The Brefeldin A-inhibited Guanine Nucleotide-exchange Protein, BIG2, Regulates the Constitutive Release of TNFR1 Exosome-like Vesicles

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The type I, 55-kDa tumor necrosis factor receptor (TNFR1) is released from cells to the extracellular space where it can bind and modulate TNF bioactivity. Extracellular TNFR1 release occurs by two distinct pathways: the inducible proteolytic cleavage of TNFR1 ectodomains and the constitutive release of full-length TNFR1 in exosome-like vesicles. Regulation of both TNFR1 release pathways appears to involve the trafficking of cytoplasmic TNFR1 vesicles. Vesicular trafficking is controlled by ADP-ribosylation factors (ARFs), which are active in the GTP-bound state and inactive when bound to GDP. ARF activation is enhanced by guanine nucleotide-exchange factors that catalyze replacement of GDP by GTP. We investigated whether the brefeldin A (BFA)-inhibited guanine nucleotide-exchange proteins, BIG1 and/or BIG2, are required for TNFR1 release from human umbilical vein endothelial cells. Effects of specific RNA interference (RNAi) showed that BIG2, but not BIG1, regulated the release of TNFR1 exosome-like vesicles, whereas neither BIG2 nor BIG1 was required for the IL-1β-induced proteolytic cleavage of TNFR1 ectodomains. BIG2 co-localized with TNFR1 in diffusely distributed cytoplasmic vesicles, and the association between BIG2 and TNFR1 was disrupted by BFA. Consistent with the preferential activation of class I ARFs by BIG2, ARF1 and ARF3 participated in the extracellular release of TNFR1 exosome-like vesicles in a nonredundant and additive fashion. We conclude that the association between BIG2 and TNFR1 selectively regulates the extracellular release of TNFR1 exosome-like vesicles from human vascular endothelial cells via an ARF1- and ARF3-dependent mechanism.

Tumor necrosis factor (TNF) is an important regulator of inflammation, apoptosis, and innate immune responses. TNF signals through the type I 55-kDa (TNFR1, TNFRSF1A, CD120a) and type II 75-kDa (TNFR2, TNFRSF1B, CD120b) TNF receptors (1–3). TNFR1, which contains death domains in its intracytoplasmic tail, is considered the major receptor for TNF signaling (3, 4). TNFR1 is also released from cells to the extracellular space and thereby modulates TNF bioactivity. Release of TNFR1 to the extracellular space is mediated by two distinct mechanisms. The first involves the proteolytic cleavage of TNFR1 ectodomains by a receptor sheddase that results in the shedding of soluble TNFR1 ectodomains (5–9). The major site of TNFR1 cleavage is in the spacer region adjacent to the transmembrane domain between Asn-172 and Val-173, and a minor site is between Lys-174 and Gly-175 (8, 10, 11). TACE was identified as a TNFR1 sheddase because TACE-deficient cells had lower ratios of shed to cell surface TNFR1 than did TACE-reconstituted cells (12). Proteolytic cleavage and shedding of soluble TNFR1 can be induced by diverse stimuli, such as phorbol ester, interleukin-1β, and proteasome inhibitors (13–15).

The second mechanism is the constitutive release of full-length TNFR1 from cells within membranes of exosome-like vesicles (16). Exosomes are small membrane-enclosed vesicles, 30–200 nm in diameter, that correspond to the internal vesicles of endolysosome-related multivesicular bodies and are released via exocytic fusion with the plasma membrane (17–20). TNFR1 exosome-like vesicles were initially identified in conditioned medium from human umbilical vein endothelial cells (HUVEC), which contained 20–50-nm exosome-like vesicles that were pelleted by high speed centrifugation, sedimented to a density of 1.1 g/ml, and were capable of binding TNF (16)...

