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Adenovirus Tales: From the Cell Surface to the Nuclear Pore Complex

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Introduction

Despite lingering safety concerns [1] and potential restrictions imposed by the host immune response, including the innate immune pattern recognition receptors (PRR), replication-defective and conditionally replicating human and nonhuman adenovirus (AdV) vectors continue to be a favorite vehicle for short-term (e.g., vaccine) and long-term gene delivery. This is due in part to several desirable features of AdV including their broad tissue tropism, their ample capacity for foreign gene insertion, and their LEGO-like structural adaptability (Fig 1A) to add, delete, and swap proteins and motifs from other viruses or host molecules.

While much is known about the molecular genetics and replication of AdVs, many investigators are continuing to decipher the captivating intracellular events of the first thirty minutes in the virus life cycle. This Pearl accentuates the strikingly diverse mechanisms for AdV entry, comparing human AdV type 5 (HAdV-C5) in epithelial cells and canine type 2 (CAdV2, or commonly referred to as CAV-2) in neurons. Similar viral and cellular proteins are used, and although the function of the cellular protein varies among cell types, these cell protein—virus associations promote similar outcomes. We also highlight some outstanding questions and hurdles needed to improve vector-mediated gene and vaccine delivery and treatments for AdV disease. The take home message is that one may be able to take advantage of a better understanding of these cell entry variations to control AdV pathogenesis and vector tropism for gene therapy.

Mi Casa Es Su Casa: The Cellular Determinants That Dictate AdV Tropism

Of the more than 60 human AdV (HAdV) types that make up the current seven species (denoted as A–G), the most extensively studied are species C type 2 (HAdV-C2) and HAdV-C5. Many human and some nonhuman AdVs, including CAV-2, use the coxsackievirus and adenovirus receptor (CAR) [2–4] for high affinity attachment to host cells via the capsid fibre protein. On polarized epithelial cells, the predominant CAR isoform is targeted to the basolateral surface and in tight junctions. A minor exon 8-containing CAR isoform may be targeted to the apical surface [5] of some epithelial cells and allow easier access of CAR-tropic virus attachment. Other HAdV types from species B use desmoglein 2 or CD46, a member of the complement regulatory protein family, while species D HAdVs appear to use CAR, sialic acid, the GD1a glycan, and/or CD46 (for a recent review on AdV receptors see [6]). For HAdVs that use CAR as an attachment molecule on epithelial cells, engagement of the αv integrin is needed for efficient internalization (Fig 1B). This engagement occurs through association of the integrin

