Novel mRNA-containing cytoplasmic granules in ALK-transformed cells

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ABSTRACT

In mammalian cells, non-translating mRNAs are concentrated in different cytoplasmic
foci, such as processing bodies (PBs) and stress granules (SGs), where they are either degraded or
stored. In the present study, we have thoroughly characterized cytoplasmic foci, hereafter called AGs
for ALK granules that form in transformed cells expressing the constitutively active anaplastic lymphoma kinase (ALK). AGs contain polyadenylated mRNAs and a unique combination of several RNA binding proteins that has not been so far described in mammalian foci, including AUF1, HuR and the poly (A) binding protein PABP. AGs shelter neither components of the mRNA degradation machinery present in PBs nor known markers of SGs, such as translation initiation factors or TIA/TIAR, showing that they are distinct from PBs or SGs. However, AGs and PBs both move on microtubules with similar dynamics and frequently establish close contacts. In addition, in conditions in which mRNA metabolism is perturbed, AGs concentrate PB components with the noticeable exception of the 5’ to 3’ exonuclease XRN1. Altogether, we show that AGs constitute novel mRNA-containing cytoplasmic foci and we propose that they could protect translatable mRNAs from degradation, contributing thus to ALK-mediated oncogenicity.

INTRODUCTION

A key aspect of gene regulation in eukaryotes is the cytoplasmic control of mRNA degradation and translation. A number of cytoplasmic granules containing messenger ribonucleoproteins (mRNPs) have been identified in the past few years, including stress granules (Anderson and Kedersha, 2009), neuronal granules (Kiebler and Bassell, 2006), germ cell specific granules (Seydoux and Braun, 2006) and processing bodies (PBs) (review in Balagopal and Parker, 2009; Eulalio et al., 2007; Franks and Lykke-Andersen, 2008; Kulkarni et al., 2010; Moser and Fritzler, 2009). PBs, also known as GW bodies, are involved in deadenylation-decapping-5’-3’ decay pathway as well as in miRNA-mediated silencing, non sense-mediated mRNA decay (NMD) and AU-rich mRNA decay (AMD) (review in Eulalio et al., 2007; Franks and Lykke-Andersen, 2008; Garneau et al., 2007; Kulkarni et al., 2010). Although it is still questionable whether mRNA decay happens within PBs, the fact that they concentrate decapping enzymes (DCP1a-b and DCP2), activators of decapping (LSM1-7, RCK/p54, EDC1-3) and the 5’ to 3’ exonuclease XRN1 but neither ribosomal proteins nor most of the translation factors strongly suggests that PBs are active sites of mRNA degradation (Cougot et al., 2004; Sheth and Parker, 2006). However, PBs have also
been shown to be storage sites for translationally arrested mRNAs that can return to translation (Bhattacharyya et al., 2006; Brengues et al., 2005; Sheth and Parker, 2006; Teixeira et al., 2005).

Mammalian cells also contain cytoplasmic foci that appear in response to environmental stresses and are thus called stress granules (SGs) (review in Anderson and Kedersha, 2008). SGs accommodate mRNAs, proteins of the small ribosomal subunit, several translation initiation factors (Kedersha et al., 2002; Kedersha et al., 2005), PABP (Kedersha et al., 1999) and repressors of translation, such as TIA/TIAR (Piecyk et al., 2000) and CPEB1 (Wilczynska et al., 2005), as well as other components, including the AU-binding protein (AU-BP) HuR (Gallouzi et al., 2000). Since SGs do not include components of the mRNA degradation machinery, these granules are believed to be sites of mRNA storage (review in Anderson and Kedersha, 2009). Although SGs and PBs are clearly distinct structures, they share proteins, in particular TTP and BRF1, two AU-BPs involved in AMD (Fenger-Gron et al., 2005; Franks and Lykke-Andersen, 2007), and the same reporter mRNA (Kedersha et al., 2005), leading to the proposal that PBs and SGs might exchange mRNPs (Balagopal and Parker, 2009). The recent observation that PBs and SGs move within the cytoplasm in a microtubule-dependent manner gives support to this hypothesis (Aizer and Shav-Tal, 2008; Chernov et al., 2009; Nadezhdina et al. 2010) and strongly suggests that cycling of mRNPs between polysomes, PBs and SGs requires an intact microtubule network. Studying NPM-ALK transformed cells, we and others have recently detected cytoplasmic foci that concentrate this oncogenic tyrosine kinase (Fawal et al., 2006; Honorat et al., 2006). NMP-ALK is a chimeric protein resulting from the (t2;5)(p23;q35) chromosomal translocation bringing the nucleophosmin (NPM) gene at 5q35 in juxtaposition with the anaplastic lymphoma kinase (ALK) gene at 2p23, which encodes a receptor tyrosine kinase expressed almost exclusively in the central nervous system during embryogenesis (Morris et al., 1997). Even though NPM-ALK is the most frequent chromosomal translocation observed in Anaplastic Large Cell Lymphomas (ALCLs) (Benharroch et al., 1998; Morris et al., 1997; Pulford et al., 1997), other less common N-terminal-fused partners of ALK, such as ATIC (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase) or TPM3 (tropomyosin 3), have been described (Lamant et al., 1999; Touriol et al., 2000; Trinei et al., 2000).
In those cases, the N-terminal partners of ALK share an oligomerization domain triggering the constitutive auto-phosphorylation of ALK and activation of its oncogenic properties (Pulford et al., 2004a). This, in turn, activates various signaling pathways (Bai et al., 2000; Zamo et al., 2002) and leads to unregulated growth of X-ALK expressing cells (Duyster et al., 2001; Pulford et al., 2004b).