Recent investigations have identified previously unrecognized pathways for regulation of TNFR1 release from cells to the extracellular space. For example, histamine has been reported to induce redistribution of TNFR1 to the cell surface from an intracellular storage pool, such as the Golgi system, which served as a source of proteolytically cleaved receptors (21). We showed that calcium-dependent formation of a complex comprising ARTS-1 (aminopeptidase regulator of TNF receptor shedding), a type II integral membrane aminopeptidase, and NUCB2 (nucleobindin 2), a putative DNA- and calcium-binding protein, associates with cytoplasmic TNFR1 prior to the commitment of TNFR1 to...
pathways that result in either the constitutive release of TNFR1 exosome-like vesicles or the inducible proteolytic cleavage of TNFR1 ectodomains (22, 23). Taken together, these findings suggested the probable involvement of intracytoplasmic vesicular trafficking between ER/Golgi and cell surface plasma membranes in these processes. Initiation of vesicle formation from a donor membrane (e.g. Golgi) requires activation of a 20-kDa ADP-riboseylation factor (ARF) by interaction with a guanine-nucleotide exchange protein (GEP) that accelerates release of GDP and thereby GTP binding (24). Accumulation of ARF-GTP and coatomer (plus other proteins) leads to membrane deformation or budding. A vesicle, released by sealing off the bud at its base, is translocated to and fuses with a target membrane. Vesicular transport from the Golgi or trans-Golgi network to the plasma membrane in mammalian cells is known to involve the class I ARFs 1 and 3, which are activated by the brefeldin A-inhibited ARF-GEPs, BIG1 and BIG2 (25, 26). The experiments reported here demonstrate that BIG2, but not BIG1, was required for the constitutive release of full-length TNFR1 in exosome-like vesicles from human endothelial cells.

**MATERIALS AND METHODS**

**Cells and Reagents**—HUVEC (passages 3 and 8) and EGM-2 medium were purchased from Cambrex BioScience (Walkersville, MD). Recombinant human IL-1β was from R & D Systems (Minneapolis, MN) and brefeldin A (BFA) was from MP Biochemicals (Aurora, OH).

**Antibodies**—Chicken polyclonal anti-NUCB2 antibodies were generated against a glutathione S-transferase fusion protein with sequence corresponding to amino acids 326–420 of the NUCB2 C-terminal leucine zipper domain (Sigma Genosys) (23). Murine IgG2b monoclonal (H5) and goat polyclonal (C20) antibodies that reacted with TNFR1 were from Santa Cruz Biotechnology (Santa Cruz, CA), as were antibodies against β-tubulin (D10). Rabbit polyclonal antibodies against ARTS-1, BIG1, and BIG2 were used as previously described (27). Specific murine monoclonal antibodies reactive with human ARF1, ARF3, ARF5, and ARF6, respectively, were from Stressgen Bioreagents (Victoria, Canada), BD Biosciences (Palo Alto, CA), Abnova Corp. (Taipei, Taiwan), and Chemicon International (Temecula, CA).

**RNA Interference and Quantitative Real Time RT-PCR**—Individual siGENOME RNA duplexes for BIG1 and BIG2, siGENOME SMARTpool RNA duplexes for ARF1, ARF3, ARF5, and ARF6, as well as siCONTROL nontargeting siRNA 1 were purchased from Dharmacon (Lafayette, CO). HUVEC were transfected with siRNA (50–100 nM) using DharmaFECT 1 transfection reagent (Dharmacon) for 3 days. RNA was isolated with an RNasea Mini kit (Qiagen, Valencia, CA), and cDNA templates were prepared with a ProSTAR Ultra-HF RTPCR system (Stratagene, Cedar Creek, TX). Sequences of RT-PCR primers are listed in supplemental Table S1. Quantitative real time RT-PCR was performed using the iCycler iQ thermocycler, iQ SYBR green PCR supermix, and iCycler iQ data analysis software (Bio-Rad).

**BIG2, ARF1, and ARF3 Expression Plasmids**—Mammalian expression plasmids encoding full-length human BIG2, ARF1, or ARF3 were purchased from Origene Technologies (Rockville, MD). The BIG2 cDNA clone contained 7 nucleotide substitutions that differed from the ARFGEF2 reference sequence (NCBI accession NM_006420). Among these putative polymorphisms, two represented silent mutations (C3663T, C4131T), while 5 represented nonsynonymous mutations (A619G, G620A, A2884G, A3145G, and A5287G) that resulted in 4 amino acid substitutions. The 5 nonsynonymous mutations were corrected by site-directed mutagenesis using the QuikChange Multi Site-directed Mutagenesis kit (Stratagene). All plasmids were sequence-verified.

