Membrane-associated STAT3 and PY-STAT3 in the Cytoplasm*

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Signal transduction from the plasma membrane to the nucleus by STAT proteins is widely represented as exclusively a soluble cytosolic process. Using cell-fractionation methods, we observed that ∼5% of cytoplasmic STAT3 was constitutively associated with the purified early endosome (EE) fraction in human Hep3B liver cells. By 15–30 min after interleukin-6 (IL-6) treatment, up to two-thirds of cytoplasmic Tyr-phosphorylated STAT3 can be associated with the purified early endosome fraction (Rab-5–, EEA1–, transferrin receptor–, and clathrin-positive fraction). Electron microscopy, immunofluorescence, and detergent dissection approaches confirmed the association of STAT3 and PY-STAT3 with early endosomes. STAT3 was constitutively associated with clathrin heavy chain in membrane and in the 1–2-MDa cytosolic complexes. The membrane association was dynamic in that, within 15 min of treatment with the vici nal-thiol cross-linker phenylarsine oxide, there was a dramatic increase in bulk STAT3 association with sedimentable membranes. The functional contribution of PY-STAT3 association with the endocytic pathway was evaluated in transient transfection assays using IL-6-inducible STAT3-reporter-luciferase constructs and selective regulators of this pathway. STAT3-transcriptional activation was inhibited by expression constructs for dominant negative dynamin K44A, epsin 2α, amphiphysin A1, and clathrin light chain but enhanced by that for the active dynamin species MxA. Taken together, these studies emphasize the contribution of the endocytic pathway to productive IL-6/STAT3 signaling.

Although until recently it was considered that latent STAT proteins in the cytoplasm were monomeric, work from this and other laboratories showed that latent STATs exist in the cytosol already in the form of at least dimers and included higher order complexes (200–400 kDa statues I and 1- to 2-MDa statues II complexes) (4–8, reviewed in Refs. 9 and 10). The absence of free STAT monomers in the cytoplasm has now been extensively confirmed (11–14). Moreover, recent fluorescence transfer and fluorescence correlation spectroscopy data confirm the existence of STAT3 dimers and higher order statues complexes (200–400 kDa and 1–2 MDa) in the cytoplasm of live cells (15–17).

That different growth factor and cytokine receptors are associated with the endocytic pathway and can even maintain their ongoing signaling function from this membrane-bound compartment has been clearly delineated (18–20). Nevertheless, today, the widely represented model of IL-6/STAT3 signaling is that of an activation process at the cytosolic face of the plasma membrane with departure of “activated” STATs into the free soluble cytosol with little consideration of membrane-associated transit through the cytoplasm (see Ref. 2 for a depiction of this model). We earlier reported that the IL-6-activated STAT3 transcription factor had a cytoplasmic punctate appearance in human liver Hep3B cells in immunofluorescence studies (21, 22). In cell fractionation experiments we reported the association of Western-blottable STAT3 and PY-STAT3 as well as DNA-binding competent PY-STAT3 with a large granular membrane fraction (the “P15 fraction”) as well as a P100 sedimentable cytoplasmic fraction (6, 7). These data were at variance with the idea of IL-6/STAT3 signaling as exclusively a soluble cytosolic process. In this report we demonstrate the association of STAT3 and PY-STAT3 with the purified early endosome fraction and provide evidence for a significant role of this pathway in the transcriptional activation function of STAT3.

Diverse cytokines and growth factors, including various interleukins and interferons, signal to the cell nucleus by activating the JAK2/STAT (Janus kinase/signal transducers and activators of transcription) pathway at the plasma membrane at the level of raft microdomains (reviewed in Refs. 1–3). This signal transduction from the plasma membrane to the nucleus by Tyr- and/or Ser-phosphorylated STAT proteins is widely represented exclusively as a soluble cytosolic process.