Searching for NPM-ALK interacting partners, we found that AUF1/hnRNPD, a protein implicated in AMD (Bevilacqua et al., 2003; Zhang et al., 1993), was immunoprecipitated with NPM-ALK, both in ALCL-derived cell lines and in NIH3T3 cells stably expressing NPM-ALK that recapitulate most of the tumorigenic properties of ALCLs (Armstrong et al., 2004). In addition to their localization in the nucleus, both NPM-ALK and AUF1 were found to concentrate within discrete cytoplasmic foci in NPM-ALK-expressing NIH3T3 and ALCL-derived cells (Fawal et al., 2006; Honorat et al., 2006). We also found that several AUF1-target mRNAs encoding key regulators of cell proliferation are stabilized in these cells, giving those cells a survival advantage that could contribute to their oncogenic properties (Fawal et al., 2006). We thus hypothesized that NPM-ALK cytoplasmic bodies, hereafter called AGs for ALK Granules, could act to control cytoplasmic mRNA fate. In this work, we have further characterized AGs. We show that they concentrate the active, phosphorylated form of NPM-ALK. They do contain mRNAs but do not include components of mRNA translation or degradation machineries. Using live cell imaging, we have visualized the dynamics of AGs in the cytoplasm and found it similar to PBs one. Indeed, most AGs are mobile and require intact microtubule network for their movement. Altogether, our results highlight an unexpected function of cytoplasmic NPM-ALK in assembling large mRNP structures. We propose that through their ability to scan the cytoplasm and capture mRNAs, AGs may be potent actors of cell transformation.

RESULTS

*X-ALK fusion proteins are concentrated in cytoplasmic foci in their active phosphorylated form*

In cells derived from ALCLs, NPM-ALK is expressed in the nucleus, including nucleolus (Pulford et al. 1997) and in small cytoplasmic foci (AGs) (Fawal et al., 2006; Honorat et al., 2006).
AGs are also detected in NPM-ALK expressing NIH3T3 cells (Fawal et al. and Fig. 1A, left) that are used as a convenient model to study NPM-ALK oncogenic properties (Armonstrong et al. 2004). In those cells, NPM-ALK expression is weaker than in ALCL-derived cell lines (Fig. 1B), showing that AG nucleation does not result from aberrant NPM-ALK overexpression. Most NPM-ALK NIH3T3 cells and ALCLs-derived cells (Cost and Karpas) contain AGs with an average of 8 cytoplasmic foci per cell (Fig. 1A, supplementary material Fig. S1A for quantification and S4B for Karpas AGs). Such cytoplasmic foci were not observed in NIH3T3 cells transiently transfected with vector encoding NPM-eGFP (Grummitt et al., 2008) or the full-length ALK receptor (Fig. S1B), showing that NPM-ALK fusion is required for AG nucleation. We previously established that a kinase dead mutant of NPM-ALK protein could not nucleate AGs (Fawal et al. 2006). Consistent with this result, we observed that AGs disappear when NPM-ALK NIH3T3 cells are treated with an ALK kinase specific inhibitor (see Materials and Methods) that does not modify the level of NPM-ALK but inhibits its kinase activity (Fig. 1C and Fig. S1B).