The T31N mutations were introduced using the Quik-Change Multi Site-directed Mutagenesis kit and the following primers; ARF1: 5′-CTGAGGGAAGAAGCAGTCTCTACAG-3′ and ARF3: 5′-CGCAGGAAACGAACTCATCTCTACAA-3′. RNAi-resistant BIG2, ARF1, and ARF3 mutant plasmids, which contained synonymous mutations, were generated by site-directed mutagenesis using the following primers; BIG2: 5′-ATTATGTGCAAAGTTATGTTGGATTTAATCTGC-3′, ARF1: 5′-CAGCAATGACAGGACGATGAAACGAGGCCGGCC-3′, and ARF3: 5′-CAGAAAAGAGCAGCATACTGTACAAGCTGAAAAG-3′. Mutations were confirmed by sequencing. HUVEC, grown in 6-well plates, were transfected with plasmids using FuGEN6, according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN). For co-transfection, individual siGENOME siRNA duplexes (Dharmacon) targeting BIG2 (5′-CACACUCUGACUGAUAUU-3′) ARF1 (5′-ACAGAGAGCUGUGUAAG-3′), or ARF3 (5′-GGAAGACCACCUCCAUUAU-3′) were transfected at a concentration of 100 nM using the DharmaFECT 1 transfection reagent.

**Immunoblotting**—HUVEC were lysed in buffer containing 1% Triton X-100, 1% n-octyl β-D-glucopyranoside, 50 mM Tris, pH 7.5, and 120 mM NaCl (Sigma), supplemented with Complete™ protease inhibitor (Roche Applied Science, Indianapolis, IN). For immunoblotting, conditioned medium was cleared of cells and debris by sequential centrifugation at 200 × g for 10 min, 500 × g for 10 min, 1,200 × g for 20 min, and 10,000 × g for 30 min. Immunoblots of cellular proteins (50 μg per lane) or medium (26 μl per lane) were performed as previously described, using NIH Image Software (version 1.63) for densitometry (23).

**Immunoprecipitation**—HUVEC were lysed in buffer containing 0.1% Triton X-100, 50 mM Tris, pH 7.5, and 120 mM NaCl (Sigma) supplemented with Complete™ protease inhibitor. Samples (200 μg) of HUVEC lysates were incubated for 2 h with 8 μg of rabbit anti-BIG2 antibodies immobilized on 100 μl of protein A/G beads (Pierce) that had been blocked with 1% ovalbumin in PBS. Controls were protein A/G beads incubated with preimmune serum or without bound antibodies. Beads were washed six times with cold lysis buffer, and immunoblots were performed as previously described (23). Proteins from HUVEC supernatants after
immunoprecipitation were precipitated with 10% trichloroacetic acid for immunoblotting.

**Immunofluorescence Confocal Laser Scanning Microscopy**—
HUVEC grown on collagen I-coated slides (BD Biosciences) were fixed in 4% paraformaldehyde for 10 min, washed three times with PBS, permeabilized with 0.1% saponin in PBS (5 min), washed three times with PBS, and blocked with PBS containing 10% donkey and 10% goat serum for 1 h. Cells were incubated overnight at 4 °C with primary antibodies diluted in PBS containing 1% donkey and 1% goat serum, as follows: goat anti-TNFR1 (C20) polyclonal antibodies (2 μg/ml), rabbit polyclonal anti-BIG2 antibodies (diluted 1:500), and mouse monoclonal anti-p230 trans-Golgi antibody (1:200). After washing three times in PBS containing 0.1% bovine serum albumin and incubation with species-specific secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor® 568, (Molecular Probes, Eugene, OR, 1:200 dilution), cells were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and visualized using a Leica SP Laser Scanning Confocal Microscope (Leica, Heidelberg, Germany).

**Quantification of Extracellular TNFR1 by ELISA**—HUVEC were transfected with siRNA for 3 days, and then fresh, exosome-depleted medium was added for 24 h. Medium cleared of cells and debris as described for immunoblotting was analyzed for TNFR1 using a Quantikine sandwich ELISA kit with a sensitivity of 7.8 pg/ml (R & D Systems).

**Statistical Analyses**—Data were analyzed by a paired Student’s *t* test with a Bonferroni correction for multiple comparisons or analysis of variance. A *p* value ≤0.05 was considered significant.