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§The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activator of transcription; CHC, clathrin heavy chain; EE, early endosome; EEA, early endosome antigen; EGFR, epidermal growth factor; EGF, epidermal growth factor receptor; HM, heavy membrane; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; IL, interleukin; LE, late endosome; PAO, phenylarsine oxide; PY, tyrosine-phosphorylated; Smad, anchor for receptor activation; pAb, polyclonal antibody; mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

MATERIALS AND METHODS

Cell Growth and Fractionation—Human liver Hep3B cells were grown as described earlier (5–7, 23). In different experiments cells were treated with IL-6 (10 ng/ml, R&D Systems Inc., Minneapolis, MN), phenylarsine oxide (PAO, 5 μM, Sigma-Aldrich), or 2,3-mercaptothanol (50 μM, Sigma) as indicated. Unless indicated otherwise, cell fractionation into a nuclear fraction and the post-nuclear P15 large granular membrane fraction, the P100 and S100 cytoplasmic fractions, DNA shift assays for activated STAT3 using the m67-SIE probe, Western blotting and enzymatic assays for 5′-nucleotidase, and lactate dehydrogenase were carried out using methods previously described (5).

Purification of Early Endosomal Fraction—This was carried out using the procedure of Aniento et al. (26). Briefly, Hep3B cells were scraped into 2 ml of buffer H (3 mM imidazole, 1 mM EGTA, 8.55% sucrose, pH 7.4), broken by Dounce homogenization, the postnuclear supernatant adjusted to 40.6% sucrose, and subjected to equilibrium flotation through layers of 35%, 25%, and 8.55% sucrose. As previously characterized (26), the visible band at the 35/25% interface corresponds to the early endosome (EE) fraction, that at the 25/8.55% interface to late
endosomes (LE), and the one at the 40.6/35% interface to heavy membranes (HM). Aliquots of the soluble (S) and the pellet (P) fractions were also collected from within the 40.6% loading region (1 ml out of 4 ml loaded) and the bottom, respectively.

**Immunosolation Using Anti-STAT3 pAb-coupled Magnetic Beads—** Anti-STAT3 rabbit IgG (H190X, gelatin-free, Santa Cruz Biotechnology, Santa Cruz, CA) was coupled to tosyl-activated 2.8-μm paramagnetic Dynabeads (Dynal Biotech Inc., Brown Deer, WI) using procedures provided by the manufacturer (100 μg of IgG coupled to 4 × 10^8 beads). As a control, non-immune rabbit IgG (Sigma) was also coupled to Dynabeads in parallel. Intact membrane elements were isolated using these beads in buffer H while protein complexes were isolated in detergent-containing buffers (e.g. 0.05–0.5% Triton X-100 and 0.1% SDS) as described earlier (7, 25). Alternatively immunopanning for protein complexes was carried out using anti-STAT3 pAb (C-20) in buffer adjusted to 0.5% Triton X-100 and 0.1% SDS and Protein A-magnetic beads.

**Electron Microscopy of STAT3-carrying Membrane Elements—** Immunosolates derived from purified early endosome fractions using anti-STAT3- or IgG-coupled Dynabeads (2.8 ± 0.2-μm diameter) in buffer H, and fixed with 4% paraformaldehyde and 0.1% glutaraldehyde. Whole mount negatively stained specimens spotted on Formvar-coated grids were examined using an Hitachi H7000 electron microscope.

**Confocal Immunofluorescence Microscopy—** This was carried out as described previously using several different fixation protocols as indicated in respective figures (methylene-acetone fixation, fixation in cold paraformaldehyde/Triton, or with formaldehyde (37 °C)/Triton (21, 22, 25)) and various primary rabbit pAb and murine mAb. Secondary antibodies included corresponding AlexaFluor 488-, AlexaFluor 594-, and AlexaFluor 647-tagged antibodies (Molecular Probes, Eugene, OR). Images were collected using an MRC 1024 ES (Bio-Rad) confocal microscopy system. All data within each experiment were collected at identical imaging settings. Deconvolution of collected images was carried out using the Iterative Deconvolve 3D plugin (DAMAS3 algorithm) for National Institutes of Health (NIH) Image J.