To test whether other ALK fusions nucleate AGs, we stained previously described TPM3- and ATIC-ALK NIH3T3 cells (Armstrong et al., 2004) with anti-ALK and anti-phospho-ALK antibodies. Confocal microscopy analysis showed that both cell types concentrate the ALK fusion proteins in cytoplasmic foci in their phosphorylated, i.e., active kinase form (Fig. 1A and data not shown for ATIC-ALK). Altogether, these results indicate that ALK is present in its phosphorylated form in AGs and that AG formation relies on the active tyrosine kinase domain of ALK fusions.

**AGs contain polyadenylated mRNAs**

It is well established that cytoplasmic foci such as PBs and SGs accumulate mRNAs together with mRNA-binding proteins. Since AGs contain the RNA-binding protein AUF1 (Fawal et al. 2006), we further asked whether AGs also contained mRNAs. To test this hypothesis, we first used ethidium bromide (EtBr) to fluorescently label endogenous RNAs in NPM-ALK NIH3T3 cells, as previously described (Tang et al., 2001). When cells were simultaneously stained with anti-ALK antibodies, we observed that ~ 50% of ALK-containing cytoplasmic foci also concentrate EtBr (Fig.
2A). Second, PABP was detected in AGs of NPM-ALK 3T3 cells stained both with anti-PABP and anti-phospho-ALK antibodies (Fig. 2B). Those results were indicative of the presence of mRNAs in AGs. To directly test this hypothesis, we transiently expressed both a lacZ reporter mRNA containing MS2-binding sites in its 3’ UTR (lacZ-3’MS2) and an YFP-MS2 fusion protein. When expressed alone, the YFP-MS2 protein was found exclusively in the nucleus, due to its nuclear localization signal (Rook et al., 2000). In contrast, co-expressing lacZ- 3’MS2 mRNA and YFP-MS2 allowed YFP-MS2 tethering to lacZ-3’MS2 mRNA and its subsequent export from the nucleus to the cytoplasm where it concentrates in PBs (Kedersha et al. 2005). In NPM-ALK transformed NIH 3T3 cells, the reporter mRNA is both visualized in PBs revealed by anti-DCP1 antibody and, although at a weaker intensity (1.6 fold lower), in most AGs (90%) with a diameter > 0.1 μm (Fig. 2C, D). These data are in agreement with our previous findings showing that PBs and AGs are distinct structures (Fawal et al. 2006) and reveal that AGs and PBs can harbor the same mRNA species. Finally, to know whether mRNAs contained in AGs were polyadenylated, we performed in situ hybridization using oligo dT probe. Concomitant immunodetection of AGs using anti-phospho-ALK antibodies revealed that most large AGs (90%) contained both NPM-ALK and oligodT (Fig. 2E). All together, those different approaches show that AGs concentrate mRNAs that are polyadenylated.

**AG formation and movements**

To further characterize AG formation, we transiently expressed NPM-ALK as a GFP (NPM-ALK-GFP) or Halo (NPM-ALK-Halo) fusion protein in NIH3T3 cells. Those fusion proteins conserve their phosphorylation status as shown by Western blot analysis (Fig. S2A) and nucleate cytoplasmic foci whose number, shape and size were identical to those observed in NPM-ALK stably transformed NIH3T3 cells (compare Fig. S2B to Figs. 1A and 6A). We then visualized AG formation and movements in the cytoplasm conducting both short (2 min) and long term (24h) time-lapse analysis to track AG movements, as previously described for PBs (Aizer and Shav-Tal, 2008). Using GFP-NPM-ALK transiently transfected cells, we observed that AGs rapidly grow to reach
their standard size (0.3-0.5μm diameter within 10 minutes) (Fig. S2C). Single particle tracking from short-range movies (2 min, supplementary movie 1) allowed us to classify AGs in three categories according to their roamed distances: <5 μm, 5-10 μm or > 10 μm (Fig. 3A, C, D). The latter could be tracked for distances up to 18 μm (Fig. 3 and Fig. S3). To compare AGs and PBs movements in the same cellular context, we transfected NIH3T3 cells with a GFP-DCP1a construct and observed similar tracking profiles for AGs and PBs although AGs move globally slightly faster than PBs (Fig. 3 and S3).