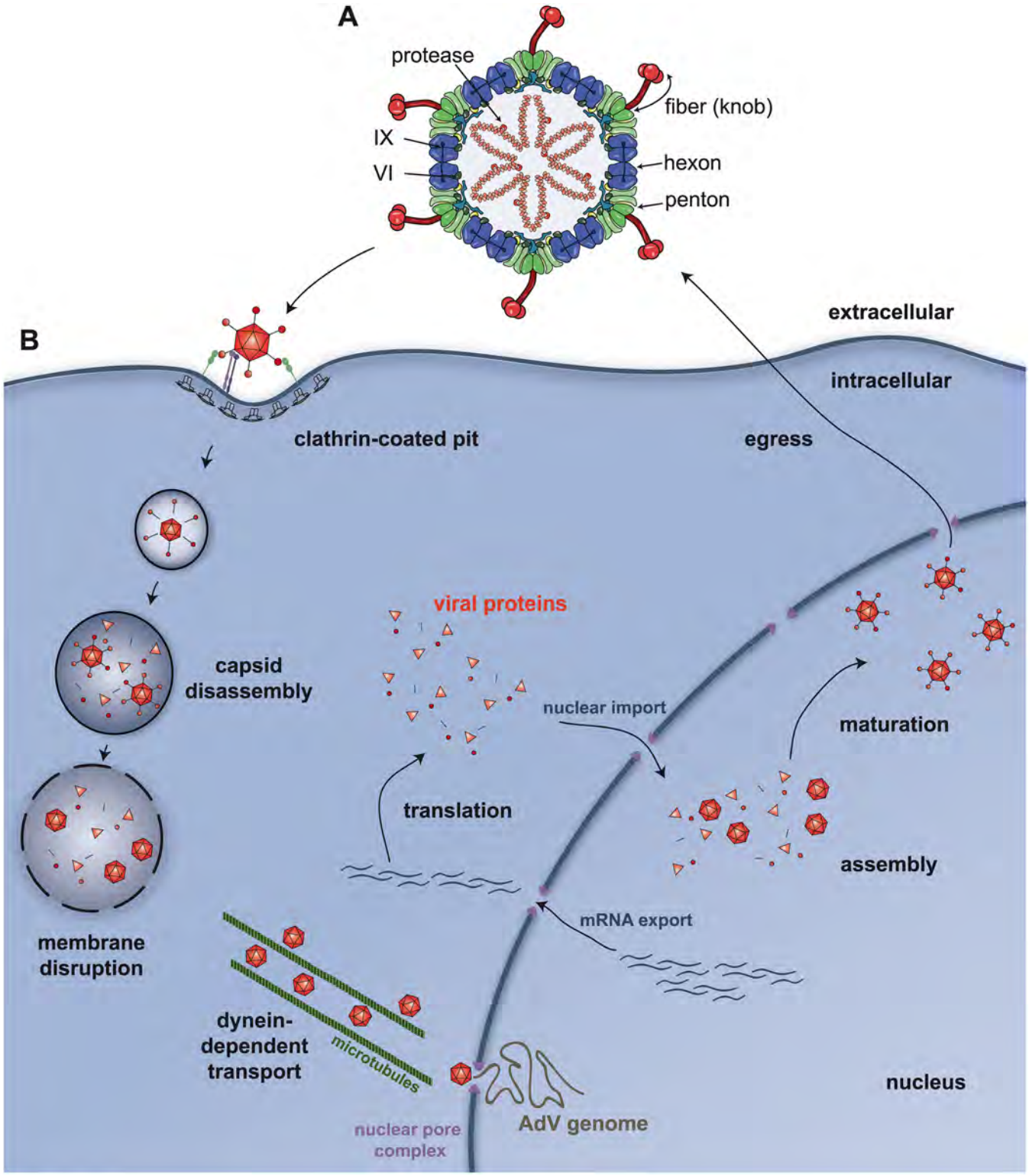

Fig 1. Adenovirus structure and trafficking. A) An illustration of the cross-section of a prototype 90 nm AdV capsid showing the location of the principal capsid proteins (hexon, penton, protein VI, protein IX, protease, and the fibre—the knob is the globular head of the fibre) involved in trafficking. B) An illustration showing the quintessential steps of AdV trafficking in epithelial cells. Via the knob region of the fiber, the capsid engages the cellular receptor. In some cell types, fibres are lost from the metastable* capsid during internalization in clathrin-coated pits. Postinternalization, the capsid continues to dissociate and releases protein VI, which allows the capsid access to the cytosol and interaction with dynein, then dynein-dependent transport along

microtubule to the nuclear pore complex. *Metastable is a common term used to describe the biophysical state of fully mature nonenveloped virions. Overall, the particle is stable to the environment; however, it is able to respond to cellular cues to undergo conformational changes during cell entry.

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with a consensus integrin interacting motif (RGD in most AdVs) located on an extended loop on the penton base [7]. Integrin ligation triggers signaling events that promote virus entry into early endosomes via clathrin-mediated endocytosis (Fig 1B). In epithelial cells, it seems that CAR facilitates attachment but not cell entry [8]. However, it is still unclear how significantly the integrin repertoire involved in membrane penetration influences different AdV types. Moreover, when injected intravenously in mice, some AdVs can interact with specific coagulation factors [9] that alter tissue tropism by preventing binding of naturally occurring antibodies and then by acting as a bridge to attach to proteoglycans on liver cells [10]. That coagulation factors influence HAdV tropism in rodents is clear, but its relevance for HAdV disease and HAdV vector administration in humans is unknown. As discussed below, AdV trafficking into neurons follows a pathway different from that of epithelial cells. Thus, the routes and modes of AdV cell entry are variable and cell-type dependent.