**Colocalization Image Analyses—** Dual channel colocalization analysis was performed on randomly selected sections of the images representing cytoplasmic signals using the NIH Image J software and the Colocalization Threshold and Colocalization Test plug-ins. The Colocalization Threshold tool calculates the Mander’s coefficients M1 and M2 representing the number of colocalized pixels expressed as a fraction of the number of the pixels within the respective channels. Zero-zero pixels are ignored for purposes of this calculation. The Mander’s coefficients range from 0 to 1 and are independent of the pixel intensities within the individual channels (27). Pearson’s correlation co-efficient (R), a well known index to determine pattern similarity (28), was determined by both plug-ins and matched well.

The Colocalization Test tool was used to generate random images using an approach described by Costes et al. (29). Briefly, scrambled images from the second channel are compared with the first channel to determine if the colocalization occurs as a matter of chance. To generate the scrambled images, blocks of pixels are rearranged at random and the Pearson’s R is calculated for each new random image. For each comparison, 3000 such random images were generated, and the values of the random R (R(rand)) were compared with the actual R.

**Plasmids, Transient Transfection, and Luciferase Reporter Assays—** The pSTAT3/Luciferase reporter was a gift from Dr. David Levy, New York University and carried two tandem repeats of the acute phase response element of α-macroglobulin (30). The p950M4/Luciferase reporter, containing four copies of the STAT3-binding DNA element from the human angiotensinogen promoter (5′-CGTTTCTGG-GAACCT-3′) cloned into pBLuc, was a gift from Dr. Ashok Kumar, New York Medical College (7). The expression vector for dynaminK44A and for clathrin light chain were gifts from Dr. Lois Greene, NHLBI, National Institutes of Health (31). Vectors for a truncated amphipysin A1 dominant negative mutant and for epsin2a were provided by Dr. Richard Jove, University of South Florida (32), and that for the MxA dynamin species was a gift from Dr. Otto Haller (Universität Freiberg, Freiberg, Germany) (33). Transient transfection assays in cultures of Hep3B cells in 6-well plates were carried out as described earlier (7). Transfections were carried out at least in triplicate for each variable, and luciferase activity is expressed in arbitrary units (as mean ± S.E.) after normalization with the β-galactosidase activity among different samples. The Student’s t test was used to assess statistical significance.

**Antibodies—** Rabbit polyclonal IgGs to STAT3 (C20, H190, and H190X), and respective immunoproteins to which pAbs C20 (STAT3) and sc-894 (caw-1) had been raised, and murine mAb to PY-STAT3 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit pAb to PY-STAT3 was purchased from Cell Signaling, Beverly, MA, whereas murine mAbs against STAT3, clathrin heavy
STAT3 in Early Endosomes

FIGURE 2. STAT3 and PY-STAT3 in purified early endosome fractions. Purified early endosome (EE) and late endosome (LE) fractions were prepared from control and IL-6-treated Hep3B cells by flotation of the post-nuclear supernatant up a differential sucrose gradient as described under “Materials and Methods.” Additionally, aliquots of the very top of the gradient (light layer, LL), the heavy membrane (HM) fraction at the 40.6/25% interface, the soluble material (S), and the pellet fraction (P) were also collected. The HM fraction was washed once in Buffer H to remove soluble material. A, compartment-matched aliquots of the LL, LE, EE, and HM fractions as indicated were immunopanned using anti-gp130 pAb or anti-STAT3 pAb, Western-blotted and the blots probed using anti-STAT3 and anti-PY-STAT3 mAbs. B, compartment-matched aliquots of each indicated fraction (except the S fraction, which was 25% of the matched amount) were Western blotted for respective markers for early endosomes and for STAT3. For PY-STAT3, aliquots (500 µl) were first immunopanned using anti-STAT3 pAb and Western blotted, and then the blot was probed using an anti-STAT3 mAb. Densitometric analyses of the PY-STAT3 blot indicated that ~68% of the total PY-STAT3 was in the EE fraction, while >90% of the STAT3 was present in the soluble (S) fraction. C, whole mount electron microscopy of the negatively stained, purified LE and EE fractions derived from IL-6-treated Hep3B cells after subjecting these to immunopurification using non-immune IgG (not shown) or anti-STAT3-coupled Dynal beads. Scale bars — 10 nm in LE depiction; 25 nm in EE depiction.