**RESULTS**

**BIG2 Regulates the Constitutive Release of TNFR1 Exosome-like Vesicles**—The role of BIG1 and BIG2 in the constitutive release of TNFR1 exosome-like vesicles from HUVEC was assessed using RNA interference. Small interfering RNA duplexes (siRNA) specifically decreased levels of BIG1 and BIG2 mRNA and protein (Fig. 1, *A* and *B*), but did not alter the
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quantities of mRNA (data not shown) or protein (Fig. 1B) encoding NUCB2 or β-tubulin. As quantified by ELISA, TNFR1 constitutively released into the medium from cells transfected with siRNA targeting BIG2 was 63% less than that from cells transfected with control non-targeting siRNA (Fig. 1C). Similarly, Western blots showed that medium from cells transfected with siRNA targeting BIG2 contained 69% less 55-kDa TNFR1 than did medium from cells transfected with non-targeting siRNA (p = 0.017, n = 4) (Fig. 1D). A ~40-kDa TNFR1 band was also seen, as has been previously described, which was similarly decreased (23). In contrast, no effects of siRNA targeting BIG1 were found.

Neither BIG1 nor BIG2 Affects the IL-1β-induced Proteolytic Cleavage of TNFR1 Ectodomains—In experiments performed to assess the role of BIG1 or BIG2 in the inducible proteolytic cleavage of TNFR1 ectodomains, transfection of HUVEC with siRNA targeting BIG1 or BIG2 did not reduce the quantity of TNFR1 released into medium following 2-h stimulation with IL-1β, as measured by ELISA (Fig. 2A) or Western blotting (Fig. 2B). The quantity of full-length 55-kDa TNFR1 released in exosome-like vesicles into medium in 2 h was below the limit of detection by Western blotting.

Endogenous BIG2 Associated with TNFR1 in HUVEC—To determine whether the action of BIG2 in the constitutive release of TNFR1 exosome-like vesicles involved an association between the endogenous proteins, BIG2 was immunoprecipitated from HUVEC homogenates, and the full-length, 55-kDa TNFR1 was detected by immunoblotting, but neither ARTS-1 nor NUCB2 were present (Fig. 3A). The BIG2 antibodies immunoprecipitated 48% of BIG2, but only 16% of the TNFR1 was pulled-down (Fig. 3B), consistent with the association of a subpopulation of endogenous TNFR1 with BIG2.

The association between BIG2 and TNFR1 in HUVEC was also characterized by confocal microscopy. As shown in Fig. 3C, TNFR1 and BIG2 were co-localized in diffusely distributed cytoplasmic vesicles. Although BIG2 had been described in the trans-Golgi network, neither BIG2 nor TNFR1 co-localized with the trans-Golgi marker, p230 (data not shown).

Because BIG2 is a BFA-inhibited activator of ARF, we looked for a BFA effect on the association between endogenous BIG2 and TNFR1. As shown in Fig. 4, no full-length, 55-kDa TNFR1 was found among proteins immunoprecipitated by BIG2 antibodies from cells incubated for 15 min with BFA. This showed that the association between endogenous BIG2 and TNFR1 was disrupted by BFA.

BIG2-dependent Release of TNFR1 Exosome-like Vesicles Involves ARF1 and ARF3 in a Nonredundant and Additive Fashion—BIG2 activates both ARF1 and ARF3 (25, 28). To assess the roles of individual ARFs in the constitutive release of TNFR1 exosome-like vesicles, ARF1, ARF3, ARF5, or ARF6 mRNA and protein were selectively depleted using RNA interference (Fig. 5, A and C), while neither TNFR1 nor β-tubulin mRNA (data not shown) and protein (Fig. 5C) were decreased. Treatment of HUVEC with siRNA targeting ARF1 or ARF3 decreased by 59 and 60%, respectively, the amount (quantified by ELISA) of TNFR1 released relative to that from cells treated with control, non-targeting siRNA (Fig. 5B). In contrast, siRNA-mediated depletion of ARF5 or ARF6 had no
effect. Quantification of Western blots showed that the siRNA-mediated depletion of either ARF1 or ARF3 reduced the amount of constitutively released 55-kDa TNFR1 exosome-like vesicles into the culture medium by 67% ($p = 0.008$, $n = 3$) and 55% ($p = 0.016$, $n = 3$) respectively, from that released by cells transfected with non-targeting siRNA (Fig. 5C).