Houston, We Have a Problem: The Escape Route HAdV Uses to Reach the Nucleus

Internalization of AdV particles is a primordial event for infection—but it is only the beginning of the journey to the nuclear pore complex (NPC). The ligation of CAR and integrins on the cell surface induces distinct membrane trafficking processes that produce a mechanical force to initiate partial capsid disassembly [11]. Analyses using atomic force microscopy are consistent with this model and indicate that integrin ligation by the virus is sufficient to loosen the vertex region(s) of the capsid [12]. Once inside most cells, the "metastable" virions ultimately need to escape a vesicular compartment to be translocated via a dynein-dependent mechanism to the NPC (Fig 1B). Removal of the vertex region—composed of the penton base, fibre [13], and likely the peripentonal hexons—allows release of the membrane lytic protein VI from the inner surface of the virus capsid [14]. Interestingly, α defensins HD5 and HNP1 bind to the vertex region of the virus capsid and prevent its disassembly. This restricts release of protein VI and membrane destruction. Exposure of the inner core of the virus apparently starts in the endosome as monitored by antibody detection of the viral genome at early time points [15]. Protein VI release is associated with increased endosomal membrane destruction [13], and a single point mutation (L40Q) in the amphipathic helical domain of protein VI significantly attenuates membrane insertion, membrane destruction, and cell infection [16]. Protein VI insertion into a lipid bilayer causes positive membrane curvature [17], and this may impart stress and global membrane destruction allowing passage of the partially disassembled capsid into the cytosol. If there are structural rearrangements that occur in protein VI after its release from the HAdV capsid, these could be druggable targets.

Partially uncoated virions also escape endocytic vesicle concomitant with a drop in pH. Yet, the precise role of pH in virus disassembly and/or engagement of molecular motors is enigmatic [18]. Partially uncoated virions can associate with dynein motor proteins that recognize the hexon [19] or possibly protein VI [20]. This association is instrumental in transporting the virions along microtubules, and numerous laboratories have seen that, in superinfected cells, AdV capsids can accumulate at the microtubule-organizing center. However, it is unclear whether the microtubule-organizing center is a launching pad for NPC engagement, an artifact of superinfected cells, or a cul-de-sac. The association of the virion with proteins at the NPC likely facilitates further uncoating of the virion, thereby allowing the genome to be translocated into

the nucleus, although the precise mechanisms involved are still being investigated. Afterwards, the AdV genome is delivered to the nucleus to initiate a new round of propagation.

Whether capsid disassembly is initiated at a unique or specific vertex—for example, a "portal vertex" where the DNA is inserted during particle maturation—is unknown. Could a unique portal vertex be used for initial disassembly or protein VI release and nuclear import during infection and DNA packaging? If a single vertex pops off from the disassembled HAdV-C5 capsids, how would the genome become available to detection by cytosolic PRRs in some cell types? Clearly, cell type-specific trafficking influences the interaction with PRRs and therefore may provide an opportunity for vector optimization.

The Penthouse Please: How Does CAV-2 Enter Neurons?

We all know that cells differ in their morphology and functions, and it would be surprising if they interacted with pathogens in identical manners. A distinct variation on AdV trafficking has been observed in neurons. CAV-2 has found an unlikely niche as a vector for gene transfer to central (brain and spinal cord) and peripheral (e.g., motor and sensory) neurons [21–23]. The basis of CAV-2's preferential targeting to neurons is likely due to the exclusive use of CAR as both the attachment and entry molecule, as well as the selective expression of CAR on neurons in the brain parenchyma (versus microglia, astrocytes, and oligodendrocytes) and at neuromuscular junctions [24]. In addition to the preferential neuronal tropism, CAV-2 vectors are efficiently taken up at axon termini and transported back to the soma via retrograde transport [24,25]. While numerous studies of AdV trafficking in epithelial-like cells (including CAV-2 [26]) have led to a well-recognized pathway, CAV-2 trafficking in neurons accentuates AdV trafficking adaptability.