RESULTS

Association of STAT3 and PY-STAT3 with the Purified Early Endosome Fraction—Three observations, taken together, prompted us to investigate the association of STAT3 with cytoplasmic membrane elements. First, we observed the association of STAT3 and IL-6-activated PY-STAT3 with cytoplasmic membrane fractions, which lacked detectable levels of the plasma membrane marker 5'-nucleotidase and of the cytosolic marker lactate dehydrogenase (Fig. 1A). Second, immunopanning analyses using anti-STAT3 pAb covalently coupled to paramagnetic beads, followed by SDS-PAGE, Coomassie staining, and mass spectrometry-MALDI TOF-based identification of proteins led to the observation that membrane-associated STAT3 was in complexes with CHC (Fig. 1, B and C). Moreover, this association between STAT3 and CHC was also observed in the soluble cytosol S100-derived 1- to 2-MDa statosomes II complexes previously reported by us (Fig. 1D), reminiscent of the constitutively cycling membrane-associated and soluble pools of CHC in the endocytic pathway. Third, STAT3 and PY-STAT3 showed punctuate cytoplasmic immunofluorescence (21, 22).

Early and late endosome fractions were purified from control and IL-6-treated Hep3B cells using an established approach (26). Fig. 2A provides evidence for the constitutive association of STAT3 with both the early and late endosome fractions as well as the clear association of PY-STAT3 with purified early endosomes. It is also clear that in these fractions most of the STAT3 and PY-STAT3 was not associated with the gp130 chain of the IL-6 receptor per se. Fig. 2B illustrates the characterization of the purification scheme used to obtain the early endosome fraction and provides a “bookkeeping” account of the distribution of STAT3 and PY-STAT3 and various markers in different fractions. As expected, the purified EE fraction was positive for the early endosome markers EEA1 and Rab5, for CHC and for the transferrin receptor. Remarkably, although >90% of bulk cytoplasmic STAT3 was present in the soluble fraction, up to two-thirds of cytoplasmic PY-STAT3 was in the EE fraction. Fig. 2C shows negative-stained whole mount electron microscopy of anti-STAT3-immunopurified vesicles from the LE and EE fractions. These data show that the STAT3-bearing vesicles in the LE and EE fractions are different in appearance and that those in the EE fraction are clearly “coated.” As controls, non-immune rabbit IgG-coupled beads failed to pull out any detectable vesicular elements (data not shown). The anti-STAT3 immunosolutes from the EE fraction were positive for CHC, and the hepatocyte growth factor regulated tyrosine kinase substrate (HRS) (34) but not for the Smad anchor for receptor activation as assayed by Western blotting (data not shown).

