—caution, highly corrosive), the surface was activated by immersion in a saturated solution of 3-(maleimido)propionic acid [Sigma-Aldrich] water. Purified maleimido-RGD was lyophilized as a white powder and stored at −20°C. Electrospray ionization mass spectrometry was a mass per charge of 883.1; calculated [M]+ was 883.

Surface binding assays
Label-free monitoring of protein binding to surfaces was performed by reflectance interference spectroscopy using a home-built setup as described previously (Piehler et al., 2011). Nonpatterned, maleimide-functionalized surfaces (prepared as described in the previous paragraph) were irradiated in the presence of maleimido-biotin for different exposure times. After mounting and equilibrating in Hepes-buffered saline, the amount of immobilized biotin groups was quantified by monitoring binding of 100 nM streptavidin in real time under continuous flow through fluorescence microscopy. For probing binding of HaloTag protein to micropatterned surfaces, 100 nM purified HaloTag-SGFP was incubated for 15 min at 37°C followed by three washing steps with medium. Subsequently, EGFP was imaged by confocal laser scanning microscopy [Fluoview 1000; Olympus; as well as penicillin and streptomycin (PAAR Laboratories) to a density of ~50% confluence and transfected by calcium phosphate precipitation as described earlier (Muster et al., 2010). Cells were plated on micropatterned functionalized coverglass 48 h after transfection and cultured for 15–20 h. For live cell imaging, the medium was exchanged for medium without phenol red. Immunostaining was performed after fixing cells with paraformaldehyde by standard protocols using a pk Handbook of Cell Biology (Santa Cruz Biotechnology, Inc.).

Fluorescence imaging was performed using a microscope [CellTIRF Xcelsure; Olympus] equipped with lasers at 405, 488, 561, and 640 nm as well as a back-illuminated electron-multipled charge-coupled device camera [C9100-13; Hamamatsu Photonics]. A 60× (U Apochromat N 60x/1.45 NA; Olympus) or 150× TIR objective (U Apochromat N 150x/1.45 NA; Olympus) was used depending on the resolution needed in the respective experiment. To avoid spectral cross-talk, each channel was interrogated separately by using excitation only with the required laser line in combination with the corresponding single-band emission filter. Data acquisition was performed with the acquisition software Xcelsure rt version 1.2 (Olympus).

Fluorescence imaging of transmembrane microdomains was performed at 37°C in an incubation chamber (Olympus). Imaging was performed with a 60× objective and excitation at 405, 488, 561, and at 640 nm with a typical power output of 0.1–1 mW at the objective. For ensemble ligand binding experiments, 10 nM of wild-type or mutant AT655IFNα2 was added to the medium. Single-molecule ligand binding experiments were performed in the presence of 2.4.5 M [DA2 M1/4BA-NY] or [DA2 M1/4BA-A] by TIRF imaging as described previously (Waichman et al., 2011). Imaging was performed at 640 nm with a laser power of 2 mW at the objective in the presence of an oxygen scavenger and a redox-active photoprotectant (0.5 mg/ml glucose oxidase [Sigma-Aldrich], 0.04 mg/ml catalase [Roche], 5% wt/vol glucose, 1 µM ascorbic acid, and 1 µM methyl viologen) to minimize photobleaching (Vogelsang et al., 2008). Under these conditions, the bleaching time constant was typically >100 s, thus excluding significant bias by photobleaching. Ligand dissociation kinetics was probed by chasing with 1 µM unlabeled IFNα2/βA1/YN, which binds IFNAR with substantially higher affinity (Levin et al., 2011).

Image analysis and image processing were performed using ImageJ (National Institutes of Health). Image processing comprises cropping, scaling, and rotation as well as adjustment of brightness and contrast levels. Localization and residence times of individual IFNα2 molecules were determined from trajectories obtained by the multiple target tracking algorithm (Sergé et al., 2008) as described previously (Waichman et al., 2011). After filtering for immobile trajectories (D ≤ 0.001 μm2/s), only trajectories longer than 10 frames were used to minimize erroneous trajectories. Histograms of the frequency of different residence times were fitted by a biexponential decay function to eliminate the contribution of erroneously connected trajectories.

For probing the ligand dissociation kinetics by chasing, 1 µM unlabeled IFNα2 or IFNα2/βA1/YN was added, and the pattern was imaged until no further changes were detectable. For the evaluation, all immobile single molecules within the pattern were localized and counted frame by frame. A decay of the number of localized single molecules during the chasing experiments was fitted by a monoexponential decay function. Protein diffusion in the plasma membrane was analyzed by single-molecule tracking as described previously (You et al., 2010).

FRAP experiments
FRAP experiments were performed by using the pinhole in the TIR condenser of the CellTIRF Xcelsure microscope for bleaching a circular region with a diameter of 18 μm (60X objective) or 8 μm (150X objective) using the 405-nm or the 488-nm laser for photobleaching. For experiments of cells expressing tyk2-EGFP, a 60× objective with an NA of 1.45 (U Apochromat N 60x/1.45 NA; Olympus) was applied for TIR excitation. By using pinhole controls, a circular region with a diameter of 18 μm was selected to cover the pattern and bleached by 488-nm excitation for 25 s with laser power of 11 mW at the objective. Fluorescence recovery was followed by image acquisition with the same time of 1 s and laser power of ~1 mW at the objective. For FRAP of STAT2-EGFP, a 150× objective with NA of 1.45 (U Apochromat N 150x/1.45 NA; Olympus) was used for TIR excitation. A circular area with a diameter of 8 μm was bleached by 405-nm excitation for 5 s with a laser power of 7.5 mW followed by acquisition with a cycle time of 1 s by a 1-mW, 488-nm laser excitation.

Fluorescence intensity values were quantified by using Imagent software. A rectangular region of interest (ROI) was drawn around the area of the pattern and a circular ROI within the bleached area but outside the
patterned area were chosen for obtaining intensity values per pixel over time. FRAP curves were obtained by the following equation:

\[
F(t) = \frac{F_{\text{ROI}_{\text{inside}}}(t) - F_{\text{ROI}_{\text{outside}}}(t)}{F_{\text{ROI}_{\text{inside}}}(0) - F_{\text{ROI}_{\text{outside}}}(0)}
\]

with \(F_{\text{ROI}_{\text{inside}}}(t)\) and \(F_{\text{ROI}_{\text{outside}}}(t)\) being the fluorescence intensities inside and outside the pattern, respectively, within the bleached spot. \(F_{\text{ROI}_{\text{inside}}}(0)\) is the fluorescence intensity of an unbleached ROI inside the micropattern, and \(F_{\text{ROI}_{\text{outside}}}(t)\) is the fluorescence intensity of this ROI before the bleaching experiment.

Online supplemental material

Fig. S1 shows the characterization of functional surface micropatterning. Fig. S2 shows that protein micropatterning in cells is specific and correlates with the surface micropatter. Fig. S3 shows negative control experiments confirming negligible effects of protein micropatterning on the actin skeleton, transmembrane protein diffusion, and on cytosolic protein distribution. Fig. S4 shows a negative control demonstrating specificity of Jak1 phosphorylation in micropatterns. Fig. S5 shows constitutive interaction of STAT2 with IFNAR2 bound to micropatterned IFNAR1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201206032/DC1.

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