We next assessed whether ARF1 and ARF3 function in an additive fashion to mediate the extracellular release of TNFR1 exosome-like vesicles. HUVEC transfected with siRNAs targeting both ARF1 and ARF3 showed specific depletion of both mRNAs (data not shown) and proteins (Fig. 5E), while β-tubulin mRNA (data not shown) and protein (Fig. 5E) were not decreased. As shown in Fig. 5D, the amount of TNFR1 constitutively released from cells transfected with siRNAs targeting both ARF1 and ARF3 was 82% less than that from cells transfected with control, non-targeting siRNA. The quantity of TNFR1 constitutively released into culture medium was also significantly less from cells transfected with siRNAs targeting both ARF1 and ARF3 than from cells treated with siRNAs individually targeting ARF1 or ARF3. Western blots (Fig. 5E) confirmed that the siRNA-mediated depletion of both ARF1 and ARF3 reduced the constitutive release of the 55-kDa TNFR1 exosome-like vesicles by 90% ($p = 9.04 \times 10^{-6}$, $n = 3$), from the level of cells treated with control, non-targeting siRNA. Similarly, Western blots (Fig. 5E) showed that the siRNA-mediated depletion of both ARF1 and ARF3 significantly reduced the constitutive release of the 55-kDa TNFR1 exosome-like vesicles as compared with cells treated with siRNAs individually targeting ARF1 or ARF3 ($p = 0.00013$, $n = 3$).

To confirm that BIG2 activation of ARF1 and ARF3 mediates the release of TNFR1 exosome-like vesicles from HUVEC, cells were transfected with plasmids expressing either wild-type or guanine nucleotide exchange-defective (T31N) ARF1 or ARF3 mutants. The T31N mutants exhibit minimal GTP binding and are preferentially constrained to the GDP-bound inactive form (29, 30). Quantification of Western blots of cell lysates (Fig. 6A) showed levels of immunoreactive ARF1 and ARF3, respectively, 205% ($p = 0.015$, $n = 3$) and 174% ($p = 0.043$, $n = 3$) those of cells transfected with the empty (control) plasmid. These were associated with increases of 106% ($p = 0.0009$, $n = 3$) and 116% ($p = 0.0008$, $n = 3$), respectively, in the amounts of 55-kDa TNFR1 exosome-like vesicles that were constitutively released into the culture medium (Fig. 6, A and B). Similarly, quantification by ELISA (Fig. 6C) showed that overexpression of ARF1 or ARF3 was associated with respective increases of 86% ($p < 10^{-9}$, $n = 6$) and 89% ($p < 10^{-12}$, $n = 6$) in the amount of TNFR1 released into the culture medium. In contrast, overexpression of the ARF1(T31N) or ARF3(T31N) plasmids did not alter TNFR1 exosome-like vesicle release.

Immunoprecipitations were next performed to assess the effect of the ARF1(T31N) and ARF3(T31N) mutants on the association between BIG2 and TNFR1. Consistent with the lack of a dominant-negative effect on the release of TNFR1 exosome-like vesicles, the mutants did not disrupt the association between BIG2 and TNFR1 (Fig. 6D). Although ARF1(T31N) and ARF3(T31N) mutants have previously been demonstrated to function as dominant-negative constructs, which occurs via sequestering of relevant ARF-GEFs (29), this was not the case in our system where their behavior was consistent with a GDP-bound inactive form. Our findings are similar to a prior report investigating secretory vesicle formation in pituitary cells, which also found that ARF1(T31N) functioned as an inactive ARF, rather than as a dominant-negative mutant (31). This study hypothesized that the T31N mutant could not undergo a GTP-induced conformational change, which resulted in impaired membrane localization, so that the mutant was inaccessible to compete with proteins binding to the ARF effector domain (31). The reason why overexpression of ARF1(T31N) and ARF3(T31N) mutants did not produce a dominant-negative effect in our system is unclear. Our data suggest that a sufficient quantity of BIG2 was not sequestered by the ARF (T31N) mutants and therefore remained available to mediate the association between BIG2 and TNFR1, as well as the release of TNFR1 exosome-like vesicles. This interpretation is consistent with our finding that only a subset of BIG2 associates with TNFR1 in HUVEC.