Confocal Immunofluorescence Studies—As an independent approach, we used confocal double-label immunofluorescence microscopy to identify the punctuate structures with which STAT3 was associated in the cytoplasm (21, 22). Supplemental Fig. S1 shows that this punctate cytoplasmic STAT3 immunostaining was observed using three different fixation methods. A relevant, but not an irrelevant peptide, specifically inhibited this immunostaining: three different antibodies to STAT3 all immunostained punctate cytoplasmic organelles with a perinuclear preponderance (Supplemental Fig. S1B), whereas non-immune rabbit IgG did not display any immunostaining (Supplemental Fig. S1C). IL-6 clearly and markedly enhanced immunostaining for chain (CHC), EEA1, Rab5, and LAMP1 were obtained from BD Biosciences. Non-immune rabbit IgG was purchased from Sigma.
Detergent Dissection Studies of STAT3 and PY-STAT3 in the Cytoplasm—We used the differential detergent dissection approach pioneered by Penman and colleagues (35–37) as another independent approach (complementary to the data in Fig. 2B), to address the bookkeeping questions: how much of cytoplasmic STAT3 is constitutively membrane-associated, and how much of the cytoplasmic pool of IL-6-induced PY-STAT3 is membrane-associated? Supplementary Fig. S2A shows that, when the detergent series saponin, Brij-58, and Triton/0.1%SDS was used to dissect Hep3B cells, >90% of cytoplasmic STAT3 was released by digitonin (which makes holes in the plasma membrane) but only one-quarter of IL-6-induced cytoplasmic PY-STAT3. Conversely, release of most of PY-STAT3 (~70%) required Brij-58. Release of the majority of the early endosomal antigen EEA1 also required Brij-58. It should be noted parenthetically that digitonin and saponin selectively permeabilize the plasma membrane but do not solubilize the cholesterol-poor endomembranes. Using a different detergent pair (digitonin and Brij-58), the data in Supplementary Fig. S2B represent an independent confirmation of the observation that while the bulk of STAT3 is released by digitonin alone, release of the majority of PY-STAT3 requires Brij-58.

Dynamic Constitutive Cycling of STAT3 at Cellular Membranes—Clathrin is known to dynamically associate with the cytosolic face of the plasma membrane and dissociate from internalized cytoplasmic vesicular elements in a constitutive manner with a t½ of ~30 s (31, 38, 39). Does STAT3 (which is associated with CHC; see Fig. 1, B–D) have a similar constitutive dynamic behavior? We have used the vicinal-thiol cross-linker PAO to explore this question (32, 40–42). The partitioning of STAT3 between the soluble and sedimentable membrane structures can be illustrated conveniently in an assay in which cellular STAT3 in Hep3B cells was separated into a saponin-soluble supernatant fraction and a saponin-insoluble pellet fraction. Within 15 min of PAO treatment approximately one-third to one-half of bulk total cellular STAT3 shifted from the saponin-soluble to the pellet fraction (Fig. 4A). This association appeared to be on the cytosolic face of the pelleted organelles in that the STAT3 and CHC but not LAMP1 were accessible to digestion by trypsin without the need to add Triton X-100 (Fig. 4B). This PAO-enhanced association of STAT3 with sedimentable membranes was reversible by addition of a 10-fold molar excess of 2,3-dimercaptopropanol (41) to cultures first treated with PAO for 15 min (data not shown). Fig. 4C shows a dramatic recruitment of STAT3 to sedimentable membrane elements coinciding with CHC and included fractions, which were EEA1- and LAMP1-positive. The occurrence of a marked increase in association of STAT3 with cytoplasmic membranes within 15–30 min of PAO (Fig. 4, A and C) is indicative of a rapid constitutive cycling of STAT3 at such membranes and the ability of the cross-linker PAO to inhibit the departure reaction. We suggest that STAT3 constitutively and rapidly cycles at the plasma membrane and the cytosolic face of the endocytic pathway, perhaps in association with CHC.

Role of Endocytic Trafficking in the IL-6-stimulated Transcriptional Activation Function of STAT3—We used transient transfection assays to evaluate the effects of modulators of endocytic function on transcriptional signaling by IL-6-activated STAT3. Fig. 5 is a composite summary of some of these experiments using the STAT3-responsive p95OM4 reporter construct (7); similar data were obtained using pSTAT3/luc (not shown). Vectors expressing proteins with a dominant-negative phenotype per se for dynamin, clathrin light chain, epsin 2A, and amphiphysin A1 function also had an inhibitory effect (Fig. 5, A and B). However, increasing dynamin activity by expression of the interferon-inducible MxA dynamin species further enhanced STAT3 signaling (Fig. 5C). Taken together, the data in Fig. 5 provide support for a func-
The contribution of the endocytic pathway toward IL-6/STAT3 transcriptional signaling.