Due to this similarity, we tested whether AGs, could associate to the microtubule cytoskeleton, as recently described for PBs (Aizer and Shav-Tal, 2008). Using double staining with anti-αtubulin and anti-ALK antibodies, we observed AGs decorating individual microtubule bundle in NPM-ALK NIH3T3 cells (Fig. 4A), suggesting that AG movements rely on their association with microtubules. This hypothesis was tested by treating cells with nocodazole that binds to tubulin monomers and leads to microtubule destabilization. NIH3T3 cells were transiently transfected with NPM-ALK-GFP and AG movements were observed before and after microtubule disruption. Short-range time-lapse analysis indicates that nocodazole significantly reduces AG mobility (compare Fig. 4B to Fig. 3C and Fig. 4C for statistical analysis). Comparison with PBs observed in GFP-DCP1a-expressing NIH3T3 cells shows that nocodazole treatment inhibits both AGs and PBs movements (Fig. 4D). Altogether, our results indicate that like PBs, AGs use the microtubule network to roam the cytoplasm. In addition, we observed that some AGs were in close contacts with PBs both in NPM-ALK-transfected NIH3T3 cells (Fig. 2C and S3A) and ALCL-derived Cost-1 and Karpas cell lines (Fig. S3B and data not shown). To further analyze their interaction, we used NIH3T3 cells simultaneously transfected with NPM-ALK-Halo and GFP-DCP1 constructs and observed that AGs and PBs are tethered together for up to 30 minutes (Fig. 5, Fig. S4C and Movie 2 in supplementary material). These results show that AGs and PBs can establish stable contact that might rely on their association with the microtubule network.
AGs recruit PB components upon altered mRNA metabolism

Although AGs and PBs are distinct entities, they both concentrate mRNAs and RNA binding proteins. We thus ask whether, alike PBs, AG composition could be modified upon treatment affecting mRNA metabolism. First, we used cycloheximide (CHX) and actinomycin D, two drugs that reduce the pool of cytoplasmic mRNPs, leading to PB disaggregation (Cougot et al., 2004 and Fig. 6G, H). None of these treatments induce change in AG number or size in comparison to untreated cells (Fig. 6A and 7 for quantification). Moreover, CHX treatment does not prevent polyA mRNA accumulation in AGs, as revealed by colocalisation of oligodT and NPM-ALK within AGs (Figure 6I). Remarkably, simultaneous detection of NPM-ALK and PB markers (DCP1, EDC3, LSM1, XRN1) revealed that 30% and 70% of AGs contain PB components, except XRN1, after treatment with CHX or ActD, respectively (Fig. 6 and 7). In addition, whatever the treatment, most foci (94%) containing DCP1 also contain NPM-ALK (Fig. 7 DCP1/ALK overlap). We also followed association of AGs and PBs by time-lapse using cells expressing NPM-ALK-GFP and RFP-RCK/p54, another component of PBs (review in Kulkarni et al. 2010) that does not produce artefacts when over-expressed (Mollet et al. 2008). When those cells were treated with CHX, PBs and AGs gradually increased their contact and ultimately fused (Movie 3 in supplementary material). Relocalization of PB markers into AGs does not require complete PB disassembly since it is also observed after puromycin treatment, a drug that does not dramatically prevent PB assembly (Fig. 7). All together, these results show that treatments decreasing the pool of mRNAs do not affect nucleation of AGs but lead to the capture of PB components within AGs with the noticeable exception of XRN1.

AGs are distinct from stress granules

AGs share some components with SGs, in particular mRNAs, PABP and several AU-binding proteins (Buchan and Parker, 2009), such as HuR (M. Fawal, unpublished). Thus, we wondered whether AGs could correspond to SG-like foci formed in NPM-ALK-transformed cells. We first tested whether known components of SGs, such as markers of the 40S subunit (ribosomal protein
S6), translation initiation factors eIF2 and eIF3, and TIA/TIAR (Kedersha et al., 2002; Kedersha et al., 2005) were present in AGs. Confocal analysis revealed that none of them are present in AGs in normal cell culture conditions (Fig. 8A,B, left panels and data not shown). Then, we asked whether stresses known to induce SGs could force accumulation of SG components within AGs. Neither heat shock (HS) nor arsenite treatment led to accumulation of TIAR or translation initiation factors (eIF2α or eIF3) in AGs while those factors did concentrate within SGs (Fig. 8A,B), showing that AGs are distinct from SGs. This result was confirmed by overexpressing G3BP that leads to SG formation (Tourriere et al., 2003). Immunostaining using anti-G3BP, anti-DCP1a and anti-ALK antibodies to mark SGs, PBs and AGs, respectively, revealed that all three types of granules were distinct entities (Fig. 8C). All together, these results show that AGs do not accumulate known components of SGs in NPM-ALK NIH3T3 cells either in normal cell culture conditions or after various stresses and are thus different from SGs in essence.