RNAi-resistant BIG2, ARF1, and ARF3 expression plasmids with 4 silent single base pair mutations in the region targeted by siRNA were used to confirm the specificity of the observed siRNA effects. BIG2, ARF1, and ARF3 proteins were detected in lysates from HUVEC co-transfected with siRNA and RNAi-resistant expression plasmids, whereas BIG2, ARF1, and ARF3 protein levels were markedly diminished in cells co-transfected with siRNA and plasmids expressing wild-type BIG2, ARF1, or ARF3 (Fig. 7, A–C). We next assessed if the effect of BIG2, ARF1, and ARF3 siRNAs on TNFR1 exosome-like vesicle release were specific and not caused by off-target effects. Medium from HUVEC co-transfected with the empty (control) plasmid and siRNA targeting BIG2, ARF1, or ARF3 contained significantly less TNFR1, quantified by ELISA, than those transfected with the empty (control) plasmid in the absence of siRNA (Fig. 7, D–F).
Reductions in TNFR1 release by siRNAs targeting BIG2, ARF1, or ARF3 were rescued by co-transfection with RNAi-resistant, but not the wild-type, BIG2, ARF1, or ARF3 plasmids. Western blots confirmed that the siRNA-mediated knock-down of BIG2, ARF1, or ARF3 reduced the constitutive release of 55-kDa TNFR1 exosome-like vesicles (data not shown). These rescue experiments confirm the specificity of the effects of exogenous siRNAs targeting BIG2, ARF1, or ARF3 on the release of TNFR1 exosome-like vesicles.

DISCUSSION

TNFR1 can be released from cells to the extracellular compartment via two mechanisms, the constitutive release of full-length TNFR1 within the membranes of exosome-like vesicles and the induced proteolytic cleavage of TNFR1 ectodomains that occurs acutely in response to inflammatory stimuli, e.g., IL-1β (16, 23). Both processes involve calcium-dependent formation of NUCB2-ARTS-1 complexes that associate with TNFR1 before it enters pathways leading to either the constitutive release of TNFR1 exosome-like vesicles or the induced proteolytic cleavage and shedding of TNFR1 ectodomains (22, 23). The ARTS-1-NUCB2 complex appears to exist in cytoplasmic vesicles, consistent with a critical role for vesicular trafficking in the regulation of TNFR1 release from cells to the extracellular compartment (23).

ARFs are 20-kDa guanine nucleotide-binding proteins that have important roles in intracellular vesicular membrane trafficking via the recruitment of cytosolic coat proteins, as well as cytoskeleton dynamics (24). ARF activation requires the replacement of bound GDP with GTP, which is accelerated by ARF-GEPs. These proteins contain a ca. 200-amino acid Sec7 domain that catalyzes the exchange of ARF-GDP with GTP and can be inhibited by BFA (32, 33). The mammalian BFA-inhibited GEPs, BIG1 and BIG2, were initially purified as components of an ~670-kDa macromolecular complex from bovine brain and later cloned from a human cDNA library (25, 34). Mutations in ARFGEF2, which encodes BIG2, are associated with autosomal recessive periventricular heterotopia and microcephaly, a severe malformation of the cerebral cortex characterized by developmental delay and recurrent infections, which occurs as a consequence of impaired neuronal cellular proliferation and migration during human cerebral cortical development.
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FIGURE 6. Overexpression of ARF1 or ARF3 increases the constitutive release of TNFR1 exosome-like vesicles from HUVEC. HUVEC were transfected with plasmids expressing either wild-type (WT) or mutant (T31N) ARF1 or ARF3 for 3 days prior to the addition of fresh, exosome-depleted medium for 24 h. A, representative Western blot, from one of three experiments, showing TNFR1 in medium and cell lysates, and ARF1, ARF3, and β-tubulin in cell lysates. B, densitometry was performed on the Western blots from A, and the quantity of the 55-kDa TNFR1 that was constitutively released into conditioned medium is presented as arbitrary densitometry units. The asterisk indicates a significant difference in the quantity of 55-kDa TNFR1 in conditioned medium from cells transfected with plasmids expressing wild-type (WT) ARF1 or ARF3 and those transfected with empty (Control) plasmid. C, TNFR1 in medium was quantified by ELISA. The asterisk denotes significant difference from cells transfected with empty (Control) plasmid. D, proteins from HUVEC that had been transfected with empty (Control) plasmid or plasmids that overexpress ARF1(T31N) or ARF3(T31N) were immunoprecipitated (IP) with antibodies against BIG2 and immunoblotted (IB) with antibodies against TNFR1 or BIG2. HUVEC that had been treated with transfection reagent alone (Mock) are shown for comparison. This blot is representative of two individual experiments that showed the same results.