**DISCUSSION**

Following the observation of punctuate pattern of cytoplasmic STAT3 immunofluorescence, the association of STAT3 with a cytoplasmic membrane fraction and its interaction with clathrin in cytosolic and membrane protein complexes, we have revisited the question of soluble versus membrane-associated STAT3 in the cytoplasm. The present studies provide direct evidence for the constitutive and IL-6-induced association of STAT3 and PY-STAT3 with early endosomes. We investigated the association of STAT3 with early endosomes using an established method to purify endosomes, immunosolubilization of STAT3-bearing vesicles in such purified EE fractions using anti-STAT3-magnetic bead panning, electron microscopy, double-label confocal microscopy, and formal colocalization image analyses. The present data identify clathrin heavy chain as a component of cytoplasmic high-molecular mass complexes of STAT3. Overall, the data emphasize the contribution of the endocytic pathway to productive IL-6/STAT3 signaling.

The importance of the endocytic pathway in the regulation of cellular signaling is becoming apparent in many experimental systems (43, 44). The formulation of a “signaling endosome hypothesis” (45–49) has drawn attention to the ability of clathrin-coated endosomal vesicles to mediate rapid and directional transit of signaling molecules through the cytoplasm. In recent years signaling by nerve growth factor, transforming growth factor-β, and epidermal growth factor (EGF) have all been shown to include a major endocytic component. Giri and colleagues (50) have recently characterized the nuclear import of ErbB-2 activated at the plasma membrane and have shown that the transcytoplasmic transit takes place along the endocytic pathway with a hand-off to the importin and nuclear pore machinery for subsequent and specific nuclear import.

With respect to membrane-associated STAT signaling, Bild and colleagues (32) have shown that EGF- and platelet-derived growth factor-activated STAT3 colocalized in the cytoplasm with vesicular elements, which also contained the α-adaptin polypeptide of the AP2 complex. PAO, and dominant-negative expression vectors for dynamin K44A, epsin 2a, and amphiphysin A1 inhibited growth factor-stimulated transcriptional activation by STAT3 (32). Independently, Scoles et al. (51) have shown a role for the endosome regulator HRS in EGF/STAT3 signaling. In these experiments, overexpression of HRS inhibited transcriptional EGF/STAT3 signaling. We have confirmed that fractions of the immunosolubilated STAT3-bearing early endosomes derived from IL-6-treated Hep3B cells (such as in Fig. 2C) contain HRS by Western blotting (data not shown). HRS, best known for targeting ubiquitinated receptor molecules (such as EGFR) for internalization, nevertheless has multiple roles in membrane trafficking, including recycling mechanisms and regulation of signaling such as that of the mitogen-activated protein kinase pathway (52).

**TABLE 1**

| Antigen pair (green/red) | Mander's coefficients | Pearson's R | Costes' randomization |
|--------------------------|-----------------------|-------------|-----------------------|
|                          | M1<sup>a</sup> | M2<sup>a</sup> | R<sup>b</sup> | R<sup>b</sup> (rand) | Iterations | R > R<sup>b</sup> (rand) |
|----------------------------|-----------------|-------------|-------|----------------|------------|---------------------|
| STAT3/CHC                  | 0.76            | 0.62        | 0.24  | −0.038         | 3000       | 100                 |
| STAT3/EAA1                 | 0.90            | 0.77        | 0.51  | 0.030          | 3000       | 100                 |
| STAT3/Rab5                 | 0.96            | 0.69        | 0.38  | 0.054          | 3000       | 100                 |
| STAT3/α-adaptin            | 0.86            | 0.67        | 0.17  | −0.025         | 3000       | 100                 |
| PY-STAT3/EAA1              | 0.86            | 0.74        | 0.21  | −0.006         | 3000       | 100                 |

<sup>a</sup> M1: Mander’s coefficient for Channel 1 (this represents the number of colocalized pixels in the green channel expressed as a fraction of the total number of non zero-zero pixels in that channel).

<sup>b</sup> M2: Mander’s coefficient for Channel 2 (this represents the number of colocalized pixels in the red channel expressed as a fraction of the total number of non zero-zero pixels in that channel).