CAV-2, like the two members of HAdV species F, does not contain an identifiable integrin-interacting motif in the penton base [27]. CAV-2 uses its trimeric fibre knob at the end of a double-hinged shaft [28] to attach to CAR with very high (1 nM) affinity. At axon termini, CAR is located in lipid rafts [29], and CAR-mediated internalization of CAV-2 appears to be induced by the disruption of intracellular homodimeric CAR interactions [29]. The number of CAR molecules needed to induce internalization is unknown, nor whether a torsional force is applied to the CAV-2 capsid during entry to induce the release of protein VI. It is also unclear whether neurons induce partial CAV-2 disassembly during internalization at, or near, the plasma membrane. The CAR-CAV-2 complex likely stays in lipid rafts, and internalization depends on actin reorganization, dynamin function, and—in contrast to HAdV-C5 internalization in epithelial cells—is clathrin-independent. Immediately postinternalization, the CAR-CAV-2 complex can be found in static $Rab5^+$ vesicles that mature into $Rab7^+$ vesicles, where active retrograde transport starts (Fig 2) [20].

The sorting platform for endocytosed vesicles is complex at the synapse: does CAV-2 influence the content of the vesicles it ends up in? Does this influence vesicular trafficking and targeting in neurons? And here is another difference between epithelial-like cells and neurons: CAV-2 does not escape from these CAR^+/pH-neutral/$Rab7^+$ multitasking compartments [30]. Of note, in epithelial cells, $Rab7^+$ is a hallmark of late endosomes that have an acidic lumen. What appears to be an intact CAV-2 (Fig 2A insert) is transported to the soma of the neurons in a vesicular structure. This long, protected retrograde journey in motor neuron axons could be more than 1 meter in humans. When the CAR-CAV-2 complex reaches the soma, CAV-2 vesicular escape also coincides with a drop in the pH [31]. Whether the pH drop and endolysosome escape of CAV-2 in neurons are mechanistically linked has not been formally tested.