Because ARF-GEPs regulate vesicular trafficking, we assessed whether BIG1 or BIG2 modulates TNFR1 release to the extracellular compartment from human vascular endothelial cells. Using RNA interference, we showed that BIG2, but not BIG1, selectively affected the constitutive release of TNFR1 exosome-like vesicles, but not the IL-1β-induced proteolytic cleavage of TNFR1 ectodomains. Furthermore, TNFR1 co-immunoprecipitated with BIG2, which is consistent with an interaction between endogenous proteins related specifically to the constitutive release of TNFR1 exosome-like vesicles. In contrast, neither ARTS-1 nor NUCB2 associated with BIG2 (22, 23). Thus, we conclude that the calcium-dependent, ARTS-1-NUCB2 complex functionally precedes the division of the two TNFR1 release pathways. These findings also identify a previously unrecognized role for BIG2 (but not BIG1) in the release pathway for TNFR1 exosome-like vesicles.

BIG2 was thought to be primarily localized to the trans-Golgi network (TGN), where it may modulate trafficking among TGN and endosomal compartments by regulating membrane association of AP-1 and GGAs via ARF activation (26, 34, 36, 37). BIG2 is also present in punctate cytoplasmic vesicles that have been identified as recycling endosomes (REs) that are positive for Rab4 and Rab11, as well as AP-1 and transferrin receptor (26–28). In addition, BIG2 has been demonstrated to modulate transferrin receptor recycling from REs to the plasma membrane (27). By confocal microscopy, BIG2 and TNFR1 in HUVEC appeared to be distributed in cytoplasmic vesicles with a predominantly perinuclear location. Furthermore, the BIG2 in HUVEC was not seen in typical Golgi structures, nor was it co-localized with the trans-Golgi marker p230. This is in contrast to its appearance in other types of cells, such as HepG2 and HeLa cells, or rat hepatocytes, where BIG2 primarily resides in the trans-Golgi network (26, 28, 37, 38).

BIG2-catalyzed ARF activation is inhibited by BFA (25). For example, BFA disrupts the association between BIG2, Exo70, an exocyst protein that is required for exocytosis, and p230, which is consistent with the BFA-induced disruption of Golgi membranes (38). Similarly, BFA inhibition of ARF activation by BIG2 impaired the delivery of β-catenin and E-cadherin from the Golgi to the cell surface, thereby interfering with their co-localization (35). Because the GEP activity of BIG2 is BFA-sensitive, we assessed the effect of BFA inhibition on the interaction between BIG2 and TNFR1 and found that it was abolished by BFA. The effect of BFA on the constitutive release of TNFR1 exosome-like vesicles, however, could not be assessed, as exposure to BFA for the time required for this assay was cytotoxic.

BIG2 preferentially activates class I ARFs (human ARF1 and ARF3) via its Sec7 domain (25, 28). Therefore, we investigated the potential function of class I ARFs in the constitutive release of TNFR1 exosome-like vesicles, showing that it was significantly decreased by siRNA-mediated knockdown of either ARF1 or ARF3, whereas the similar depletion of ARF5 or ARF6 had no effect. Similarly, overexpression of
wild-type ARF1 or ARF3 increased the constitutive release of TNFR1 exosome-like vesicles, whereas overexpression of T31N ARF1 or ARF3 mutants, which are preferentially constrained to the inactive GDP-bound state (29), did not. Thus, ARF1 and ARF3 appeared to have nonredundant roles in the constitutive release of TNFR1 exosome-like vesicles. In fact, the inhibitory effects of ARF1 and ARF3 depletion were additive, consistent with actions at different steps, or in different pathways, that lead to the constitutive release of TNFR1 exosome-like vesicles. Our findings resemble those of Shin et al. (28) who showed that inactivation of either ARF1 or ARF3 enhanced the ability of a catalytically inactive T31N ARF1 or ARF3 mutant to induce the tubulation of endosomal membranes in HeLa cells. Taken together, these findings are consistent with the conclusion that ARF1 and ARF3 may have unique and nonredundant roles in regulating specific aspects of vesicular trafficking. This is in contrast to prior studies showing that ARF1 and ARF3 have overlapping and redundant functions (39–41).

In conclusion, we have shown that the BFA-sensitive association of BIG2 and TNFR1 selectively regulates the extracellular release of TNFR1 exosome-like vesicles via an ARF1- and ARF3-dependent mechanism. In contrast, BIG2 does not modulate the induced proteolytic cleavage of TNFR1 ectodomains. This study identifies a new mechanism by which the TNFR1 release to the extracellular space is modulated and supports the conclusion that this process is regulated at the level of vesicular trafficking.

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