<sup>c</sup> R: Pearson’s correlation co-efficient (the standard Pearson’s correlation coefficient ranges from −1 to +1 and is a general indicator for pattern similarity between two images).

<sup>d</sup> R<sup>b</sup> (rand): mean of Pearson’s correlation coefficients obtained from randomized images by Costes’ method. Costes’ method for randomization involves generating scrambled images by randomly rearranging blocks in one of the channels (in a Monte Carlo fashion) for specified iterations (3000 in this case). Pearson’s R is calculated for each and the mean is stated as R<sup>b</sup> (rand).

<sup>e</sup> R > R<sup>b</sup> (rand): number of times the actual Pearson’s R is greater than the random R, within the number of iterations performed.
In the present study we have provided cell fractionation and microcopy data to validate the association of STAT3 or PY-STAT3 with an isolated early endosome fraction. What is the role of the endosomal pathway in the cytoplasmic physiology of STAT3? Inhibition of endocytosis by transfection with dominant negative amphiphysin, epsin, dynamin, and the clathrin light chain resulted in an inhibition of IL-6-stimulated STAT3 transcriptional function, indicating that endosome-mediated trafficking of STAT3 may be required for optimal signal transduction. It is unlikely that the observed inhibition of signaling is due to an alteration of surface expression of the IL-6 receptor chains, because:

(i) it has already been established that STAT3 signal generation as well as signal termination are independent of endocytosis of gp130 (53) and

(ii) the presence of a vast excess of “spare” IL-6/gp130 receptors, which in the case of hepatocytes requires a 100-fold higher of IL-6 to saturate binding sites (1 μg/ml) than the transcriptionally maximal IL-6 concentration (10 ng/ml) used in the present experiments (53).

The membrane association of cytoplasmic STAT3 may have a role in STAT3 metabolism in addition to efficient signal transduction. STAT3 contains the canonical YXXΦ motif which is known to mark cargo for internalization along the vesicular pathway (54). In light of the evidence of the association of STAT3 with endosomes, it can be hypothesized that pools of STAT3 and PY-STAT3 may associate with other cytoplasmic vesicular elements. Indeed, preliminary work from this laboratory suggests that a pool of STAT3 associates with purified lysosomes and may even undergo chaperone-mediated autophagy, a well-known pathway for degradation of cytoplasmic proteins.

STAT3 has also been shown to associate with several proteins containing the YXXQ motif such as gp130, EGF receptor (EGFR), erythropoietin receptor, leukemia inhibitory factor receptor and STAP-2/BKS (55–58). CHC also contains the YXXQ motif; however, it remains to be determined whether the STAT3-CHC interaction is mediated via these motifs. Moreover, IL-6-induced enhancement of transforming growth factor-β-signaling (which takes place along the previously well characterized raft/endocytic pathway, see Refs. 59 and 60) involved STAT3 as “scaffold” for the transforming growth factor-β-activated kinase 1/Nemo-like kinase (TAK1-NLK) kinases, specifically in the YXXQ motif-derived pathway emanating from gp130 (23). In turn, the TAK1-NLK kinases contributed to Ser-727 phosphorylation of STAT3.

To summarize, we suggest a view of cytoplasmic STAT3 dynamics in which STAT3 in association with CHC and perhaps other protein partners constitutively shuttles on and off the cytoplasmic face of vesicular elements, including early endosomes. Stimulation by IL-6 results in a more selective association of PY-STAT3 with such membranes, which could be due to a selective affinity of the Tyr-phosphorylated form for such membranes or relevant adaptors. As in the case of the ErbB2 receptor, early endosomes may serve as a platform for handing-off STAT3 from the plasma membrane to the importin-nuclear pore complex for subsequent nuclear entry.

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Note Added in Proof—The association of STAT3 with vesicular elements in the early and late endosome fractions as in Fig. 2C has been further confirmed by us using whole mount anti-STAT3 immunogold electron microscopy.

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