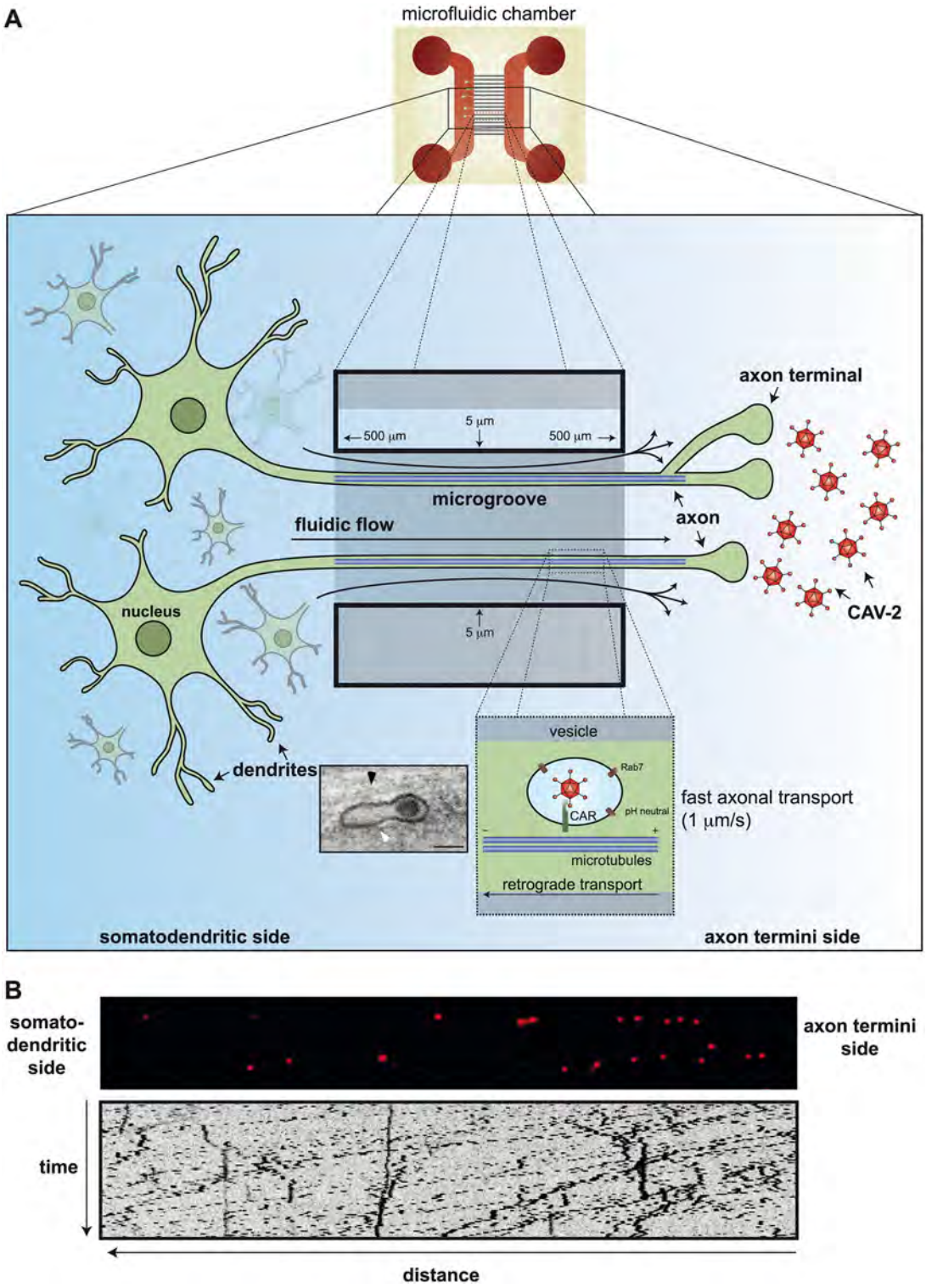

Fig 2. Axonal transport of CAV-2 in neurons. A) A schema showing the assays used to record CAV-2 directionality and speed in murine dorsal root ganglion (DRG) neurons. Neurons are cultivated in microfluidic chambers (top center) in which the microfluidic flow is from left to right. This flow of the medium in the 5-micron-wide and 500-micron-long microgroove prevents diffusion of particles and allows a physical separation between cell bodies (left) and axon termini (right). CAV-2, covalently labeled with a fluorophore (Cy3), were added for 90 min in the axonal compartment before the video was started, and axons in the middle of the microgroove were imaged at one frame/second. The rate of retrograde transport of CAV-2 in these conditions is approximately 1–2 microns/s (insert: ultrastructural electron micrograph of CAV-2 vesicular transport in motor neurons from Salinas et al. [31]). CAV-2 was mainly present in

vesicular structures (white arrow) near microtubule tracks (black arrow). B) Still images of a microgroove of the chambers containing Cy3-labeled CAV-2 (red puncta) 90 min postincubation on the axon termini side. Below is a kymograph, which gives a graphical representation of the spatial position over time, of the corresponding movie (S1 Video). Scale bars in the micrograph = 100 nm.

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Et Alors: The Implications for AdV-Mediated Gene Transfer

The modularity of the AdV capsid allows enormous adaptability for practical and theoretical vector design. With the more than 200 different AdVs partially or totally characterized to date, we have a rich reserve to create vectors for short- or long-term gene transfer. CAV-2 vector use in the brain is a perfect example [32]. Few would have predicted that CAV-2—which normally causes respiratory tract infection in some *Canidae* (e.g., dogs, wolves, foxes, bears, etc.)—would be a valuable tool with which to probe higher order brain function or as a vector to treat neurodegenerative diseases that affect the entire brain [33]. A CAV-2 vector eliminated the neuropathology in the mucopolysaccharidosis type VII (MPS VII) mouse and the MPS VII dog brain—a brain that is one-third the size of a two-year-old child. This therapeutic potential is due to the low immunogenicity, long-term transgene expression, ample cloning capacity [25], preferential transduction of neurons, and the use of the interconnectivity of neurons to reach structures throughout the brain. CAV-2 vector potential was unpredictable 20 years ago.

So, what are we waiting for? *Carpe momentum*.

Supporting Information

S1 Video. Mouse DRG neurons were cultivated in microfluidic chambers in which the microfluidic flow is from left to right. This fluidic flow of the medium in the 5-micron-wide and 500-micron-long microgroove prevents diffusion of particles and allows a physical separation between cell bodies (left) and axon termini (right). CAV-2 particles covalently labeled with a fluorophore (Cy3) were incubated for 90 min in the axonal compartment before the video was started, and axons in the middle of the microgroove were imaged at one frame/second x 100 seconds.
(MOV)

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