DISCUSSION

In this study, we have further characterized AGs, which are cytoplasmic bodies different from PBs and SGs. AGs were observed in ALCLs (Honorat et al. 2006; Fawal et al. 2006 and this study) as well as in the murine NIH3T3 cell line model expressing the NPM-ALK translocation (Fawal et al. 2006) or other X-ALK fusion proteins (this study). We show that AG nucleation is independent of the N-terminal partner of the fusion protein as AGs are found in NPM-, ATIC- or TPM3-ALK-expressing cells. However, the N-terminal part promotes dimerization and allows ALK fusion kinases to transphosphorylate themselves (Chiarle et al. 2008) and is thus indirectly required for AG formation. By contrast, cells expressing full-length ALK protein do not harbor AGs, possibly because the receptor, during its trafficking, is addressed to different subcellular compartments and/or forms holo- or hetero-complexes with a different subset of adaptor proteins.

As revealed by time-lapse analysis, AGs are rapidly formed and move in the cytoplasm. They decorate microtubule network and their movement is reduced upon microtubule depolymerisation, indicating that their mobility relies on an intact microtubule network. However, whether
AG/microtubule association is direct or not remains to be investigated. We compared AG to PB mobility in the same cellular context and found that their track patterns are similar although the proportion of fast moving AGs is higher than mobile PBs. The distances roamed by PBs during the same given time (2 min) are in the same range than those reported by Aizer and collaborators (2-10 µm), indicating that PB movements are comparable in the murine fibroblastic NIH3T3 cells and the human osteosarcoma U2OS line (Aizer et al. 2008). However, the velocity values are different in the two studies, a discrepancy that most probably relies on different methods of measurements (see materials and methods).

It has recently been proposed a model integrating polysomes, SGs and PBs into an mRNP cycle that relies on the microtubule network (Buchan and Parker, 2009). The association of AGs to microtubules as well as the presence in these foci of mRNA (this study) and two RNA-binding proteins, AUF1 (Fawal et al. 2006) and HuR (not shown), suggest that AGs could also participate in the mRNP cycle. The presence of PABP as well as polyadenylated mRNAs but not PB components in AGs in normal cell culture conditions indicates that AG-associated mRNAs are neither deadenylated nor engaged in a degradation process, like those found in PBs, but still competent for translation. However, AGs do not concentrate components of the translation machinery or ribosomal subunits, indicating that the mRNAs stored or trafficking in AGs are most probably not stalled in the process of translation initiation, like those accumulating within SGs (review in Buchan and Parker, 2009). Thus AGs may trap translatable mRNAs away from the decay machineries. Since we frequently observed close contacts between AGs and PBs, these mRNAs could be captured from nearby PBs. However, the fact that AGs, but not PBs, concentrate polyadenylated mRNAs is not in agreement with this hypothesis but rather suggests that mRNAs contained in AGs are captured from the cytosol. In addition, PB components are not normally concentrated within AGs, although cells contain a diffuse cytoplasmic pool of those components (Mollet et al., 2008 and reviewed in Aizer and Shav-Tal, 2008). Nevertheless, after treatments that alter mRNA metabolism, some PB components (LSM1, EDC3, RCK/p54 or DCP1a) are recruited in AGs. They might be captured from adjacent dissociating PBs, as suggested from video-microscopic observations. However, XRN1 does
not relocate to AGs during those treatments showing that, if it really takes place, relocalisation of components from PBs to AGs is a selective process.

All together our data demonstrate that AGs are different from PBs and SGs and thus most probably do not act like PBs or SGs whose functions, despite an abundant literature, is still under debate (Franks and Lykke-Andersen, 2008; Mollet et al., 2008; Balagopal and Parker, 2009; Bunchan and Parker, 2009; Kulkarni et al., 2010). It is thought that PBs roam the cytoplasm to collect mRNAs destined for degradation (Aizer and Shav-Tal, 2008) while SGs transiently store mRNAs engaged in the initiation step of translation. In SGs, arrested mRNAs are maintained intact, i.e. in their translatable state, until translation resumes. Since AGs do not contain components of the translation machinery but are capable to shelter polyadenylated mRNAs that are not tagged for degradation, we propose that through their ability to scan the cytoplasm, AGs capture polyadenylated mRNAs that they will store away from degradation. Because ARE-containing mRNAs have been found stabilized in NPM-ALK expressing cells (Fawal et al. 2006), AGs could function as nursery for these mRNAs, allowing their dressing with AU-binding proteins, such as HuR or AUF1.

To conclude, we have described in this paper mRNP cytoplasmic granules that are encountered in X-ALK transformed cells where simultaneous activation of various ALK-mediated signaling pathways dramatically enhances transcription (review in Pulford et al., 2004b). Permanent transcriptional activation might lead to mRNA influx that exceeds the translational capacity of these transformed cells. We would like to speculate that AGs constitute a reservoir system for translatable mRNAs that would have been degraded otherwise. Continuous availability for translation of some specific mRNAs with altered turnover could be the basis of the oncogenic properties of X-ALK expressing cells. Whether and how mRNAs are sorted before storage in ALK granules are important questions for future studies.
MATERIALS AND METHODS

Cell culture

X-ALK stably transfected NIH3T3 cells and ALCLs-derived cell lines are described in (4, 17). Actinomycin D (2 µg/ml final concentration), cycloheximide (5 µg/ml), puromycin (20 µg/ml) and Nocodazole (40 µM) (all from Sigma) were added to cell culture medium for the indicated times. The ALK inhibitor [Racemic PF-2341066 [3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine] that resulted in the abolition of NPM-ALK autophosphorylation was synthesized according to the method described in the patent international application WO 2006/021881 and used at a final concentration of 10^{-5}M.

Plasmids and transfection

GFP-ALK and ALK-Halo vectors were generated by cloning the full open reading frames of human NPM-ALK into HindIII/EcoRI sites of the pEGFP N3 vector (Clontech) or HindIII/EcoRV sites of Halotag pHT2 vector (Promega). βgal MS2 (RSV-Z-MS2-24), YFP-MS2 (L_{30-}YFP-MS2_{nls}), GFP-DCP1 and RFP-RCK/p54 were a generous gift from E. Bertrand, B. Seraphin and D. Weil. NIH3T3 cells were transiently transfected using Lipofectamine® following the manufacturer’s instructions (Invitrogen). Double transfections were done with a 1:1 ratio, except for YFP-MS2/LacZ-MS2-24 which was performed with a ratio of 1:10. NIH3T3 cells stably transfected with full length ALK cDNA were generously provided by I. Janoueix and O. Delattre.

Immunofluorescence and live cell imaging

For IF analysis, NIH3T3 cells were seeded on cover glasses (10^4 cells/ml), cultured overnight, fixed, permeabilized and stained as described (Fawal et al. 2006). The antibodies used were: monoclonal anti-ALK1 (DakoCytomation), rabbit anti-phospho-ALK (Cell Signaling), rabbit anti-hDCP1a (generous gift of B. Séraphin), anti-Edc3 and anti-Xrn1 (gift of Lykke-Andersen) and anti-
PABP (Sigma). The secondary antibodies were goat anti-rabbit antibody (Alexa 566, Molecular Probes) or goat anti-mouse (Alexa 488, Molecular Probes). Slides were mounted in Mowiol and analyzed with a Leica SP2 confocal microscope equipped with helium-neon lasers and appropriate filter combinations or a wide-field Leica DMIRE2 microscope equipped with a micromax Princeton CCD camera. Unless specified otherwise, all images are single Z section from a series of confocal images. The images were acquired using Leica or metamorph software and edited with Adobe Photoshop.

For time lapse analysis, NIH3T3 cells were seeded on Nunc 4 cm glass-bottom dishes. Cells were transfected as described above and observed 36 to 48 h post-transfection. Cells were maintained at 37°C and 5% CO2 and signals were detected by a LEICA DMIRE 2 microscope driven by the Metamorph software (Universal imaging). A 63X oil-immersion inversed objective was employed. Images were acquired every 6 minutes and represent the maximal projection of 14 planes with 0.16 µm optical sections. For short range movies, images were acquired every 2 sec. and represent a maximal projection of 7 planes with 0.16 µm optical sections. For statistical analysis, the distance of granules (AGs and PBs) were measured on a minimum of 100 granules from at least 15 different cells/movies using Metamorph software utility, Track objects. Tracks were recorded. Image J was used to calculate the area corresponding to the best fit ellipse covered by a given track. To calculate velocity, we divided the distance roamed during the time of observation (2 min plus the time for collecting data between sections). In Aizer's study, instantaneous velocities were reported on the basis of total distance and mean square displacements that allow calculating diffusion coefficients (Aizer et al. 2008).

Detection of polyadenylated mRNAs within AGs

NPM-ALK 3T3 cells seeded on cover glass were incubated 15 min. in PFA 4%, 5 min in 0.1 % Triton X-100 rinsed in PBS and incubated 30 min. in 2 X SSC/50% formamide. Polyadenylated mRNAs contained in AGs were detected by in situ hybridization using a digoxigenin-labelled DNA oligo dT probe. Hybridization of 50ng oligo dT probe was performed in 50 µl hybridization solution
(2 X SSC/ 50% formamide/10% dextran sulfate/ 0.5 mg/ml yeast tRNA/ Rnase inhibitor (Promega)) at 37°C overnight. Cells were then washed twice for 30 minutes in 2 X SCC/ 50% formamide at 37°C and 20 min. in SSC 1X. Oligo dT signal was detected using anti-digoxigenin sheep antibodies that were recognized by an donkey anti-sheep antibodies (Alexa 654, Molecular Probes). AGs were detected using either anti-ALK or anti-phospho-ALK antibodies that were incubated together with the anti-digoxigenin antibodies. Cells were analyzed under a wide field microscope and images treated using Adobe Photoshop.

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REFERENCES


Morris, S. W., C. Naeve, P. Mathew, P. L. James, M. N. Kirstein, X. Cui, and D. P. Witte. 1997. ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). Oncogene 14:2175-88.


Figure Legends

Figure 1: AGs correspond to cytoplasmic foci containing an active X-ALK kinase domain.

A. Confocal analysis of NIH3T3 cell lines stably expressing NPM- or TPM3-ALK using anti-ALK and anti-phospho ALK antibodies. The merge view shows localization of phosphorylated fusion-ALK proteins in AGs.
B. Western blot analysis of NPM-ALK level of expression in NPM-ALK NIH3T3 and ALCL-derived Karpas and Cost cell lines using anti-phospho (P-ALK) and anti-ALK (ALK) antibodies.

C. ALK inhibitor prevents ALK phosphorylation. NPM-ALK NIH3T3 cells were treated with the ALK inhibitor, fixed at different times after treatment and analyzed for immunofluorescence using anti-ALK antibody with a wide-field microscope to determine the kinetics of the loss of AGs. While 85% of untreated cells (0h) contained more than one AG, there were only 22% at the peak of NPM-ALK dephosphorylation (15h). Most probably due to the transient activity of the inhibitor and concomitant to NPM-ALK rep phosphorylation (supplementary Figure 1C), AGs reappear and approximately 70% of cells contained AGs 24h after treatment. Results shown were obtained in three independent experiments. The scale bar represents 5 µm.
Figure 2: AGs contain mRNAs
A. Stably NPM-ALK-expressing NIH3T3 cells were labelled with EtBr and stained with anti-ALK antibody and analyzed by confocal microscopy to visualize AGs.

B. NPM-ALK-expressing NIH3T3 cells were doubly stained with anti-phospho-ALK (red) and anti-PABP (green) antibodies and analyzed by confocal microscopy. The yellow spots (merge) indicate that AGs contain PABP.

C. Cells transiently expressing MS2-lacZ mRNA and YFP-nls-MS2 fusion protein were analyzed by confocal microscopy to visualize PBs labelled with anti-hDCP1 (red in the enlarged square) and AGs labelled with anti-ALK antibodies (blue). In a small percentage of cells (2-5%), both granules contain the tethered reporter mRNAs (in the merge view, green (YFP) + red (hDCP1) = yellow; green (YFP) + blue (ALK) = cyan). Arrows show juxtaposed PBs and AGs. The scale bar in A-C represents 5 µm.

D. Intensity of YFP-MS2 signals was quantified in over 50 AGs (cyan) and 50 PBs (yellow). Error bars represent standard deviation. *** = p <0.001.

E. In situ hybridization was performed to test the presence of polyadenylated mRNAs within AGs. Oligo dT probe (red) concentrates within AGs that are detected by immunofluorescence using anti-phospho-ALK antibodies (green). The merge picture shows that most AGs concentrate polyadenylated mRNAs (yellow).
Figure 3: AG dynamics resembles that of PBs
A. B. NIH3T3 cells were transiently transfected with an NPM-ALK-GFP (A) or GFP-DCP1a (B) expressing vector and observed by video microscopy. Representative images collected from 60 frames (total 2 minutes) showing tracks of multiple AGs (A) and PBs (B) are shown.

C. Tracks of approximately 200 AGs and 100 PBs were analyzed and divided in three categories following their roamed distances, < 5 μm, 5 to 10 μm and > 10 μm. For a given category, mean distances and velocities (expressed in μm^2/secondes) are also indicated. The percentage of AGs or PBs belonging to a given category is shown.

D. Examples for each category of track and relative area (in pixels) in which AGs or PBs moved are given.
Figure 4: AGs reside on microtubules

A. NPM-ALK NIH3T3 cells were doubly stained with anti-tubulin (green) and anti-ALK (red) antibodies and observed by confocal microscopy. a,b,c: successive enlargements. The scale bar represents 5 µm.

B. NIH3T3 cells transiently transfected with a NPM-ALK-GFP construct were treated with nocodazole for 30 minutes. Fresh medium was added and AG movements were subsequently observed by video microscopy, as described in Fig. 3B,C, and their tracks drawn.

C. Comparative analysis of the distances (in µm) crossed by AGs before and after nocodazole treatment. Error bars (standard deviations) and t student values are indicated on each graph.

D. Comparative analysis of the surfaces (in µm²) roamed by PBs and AGs before and after nocodazole treatment. n indicates the number of AGs or PBs counted in each experiment. t student values are indicated on each graph.
Figure 5: Close contacts between AGs and PBs

Live imaging of PB and AG communication. NIH3T3 cells were transiently co-transfected with NPM-ALK-Halo (red) and GFP-DCP1 (green) encoding plasmids. Two days later, cells were observed by wide-field microscopy and time lapse analysis performed for two consecutive hours. Images taken every 6 minutes from 0 (a) to 96 minutes (p) on a region of contact are shown.
Figure 6: AGs and PBs share components when mRNA metabolism is altered
A-F. NPM-ALK-expressing NIH3T3 cells were either untreated (A, E) or treated with cycloheximide (CHX) for 2 hours (B-D, F). CHX treatment led to expression of LSM1 (B), DCP1 (C), EDC3 (D), but not XRN1 (F), within AGs. Representative images of cells containing more than three AGs and in which the size of PBs is >0.4 µm are shown. Such cells represent approximately 5% of a given field. Scale bar: 5 µm. G-I. NPM-ALK-expressing NIH3T3 cells were either untreated (G) or treated with cycloheximide (CHX) for 2 hours (H,I). The presence of polyadenylated mRNAs within PBs or AGs was analyzed through in situ hybridization using oligo dT probe (red) and concomitant immunofluorescence study using anti-DCP1 (G, H) or anti-ALK antibodies (I) (green), respectively. Scale bar: 10 µm.
Figure 7: Merging of AGs and PBs

NPM-ALK NIH3T3 cells were doubly stained with anti-DCP1 and anti-ALK antibodies and observed under wide-field microscope. Foci that contained only DCP1 (PBs) or NPM-ALK (AGs) or both proteins (AG-PBs) were counted in untreated NPM-ALK NIH 3T3 cells (WT) or after cycloheximide (CHX) or Actinomycin D (Act.D) treatment that resulted in decreased cytoplasmic pool of mRNAs and subsequently a nearly complete loss of PBs. By contrast, puromycin (Puro) that inhibits translation by the release of mRNA from polyribosomes does not inhibit PB formation to the same extent. Numbers in the graphs represent the average foci number for each category. Results shown were obtained in at least two independent experiments and >100 cells were analyzed.
Figure 8: AGs and SGs are two independent structures
A, B. NPM-ALK-expressing NIH3T3 cells were either untreated (control), heat shocked (HS) (44°C for 30 minutes) or treated with arsenite (ARS) for 2 hours to induce SGs. Confocal analysis was undertaken to visualize TIA, eIF2α or eIF3 in AGs or SGs using concomitantly anti-phospho-ALK antibody to label AGs. In the absence of stress, we observed punctuate cytoplasmic labeling of eIF2α or eIF3 in control NPM-ALK cells, but no specific labeling of AGs by these proteins. HS and ARS treatment induced the formation of SGs, revealed by TIA concentration in SGs in control and NPM-ALK-expressing cells but not in AGs visualized by accumulation of phospho-ALK protein.

C. SGs were induced by transient GFP-G3BP overexpression in NPM-ALK expressing cells. SGs, PBs and AGs were simultaneously observed by confocal analysis using GFP fluorescence, anti-DCP1 and anti-ALK antibodies, respectively.